RISK FACTORS FOR OESOPHAGEAL CANCER IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA

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DECLARATION

I, Vikash Sewram, hereby declare that the work on this dissertation is based on my original research and has not, in whole or in part, been submitted towards another degree, at this university or elsewhere. The university is empowered to reproduce either the whole or any portion of the contents for the purposes of research.

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ABSTRACT

A multicentre hospital-based case-control study with incidence density sampling was conducted between November 2001 and February 2003 to assess the impact of social and dietary habits, and the consumption of dietary and medicinal wild plants on the risk of developing oesophageal cancer (OC) among residents of the Eastern Cape Province of South Africa. The study was conducted on 670 incident cases (98% response rate) and 1188 controls (96% response rate) attending either of the three major referral hospitals in the Province, i.e. Umtata General, Frere and Cecilia Makiwane Hospitals. Patients were interviewed using a structured questionnaire which aimed to solicit information pertaining to tobacco usage, alcohol consumption, dietary preferences with regards to the traditional Xhosa diet and the use of wild plants for dietary and medicinal purposes. Plants were also collected from the wild with the help of local residents, authenticated by comparison with reference herbarium specimens and subjected to a variety of laboratory-based assays to determine their mutagenic potential, trace metal and mycotoxin contamination levels, to bring about mechanistic evidence of biological plausibility.

Of all the eligible cases, 36% were diagnosed by histology, 60% by radiology and 4% by endoscopy. The middle third region of the oesophagus was the most frequent tumour location with 56% of the patients presenting, whilst 20% and 16% presented with tumours in the lower and upper third of the oesophagus respectively. The information on the remaining patients was either missing or unspecified. Most of the cases were in the 55 – 74 year age-group and from rural regions. With increasing years of education, a protective effect was observed. Males and females having 8 or more years of education had a 50% and 57% reduced risk of developing OC respectively (males OR = 0.50, 95% CI 0.34-0.73; females OR = 0.43, 95% CI 0.30-0.62). In addition, rural residents were more likely to develop OC than urban residents, with a significant association being observed only among males (OR = 1.56, 95% CI 1.18-2.07).

The measures of the strength of association between the different exposure variables and OC were obtained by calculation of odds ratios (ORs) with 95% confidence intervals as a direct estimation of the relative risk. Unconditional multiple logistic-regression models were fitted after adjustment for age, hospital of admission, urban/rural status and years of education. The tobacco-related variables were further adjusted for total grams ethanol consumed per week whilst the alcohol-related

variables were further adjusted for total grams tobacco smoked per day. Dietary and plant-related variables were further adjusted for total grams tobacco smoked per day and total grams alcohol consumed per week.

A monotonic dose-response was observed across the categories in all of the tobacco-related variables in both males and females. Based on total tobacco use, males and females currently smoking more than 14.5 grams of tobacco per day were observed to have approximately 4-times a greater risk of developing OC (males OR = 4.36, 95% CI 2.24-8.48; females OR = 4.56, 95% CI 1.46-14.30). Similar trends in risk were observed for the alcohol-related variables. The quantity of ethanol consumed was an important factor in OC development rather than the type of alcoholic beverage. Males and females consuming more than 371 grams ethanol per week had an almost 5-times greater risk in comparison to non-drinkers (males OR=4.72, 95% CI 2.64-8.41; females OR=5.24, 95% CI 3.34-8.23). Tobacco use and alcohol drinking had independent effects on OC risk. No evidence of statistical interaction was observed on the multiplicative scale. The attributable fractions for smoking and alcohol consumption were found to be 58% and 48% respectively. Hence, on the assumption that these exposures are causal, 58% and 48% of oesophageal cancer would be prevented if these exposures were removed from the environment.

Amongst the reported dietary items, consumption of wild plants (*imifino*) was positively associated with OC risk, while the consumption of green leafy vegetables, fruit and meat were observed to provide a protective effect against the development of OC. Principal component factor analysis revealed three distinct dietary patterns. Dietary pattern 1 , comprising sorghum, green leafy and podded vegetables, fruits and meat was found to be common amongst the more educated population and observed to confer protective effects (OR=0.54, 95% CI 0.34-0.89). Dietary pattern 2, comprising maize, *imifino* and beans was more common amongst the less educated and rural population and was associated with an increased risk (OR=1.67, 95% CI 1.04-2.67) whilst dietary pattern 3, comprising exclusively of wheat-based products was observed to confer protective effects. The effects of reported mouldy maize consumption were explored. No association between mouldy maize consumption and OC risk was observed indicating that mouldy maize consumption was not a risk factor for the development of OC in either males and females (males OR=0.98, females OR=0.86). The adjusted risk estimates associated with the use of various dietary and medicinal wild plants were also obtained. Of the 19 plants reported used

as dietary supplements, 8 were associated with a significant increase in OC risk in both males and females (ORs ranging from 1.40 to 4.12). Similarly, of the 29 medicinal plants investigated, 4 were associated with a significant risk of developing OC (ORs ranging from 1.41 to 2.26).

In addition to the risks presented by this epidemiological study, some of these plants were also found to have mutagenic potential when assayed on the *Salmonella* reverse mutation assay. Through the use of a metabolic activation system, it was possible to identify direct-acting mutagens from promutagens. The plant, *Rumex lanceolatus*, which is used as a dietary supplement by 21% of the population was associated with an almost 2-fold increased risk in males and females and was observed to alter the DNA of the bacteria through frameshift mutations, base-pair substitutions and oxidative cell damage. The plant, *Raphanus nasturtio aquatica* which was also associated with a 2-fold increased risk only displayed mutagenicity through base-pair substitution. Similarly, the mutation pathway for the other plants was elucidated.

Trace metal analysis of these plants also indicated a severe deficiency of selenium and molybdenum while carcinogenic and toxic metals such as Co, Cr, Cd, As and V was present in the dietary wild plants. With regards to the plants used as traditional medicines, the levels of these toxic metals were quite low and it is not anticipated that a risk would be imposed.

Analysis of the plants for mycotoxin contamination indicated that the plants, *Rumex lanceolatus*, *Raphanus raphinastrum*, *Zantedeschia aethiopica*, *Solanum nodiflorum*, *Dalbergia obovata*, *Catha edulis*, *Datura stramonium* and *Brunsvigia sp.* were contaminated with the mycotoxin, fumonisin B_1 (FB_1) at levels ranging between 8.6 – 1553 μg/kg of plant material. Despite the association of some of these plants with OC risk, the levels detected were comparable to the background levels in good maize, hence once again, no association to FB_1 could be made. No aflatoxin B_1 contamination was detected in the plants studied.

This study is to-date one of the largest case-control study on OC to be conducted in South Africa and reveals that the population of the Eastern Cape Province indeed fall in the high risk category as a result of the exposures they endure due to their social and dietary habits and therefore are a high-risk subset of the South African population.

PUBLICATIONS AND CONFERENCE PRESENTATIONS

Selected results from this thesis have been published in a scientific journal and presented at various national and international conferences.

1. Sewram, V., Shephard, G.S., van der Merwe, L. and Jacobs, T.V. (2006) Mycotoxin Contamination of Dietary and Medicinal Wild Plants in the Eastern Cape Province of South Africa. J. Agric. Food Chem., 54, 5688-5693. (Impact factor 2.507)

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3. Sewram, V., Sitas, F., O'Connell, D., Van der Merwe, E., Myers, J. Risk Factors For Oesophageal Cancer In South Africa: Re-Evaluating Past Evidence And Uncovering New Realities Related To Traditional Practices. 18th International Conference of Nutrition. International Convention Centre, Durban, South Africa. 19 – 23 September 2005. (Poster Presentation)

4. Sewram, V., Shephard, G.S., Van der Merwe, E., Jacobs, T.V. Cancer Risk Following Dietary And Medicinal Wild Plant Use In South Africa. Indian Society for Mass Spectrometry (ISMAS) Symposium. Tea County, Munnar, India, 28 January - 01 February 2006. (Oral Presentation)

5. Sewram, V., Sitas, F., O'Connell, D., Van der Merwe, E., Myers. Study of Dietary and Medicinal Wild Plants as Potential Risk Factors for Oesophageal Cancer in the Eastern Cape Province of South Africa. UICC World Cancer Conference 2006, Washington Convention Centre, Washington DC, USA. 8 – 12 July 2006. (Oral Presentation)

6. Sewram, V., Sitas, F., O'Connell, D., Van der Merwe, E., Myers, J. Risk Factors For Oesophageal Cancer In South Africa: Re-Evaluating Past Evidence And Uncovering New Realities Related To Traditional Practices. 27th African Health Sciences Congress, Elangeni Hotel, Durban, South Africa, 3 – 7 December 2006. (Oral Presentation)

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LIST OF ABBREVIATIONS AND SYMBOLS

OC	oesophageal cancer
SCC	squamous cell carcinoma
ICD-O	International Classification of Diseases – Oncology
AJC	American Joint Committee
ASIR	Age Standardized Incidence Rate
NCR	National Cancer Registry
IARC	International Agency for Research on Cancer
RR	relative risk
CI	confidence interval
OR	odds ratio
Pe	Prevalence of exposure
IRR	incidence rate ratio
HPV	human papillomavirus
Ab	antibody
IgG	immunoglobulin G
ESCC	esophageal squamous cell carcinoma
DNA	Deoxyribonucleic acid
PCR	polymerase chain reaction
PAH	polyaromatic hydrocarbon
TSNA	tobacco-specific nitrosamine
EPIC	European Prospective Investigation into Cancer and Nutrition (EPIC)
WCRF	World Cancer Research Fund
FB_1	Fumonisin B_1
AFB_1	Aflatoxin B_1
DTE	dietrpene ester
CHP	cumoylhydroperoxide
H_2O_2	hydrogen peroxide
2-AAF	2-acetamidofluorene
DMSO	dimethyl sulphoxide
PCB	polychlorinated biphenyl
ECRA	Ethics Committee for Research on Animals
MFO	mixed function monooxygenases
SD	standard deviation
ICP-MS	Inductively coupled Plasma-Mass Spectrometry
HPLC	High Performance liquid chromatography
MS	Mass Spectrometry

PMTDI	Provisional Maximum Tolerable Daily Intake
JECFA	Joint FAO/WHO Expert Committee on Food Additives
cm	centimetre
%	percent
viz.	namely
L	litre
ml	millilitre
ppm	parts per million
g	gram
kg	kilogram
µg	microgram
M	Molar
mM	millimolar
m/v	mass-to-volume
ng	nanogram
W	Watt
s	second
V	volt
kV	kilovolt
°C	degree Celsius
γ	Kruskal's gamma coefficient
α	alpha

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Oesophageal cancer (OC) is considered the eighth most common cancer in the world (Parkin *et al.*, 1999) with worldwide variation in incidence and mortality rates between countries, and the fourth lethal site characterised by very poor survival similar to the liver, pancreas, and lung. It has one of the lowest probabilities of cure, with 5-year survival rates estimated to be approximately 10% overall (Wong, 2000). The high mortality rate due to OC is related to the fact that symptoms first appear when the patient cannot swallow; by that time, the tumour is large, invasion of the oesophagus and surrounding tissues is advanced, and prognosis is poor (Day, 1986). It is thus a very difficult condition to treat, hence underlining the importance of understanding its aetiology for primary preventive purposes.

1.2 Pathology and Clinical Presentation

This malignancy exists in two main forms with distinct aetiological and pathological characteristics, *viz.* squamous cell carcinoma (SCC) and adenocarcinoma. However, more than 90% of oesophageal cancers worldwide are SCCs (Stoner and Rustgi, 1995; Beer and Stoner, 1998) and is thus the subject of this review. Oesophageal SCC is defined as a malignant epithelial tumour with squamous cell differentiation, microscopically characterised by keratinocyte-like cells with intercellular bridges and/or keratinization (Gabbert *et al.*, 2000) and is afforded the International classification of Diseases – Oncology (ICD-O) code 8070/3.

The most common symptoms of advanced OC are dysphagia, weight loss, retrosternal or epigastric pain, and regurgitation caused by narrowing of the oesophageal lumen by tumour growth (Goodnight *et al.*, 1996). Superficial SCC usually has no specific symptoms but sometimes causes a tingling sensation and is, therefore, often detected incidentally during upper gastrointestinal endoscopy (Endo *et al.*, 1986; Tachibana *et al.*, 1997) as a slight elevation or shallow depression on the mucosal surface, which is a minor morphological change compared to that of advanced cancer.

Cancer of the oesophagus is located predominantly in the middle and the lower third of the oesophagus with only 10-15% being situated in the upper third (Levin & Appelman, 1996). In South Africa, Mannell and Murray (1989) reported on the clinical data of 1926 cases (1438 males and 488 females) between November 1985 and

August 1988 collected by the National Study Group for OC. Of 1036 patients that could be staged from the information provided, 71% presented with stage III of the disease (American Joint Committee (AJC) System of Staging of Oesophageal Cancer, 1983). Regarding the extent of dysphagia at the time of admission, it was found that 62% of the patients complained of dysphagia for solid food, 12% had difficulty swallowing liquids and 24% reported total dysphagia. The middle thoracic region was the most common tumour site (53%) followed by the lower third (25%) and then the upper third (16%). The length of the tumour in 58% of the patients was between 5 – 10 cm with the median being 6cm. At oesophagoscopy it was found that 89% of the patients had cancer involving the entire circumference of the oesophagus. It is a common impression that African black patients with OC are admitted in a much more debilitated state than their Western counterparts.

1.3 Geographical Distribution of Oesophageal Cancer

1.3.1 International Comparison

The burden of OC in terms of morbidity and mortality varies enormously according to geographical area with about 80% of all cases and deaths occurring in less developed countries (Pisani, *et al.*, 1993). There are three regions in the world where incidence rates are particularly high. These are (A) southeast South America which includes northeastern Argentina, southern Brazil, Paraguay and Uruguay (Parkin *et al.*, 1999; Vassallo *et al.*, 1985); (B) the so-called 'Asian oesophageal cancer belt' that stretches from eastern Turkey and east of the Caspian Sea through northern Iran, northern Afghanistan, southern areas of the former Soviet Union, such as Turkmenistan, Uzbekistan and Tajikistan to northern China (Schottenfeld, 1984; Sons, 1987; Yang, 1980) and (C) southeast Africa (the former Transkei region of the Eastern Cape Province of South Africa) (Makaula *et al.*, 1996). The differences between the incidence of OC in distinct geographical areas are more extreme than observed for any other cancer. Even within these high-risk areas, there are striking local variations in risk with very high-risk regions neighbouring onto districts with a much lower risk. This situation has been studied most often in China, when comparing the Linxian region with Fanxian (Cai, 1982).

In the geographic cluster of high incidence areas of South America, as stated above, age-standardized incidence rates (ASIRs) ranged from 3.3 and 1.3 per 100 000 to 13.9 and 3.1 per 100 000 for males and females respectively (Parkin *et al.*, 2002). More specifically, data from the cancer registry in Montevideo, Uruguay has revealed

ASIRs of 10.7 and 2.5 per 100 000 for males and females respectively (Parkin *et al.*, 2002). However in the northeast region bordering with Brazil, the ASIR is reported to be 55.8 and 14.7 per 100 000 for males and females respectively (De Stefani *et al.*, 1999). The mortality rates for this cancer in Uruguay are one of the highest in America and range from 40/100 000 for males in the northeastern region that borders Brazil to 10 per 100 000 in the capital city of Montevideo (Vassallo *et al.*, 1985). The death rates are lower for females, with male/female ratio of 3.8 for the whole country. The most important histology type in this region is also SCC (De Stefani *et al.*, 1994).

In the southeastern part of the Caspian littoral, in areas surrounding Gonbad City, Iran and further to the East, rates were at least as high in women as in men, and were some of the highest rates for any single cancer ever reported anywhere in the world (ASIRs >100/100 000 per year) (Kmet & Mahboubi, 1972; Mahboubi *et al.*, 1973). It seems that the incidence of OC in Turkmen ethnicity is more than that in other inhabitants of this area (Saidi *et al.*, 2000; Islami *et al.* 2005). In addition to northeastern Iran, very high rates of OC have also been reported in several other areas of Central and East Asia (Saenko, 1975), including Turkmenistan, Uzbekistan, Karakalpakstan and Kazakhstan (Zaridze *et al.*, 1992; Kairakbaev, 1978). The northern region of Muinak in Karakalpakstan reported ASIRs of 125.96 and 150.65 per 100 000 for males and females respectively (Zaridze *et al.*, 1992). Equally high rates among females is a rare epidemiologic feature of OC but such an observation has also been made in Linxian, China, the other area of the world with registry-reported rates >100/100 000 (Ke, 2002). Furthermore, in the 1970s the annual incidence rate of OC in this area was 151/100,000 for males and 115/100,000 for females (The Coordinating Group for Research on the Etiology of Esophageal Cancer of North China, 1991). Whilst the incidence rates in China and Iran appear to have decreased during the last 3 decades, there are still hotspot areas within these countries where the ASIRs remain high. Such is the case in Cinxian where rates of 183.8 and 123.1 per 100 000 have been reported for males and females respectively (Parkin *et al.*, 2002). These rates are more than 10-times those reported for other countries where OC is reported to be high as illustrated in Figure 1.1.

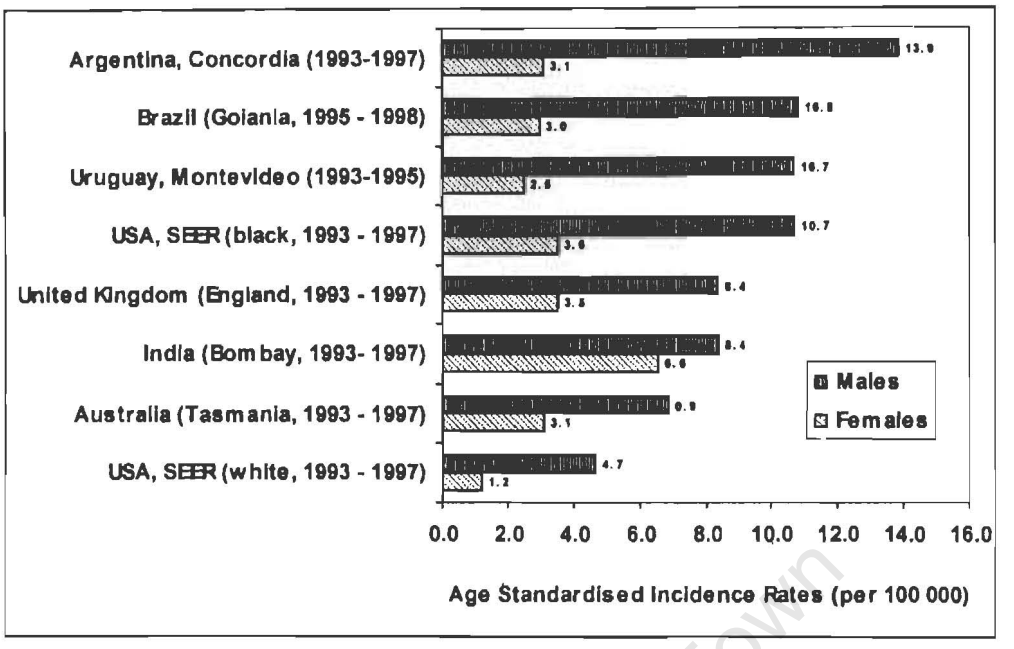


Figure 1.1. Oesophageal cancer incidence rates in selected international populations (Data Source: Parkin *et al.*, 2002)

1.3.2 Comparisons within the African Continent and South Africa

Differences in the incidence rates of OC within the African continent have also been found to exist. There are three noticeable charateristics in the occurrence of OC in Africa. The first being the geographical distribution, the second the changing pattern of frequency with time and third, the disparity in the sex ratio. The incidence in parts of East, Central and South Africa is among the highest recorded anywhere in the world while in West Africa it is virtually unknown. Sharp gradients in frequency from very high to very low within East, Central and South Africa also exist (Cook and Burkitt, 1971). In all these areas OC is reported as being more common in men than in women but the sex ratio varies from 12:1 in west Kenya to 1.5:1 in parts of South Africa. The geographical distribution suggests strongly that some environmental factor is involved in the development of OC.

Population-based cancer registry data from certain regions in Africa (Parkin *et al.*, 2002) also show the ASIRs between males and females to vary considerably (Figure 1.2) with high incidence rates in some countries, particularly in Zimbabwe and Uganda and low incidence rates in Mali, The Gambia and Algeria.

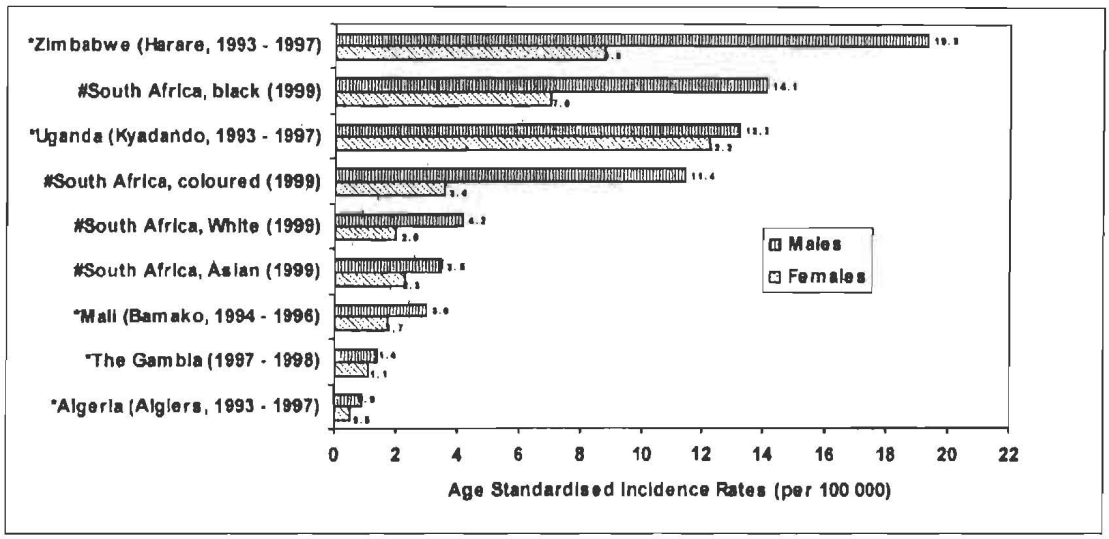


Figure 1.2. Regional and local oesophageal cancer incidence rates (* = population-based cancer registry data; # = pathology-based cancer registry data) (Data Source: Parkin *et al.*, 2002 & Mqoqi *et al.*, 2004)

The results presented for South Africa are from pathology-based data published by the National Cancer Registry (NCR) and are therefore not comparable to those of population-based registry data due to the nature of information gathering although at a glance one can note that the rates in white South Africans are close to those reported among white American (SEER rates) and rates in South African Asians (males and females) are 2.5 times lower than those reported in Bombay, India.

Based on pathology-based incidence data, the NCR ranks cancer of the oesophagus as the fifth most common cancer in all males and the most common cancer in black males. Geographic variation in the incidence of this disease has also been observed and a 15-fold difference exists between high-risk Southern African men and low-risk Western African men. In women, the difference is 20-fold between high-risk Southern Africa and China and low-risk Southern Europe (Parkin *et al.*, 1999).

In 2003, a report produced by the NCR on the incidence of histologically diagnosed cancer in South Africa between the period 1996 – 1997 showed that 3157 new OC cases were reported in males and 1665 in females (Mqoqi *et al.*, 2003). On average, black males comprised 86% of all OC cases per year and had the highest incidence rates with an ASIR ranging from 16.8 per 100 000 in 1996 to 16.2 per 100 000 in 1997. Among black females, OC was the third leading cancer and comprised on

average 6% of all black female cancers with an ASIR of 7 per 100 000 in the two years. It must be understood however that such data is not representative of the South African population as the NCR data is only pathology-based rather than population-based.

In a subsequent report published in 2004 (Mqoqi *et al.*, 2004), the NCR ranked OC the 3rd leading cancer in males. A total of 1645 and 1540 new cancer cases were reported in males, comprising 5.6% and 5.2% of all new cancer cases reported in 1998 and 1999 respectively. In women, OC ranked the fourth leading cancer. A total of 939 and 909 new cancer cases were reported consecutively in the two years and comprised 3% of all cancer cases reported in females. The crude rate in women was 4 per 100 000 and the ASIR ranged between 6 per 100 000 in 1998 and 5.5 per 100 000 in 1999.

Within the South African population, OC was the 2nd leading cancer in black men who comprised the largest proportion (83%) of all OC cases reported in men. ASIR rates ranged between 15.6 per 100 000 in 1998 and 14.1 per 100 000 in 1999. The lifetime risk of developing OC was reported to dropped from 1 in 53 in 1998 to 1 in 59 in 1999. The risk of developing OC doubled from an ASIR of 36 per 100 000 at the age of 50 – 54 to an ASIR greater than 73 per 100 000 in men older than 65 years. Coloured males had the 2nd highest incidence rates for OC of 13.4 and 11.4 per 100 000 in 1998 and 1999. In this population group, OC ranked the fifth leading cancer in 1998 and ranked 6th in 1999. The risk of developing OC was 4-times lower in Asian men than that in black men. In this group, cancer of the oesophagus ranked 8th. Asian men had the lowest OC rates of 3.5 per 100 000 in 1999.

In black females, OC was the 3rd leading cancer and comprised on average 84% of all OC cases. The ASIR rate in females was 7 per 100 000 in the two years. In 1999, the lifetime risk of developing OC in black women was 1 in 113. At the ages of 50 – 54, the ASIR was 12.6 per 100 00, three times lower than that in black males of the same age group peaking at 51 per 100 000 at the ages 70 – 74. In 1999 coloured females had the second highest rates of 3.6 per 100 000. In this group, OC ranked 8th. The lowest rates were observed among white females, with an incidence rate of 2 per 100 000 in 1999.

1.3.3 Oesophageal Cancer in South Africa: Evidence from a Historical Perspective

Despite the NCR collecting pathology-based cancer data in South Africa, a population-based cancer registry was set up as early as 1955 in the former Transkei region of the Eastern Cape Province of South Africa. The first indication that OC had become an important health problem among the Black isiXhosa speaking patients came from a publication by Burrell (1957) on information gathered in East London, the largest city near the former Transkei region. Since the beginning of 1950, there was a steep rise in OC incidence (Oettlé, 1963). Burrel reported that cancer of the oesophagus was probably unknown in the Transkei 25 years before his survey (Burrel, 1962). He gained this impression from discussion with local doctors who had worked for many years in the area and by asking elderly residents to recall their previous knowledge of the disease. In an area where cancer of the oesophagus was now so common that local people had their own name for it (isiXhosa, *umhtaza wombiza*, chronic ulceration of the gullet), and "illiterate laymen confidently diagnosed the condition" (Oettlé, 1964). Thereafter several independent surveys carried out in other parts of the previously defined homeland confirmed the presence and high frequency of this disease. In 1967 an average of 50 cases per year were diagnosed at Umtata General Hospital (Rose, 1967). At that time, the Butterworth-Kentani region of the former Transkei appeared to be the 'epicentre' of this disease.

The ratio of male to female incidence was initially reported to be only approx 1.5 to 1 for the Transkei (Rose, 1967). Cases of malignancies screened between 1960-1963 at Frere Hospital (East London, South Africa) revealed an OC frequency of 18% which represented the frequency in both sexes together and was probably only a slight underestimate (Rose, 1967). While a very high frequency of 53% (in both sexes combined) was reported from a smaller mission hospital at Glen Grey in the former Transkei (Rose, 1967) between 1957-1965 through retrospective searching of the hospital records, however it was difficult to interpret this figure without further information about the completeness of the records, or admissions policy of the hospital, or the place of residence of each patient. The situation is not entirely comparable, because Glen Grey is a small, isolated mission hospital, but the type of bias which can occur in hospital records has been well illustrated from a detailed investigation which has been made of the high frequency area around Kisumu Provincial Hospital in western Kenya. The principal catchment area of Kisumu Hospital is the Central Nyanza District. An apparent proportion of 30% of OC cases

(frequency in both sexes) was reduced to 24% when it was found that, because of the known interest of the Provincial Surgeon, patients with cancer of the oesophagus seemed to be referred to the Provincial Hospital from distant districts more frequently than patients with tumours of the other sites (Ahmed and Cook, 1969). Nevertheless, the oesophagus was still overwhelmingly the most common site of tumour growth in men in Central Nyanza. Twenty five percent of all male cancer patients had oesophageal tumours and there were almost three times as many patients with OC than with cancers of the liver or stomach.

A hospital-based study of cancer among all African black residents of Johannesburg from 1953-1955 showed OC to have a proportional frequency in men of 11% (Higginson and Oettlé, 1960). The records of 7 hospitals serving rural areas of the Northern Transvaal were also examined in the course of the same survey and showed a frequency of only 2%. Liver and lung tumours on the other hand were diagnosed with a frequency similar to that in Johannesburg (liver 22% compared with 23% and lung 7% compared with 8%). At the time, figures from South Africa showed that the gradient still existed. The figure of 28% for Johannesburg in 1962-1964 can be compared with a frequency of 6% observed at the hospital of Acornhoek in the Northern Transvaal between 1957 and 1966 (Sutherland, 1968). Oettlé followed the original study of frequency with a postal questionnaire to all general hospitals in southern Africa to see whether other similar gradients could be established (Oettlé, 1963). He asked for the number of oesophageal tumours to be expressed relative to the number of hospital beds and found a ratio which varied from 25.8 per 100 beds in the Transkei and 25.4 in Tembuland (immediately to the north of the Transkei) to only 0.2 in Swaziland. However there were certain deficiencies in the results to which Oettlé himself drew attention, such as the fact that no attempt was made to eliminate duplicates occurring in the records from the readmission of a patient to the same hospital or from multiple attendances at several hospitals, and the fact that in some towns the majority of cases had been referred from other parts of South Africa (eg. In Cape Town about 34 cases were seen each year, but the majority of those patients had been referred from the Transkei). However a more serious source of bias and one which is not mentioned, is that the denominator (number of hospital beds) itself shows considerable variation relative to the population at risk. In the Transkei there were only 0.35 hospital beds per 100 population whereas in Johannesburg there were 4.5 and in Cape Town 9.3 per 100 population.

The number and percentage of oesophageal tumours in men diagnosed at the Baragwanath hospital in Johannesburg showed a sevenfold increase between the early 1952-1954 (52 oesophageal in 542 diagnosed malignancies) and 1962-1964 (371 oesophageal in 1350 diagnosed malignancies) (Robertson, 1969). This increase was seen to be genuine and not just an artefact of a possibly ageing population since other sites which show a comparable or greater rise of frequency with age such as the stomach, colon and prostate for example did not show a comparable increase in time. In 1953-1955 the incidence in men in Johanesburg was 21.8 per 100 000 (Higginson and Oettlé, 1960; Doll, 1969).

The Zulus living in what is now the KwaZulu-Natal Province also showed similar increases during the late 1950s and early 60s (Schonland and Bradshaw, 1969). A study of the Zulu migrants to the Johannesburg goldmines showed no cases of OC prior to 1960 however the incidence of this disease between 1964-1979 when assessed from 10 widespread regions of Southern Africa was significantly raised above the mean for the Zulus from KwaZulu-Natal and for Xhosas from the former regions of the Transkei and Ciskei (Bradshaw *et al.*, 1982). This increase may possibly have been related to urbanization and possibly to more access to tobacco and alcohol. In Durban there was a five to six-fold increase in the number of cases diagnosed in men between the early 1950s and mid-1960s (Schonland and Bradshaw, 1968). At Edenvale Hospital, Pietermaritzburg in KwaZulu-Natal there was a six-fold increase in the number of hospital admissions for OC between 1953 and 1964 (Coetzee, 1966). However with regards to these observations, there were no indications of the extent to which all malignancies may have increased in frequency.

From 31 cases of OC occurring in one locality of East London between 1952 and 1956, Burrell (1957) calculated age specific incidence rates in men to be 100.8 per 100 000. Rose (1967) calculated age specific incidence rates for the Butterworth District of the Transkei (based on 151 cases seen between 1955 to 1964) by interpolation since the actual rates were given for age groups which are not directly comparable and suggested an incidence in men of 246.2 per 100 000. This figure was only slightly lower than the highest reported incidence from any part of the world in Gurjev in Kazakhstan (Doll, 1969). From the Bulawayo figures the comparable incidence in 1963-1965 was 125.8 (Doll *et al.*, 1970) and for Durban in 1964-1966, 98.9 (Doll, 1969).

Today, 49 years later after Burrell's first publication on OC (1957), this disease continues to impact on the quality of life of many people, with the former Transkei region still being considered the epicentre for this disease in South Africa. Population-based cancer registry data in this region have reported the highest incidence rates for the south of the Transkei region (Jaskiewicz, *et al.*, 1988a,b; Makaula *et al.*, 1996). Data regarding the incidence of OC during the period 1985-1990 were reported for all clinics and hospitals in four selected districts of Transkei, i.e. Centane (= Kentani), Butterworth, Lusikisiki and Bizana (Makaula *et al.*, 1996). This cancer was the most frequently recorded cancer in this region and accounted for 46.5% of the cases with mean ASIRs of 46.7 and 19.2/100 000 for males and females, respectively. The highest mean ASIR per annum in males (55.6/100 000) occurred in Centane and in females (22.3/100 000) in Lusikisiki, whereas the lowest rates in both sexes (37.0/100 000 and 11.7/100 000, respectively) occurred in Bizana. More recent data from this population-based cancer registry indicated mean ASIRs of 76.6 and 36.5/100 000 for males and females respectively (Somdyala *et al.*, 2003). These rates have been consistently higher than those reported for the USA black population; in Monetvideo, Uruguay and in Harare, Zimbabwe indicating that within the African continent, the former Transkei region is indeed a 'hotspot' for OC. This data also confirms a consistently high rate in the southwestern district of Centane during the past 35 years and progressively increasing rates in the northeastern districts of Bizana and Lusikisiki although much lower than those reported earlier for Gonbad city (Iran) and for Linxian and Cixian (China).

Pacella-Norman *et al.* (2002) also found that women born in the Eastern Cape Province were 6-times more likely to be diagnosed with OC compared to those born elsewhere in South Africa or outside the country. In relation to the duration of residence in this Province, women were once again observed to be at higher risk [1-34 years, OR=3.6 (95% CI 1.4 – 9.5); 35+ years (OR=14.7 (95% CI 4.7 – 46.0)] than men whose risk was 0.5 (95% CI 0.1 – 1.9) and 3.1 (95% CI 0.9 – 10.8) in the same two duration categories.

1.4 Risk Factors in the South African Population

Studies on cancer of the oesophagus in developed countries have consistently shown the aetiological importance of tobacco and alcohol, particularly in combination. However the geographical patterns in Africa also suggest that other factors play an important role. A few studies have been conducted in the Transkei region of the Eastern Cape Province of South Africa and in the Province of KwaZulu-Natal and it

has been shown that people at highest risk are generally poorer, have limited or restricted diets, and consume certain forms of alcohol and tobacco (smoked or chewed). A variety of risk factors has been proposed as a result of different sources of evidence (epidemiological, anecdotal, mechanistic) gathered within the affected regions and these are discussed below.

1.4.1 Tobacco and Alcohol

A substantial body of evidence gathered within South Africa and globally now show that tobacco smoke in addition to lung cancer can also cause cancer in many different organs including the oesophagus. Tobacco smoke is thus considered a multipotent carcinogenic mixture. It has been estimated that tobacco smoking is currently responsible for 30% of all cancer deaths in developed countries, and that if current smoking patterns persist, an epidemic of cancer attributable to tobacco smoking is expected to occur in developing countries (Peto and Lopez, 2001). Since publication of Monograph 38 of the International Agency for Research on Cancer (IARC) in 1986 (IARC, 1986), the evidence over the last 16 years was reviewed and the findings published in Monograph 83 (IARC, 2002a). At the 2002 IARC working group meeting on tobacco smoke and involuntary smoking, 35 case-control studies and 19 cohort studies were evaluated. Further evidence indicated that exposure to second-hand tobacco smoke (i.e. passive smoking by persons who do not smoke) is also carcinogenic.

In South Africa, long stemmed pipes were commonly smoked by both men and women in the former Transkei (Burrel, 1957). Cigarettes are however now replacing traditional methods of smoking (smoking of herbs) or are spreading where it was not formerly the custom to smoke. Lung cancer is following in their wake and has already become common in some of the bigger towns although it is still rare in most rural areas (Schonland and Bradshaw, 1968; Cook and Burkitt, 1971).

Superimposed against the background of poverty and poor nutritional status, the use of tobacco is highly evident. In 1965, Auerbach *et al.* demonstrated the damaging effects of smoking on the oesophagus similar to those found in the lung of deceased persons brought for biopsy at the Veterans Administration Hospital, East Orange, NJ, USA. They concluded that the histological changes seen in the basal squamous epithelial cells of the oesophagus of smokers were similar to those found in the trachea and bronchial tubes despite the fact that the bronchial tubes of a smoker are

directly exposed to inhaled smoke while the oesophagus is exposed to tobacco smoke products dissolved or suspended in salivary gland secretions. The experiment thus strengthened the proposed link between smoking and the development of OC.

Tobacco can be used in various ways in the former Transkei ranging from smoking either as a cigarette, in pipes or pipe tobacco rolled in paper. Smokers are also known to chew tobacco and rolled, dried tobacco leaves, while some may grind the leaves and place it in the mouth, or use it as snuff. Bradshaw and Schonland (1974) found the risk of developing cancer of the oesophagus was higher in males smoking pipes only, or cigarettes and pipes, than it was in those who smoked cigarettes only, or did not smoke at all. A ratio of 2.5 for the observed to expected number of cases was observed. These results were further strengthened by the 2002 IARC working Group where it was found that pipe smoking as well was strongly and causally related to cancers of the oral cavity, oropharynx, hypopharynx, larynx, lung and oesophagus (IARC, 2002a). The magnitude of risk was similar to that from cigarette smoking and a clear dose-response relationship was observed with the amount of tobacco smoked. Hence it is clear that independently of how it is smoked, tobacco is the causal agent.

In addition to urbanisation, the lifestyle changes for African blacks have also resulted in increased rates of smoking and alcohol consumption (Yach and Townsend, 1988; Segal *et al.*, 1988). Since the repeal of prohibitive alcohol legislation for blacks in 1961 there has been a steady shift among black drinkers from the exclusive consumption of traditional alcoholic beverages to the consumption of Western style drinks as well (Segal *et al.*, 1988).

The drinking of home-distilled spirit is common in parts of Africa but there has been no geographical evidence for an association with OC development, whilst locally this habit is not widespread (Cook, 1971). The drinking of beer made from local cereals and fruits, however, is widespread, and the distribution of maize and in particular beer made from maize is in many ways similar to the distribution of OC. Maize is an introduced crop in Africa and its spread as an ingredient of beer seems to correspond with the rise in the frequency of OC (Cook, 1971). Studies in many parts of the world have indicated that the consumption of alcoholic drinks can be a factor in the development of cancer of the oesophagus (IARC, 1988). However the mere quantity of alcohol consumed is not a sufficient factor to explain the enormous geographical variation in frequency throughout the world (Doll, 1969) and, moreover, areas of

moderate or high frequency such as India or Iran where alcohol seems to play no part in the development of OC (Wynder and Bross, 1961). Furthermore the consumption of traditional distilled spirits in areas such as the former Transkei has been less than in the urban areas of South Africa (Burrel, 1962) and there are areas such as southern and north-western Uganda where the drinking of home-made spirits is common but where the frequency of OC is low (Cook and Burkitt, 1971). Despite these anomalies however, there are sufficient pointers to alcoholic drinks in general to suggest that they could be carriers of a carcinogenic agent.

In Zambia and Malawi, it was shown that a geographical association existed between the occurrence of OC and the drinking of spirit made from maize husks and sugar (McGlashan, 1969). A negative association was also observed with the drinking of traditional beer made from millet. In the survey of the preparation and consumption of alcoholic drinks in Kenya and Uganda, the areas of high frequency of OC in west Kenya were found to be areas where maize beer is consumed, while the areas of low frequency in Uganda were areas of millet, sorghum, banana or honey beer (Cook *et al.*, 1971). Furthermore the use of maize for beer-making was found to be a recent custom, maize having replaced the traditional sorghum and millet beers of west Kenya. The association with alcoholic drinks made from maize in both Zambia and Kenya and the change to maize beer in western Kenya suggested, at that time, that a product of the fermentation of maize may be important in the development of OC. In South Africa the traditional indigenous grain, sorghum though no longer valued as a food was retained well into the present as an ingredient of beer, and sorghum continued to be grown especially for beer-making. In the former Transkei region, beer made from maize is consumed and a fermented porridge made from maize is eaten (Rose, 1967).

A number of studies in South Africa have highlighted the risk that alcohol and tobacco exposure poses on the development of OC. A case-control study examining 98 cases and 341 unmatched hospital controls (Bradshaw & Schonland, 1969) was analysed together with another study at Baragwanath Hopsital, Johannesburg where 196 male cases and 1064 age-matched controls were recruited (Bradshaw & Schonland, 1974). In both studies, OC was strongly associated with tobacco use, particularly with the use of pipe tobacco (OR in Durban=5.4, in Johannesburg=7.8, relative to non-smokers). Stratified by level of tobacco consumption, there was no significant effect of alcohol (traditional beer or western type liquors) on risk.

The hospital-based case-control study amongst the Zulu, comprising 211 OC cases with controls matched to each case by age and urban-rural background was undertaken in Durban, South Africa (van Rensburg *et al.*, 1985). An association was also found with smoking of commercial cigarettes (OR=2.6 for current smokers, 1.6 for past smokers) and pipe smoking (OR=2.1) but data for alcohol consumption was not shown.

In the Transkei, populations of the Nqamakwe and Butterworth districts, Mqanduli and Libode ditricts and Bizana and Lusikisiki as the 'high', 'medium' and 'low' incidence areas of OC respectively were compared with respect to prevalence of tobacco smoking (pipes) and consumption of different alcoholic beverages (McGlashan *et al.*, 1982). The correlation was stronger for tobacco smoking than for alcohol. In addition, a hospital-based case-control study also conducted in the former Transkei by Sammon (1992) comprising 100 cases diagnosed by barium swallow (approximately 60% of all OC admissions) and 100 controls matched for sex, age and educational status found a positive association with smoking (OR=2.6, 95% CI 1.5-5.0), but no relationship with the drinking of 'traditional' beer (OR=1.6, 95% CI 0.9-3.0). As an adjunct to this study, a further 30 cases and controls were interviewed in the same hospital and data combined (Sammon, 1998). Results once again have shown that smoking was associated with an increased risk (OR=2.69, 95% CI 1.45-4.98) whilst traditional beer did not present with a significant risk (OR 1.63, 95% CI 0.87-3.03) of developing OC.

A case-control study carried out by Pacella-Norman *et al.* (2002) at three hospitals in the greater Johannesburg area found that in males both ex- and current smokers had an increased risk of developing OC (OR=3.8 in both groups) whilst in females, the risk was higher in current smokers [OR = 3.1 (95% CI 1.7 – 5.4)]. No effect of snuff use was observed in both males and females. Frequent male drinkers were observed to have an almost two-fold increased risk [OR = 1.8 (95% CI 1.2 – 2.8)] whilst occasional and weekly drinkers did not present with an increased risk. Amongst females however an increased risk was observed in both weekly [OR=2.2 (95% CI 1.3-4.0)] and frequent drinkers [OR=1.7 (95% CI 1.0 – 2.9)]. The combined effects of tobacco and alcohol was shown to significantly increase the risk of OC in both men and women combined [OR=4.4 (95% CI 3.2-6.1)].

It can be concluded from the studies discussed above that smoking undoubtedly increases the risk of developing OC while the effect of alcohol in still unclear and needs further exploration.

1.4.2 Nutrition

1.4.2.1 Vitamins and Trace Metals

In many African populations, a restricted diet is considered as a major background factor associated with OC. Burrell (1957) noting that the Transkei was an infertile area where plants had a number of mineral deficiencies. In a comparison to areas of moderate and high risk of OC, persons living in areas of high risk had lower levels of vitamins A and C, magnesium and riboflavin (van Rensburg *et al.*, 1983). In the Transkei, populations with high OC rates appeared to eat maize as a staple eaten either as whole kernels, stamped or ground, which has low levels of niacin, riboflavin, vitamin C, zinc, calcium and magnesium. The anomaly here is that pellagra is associated with high intake of maize and acute oesophagitis but in the former Transkei no association with a history of pellagra was found (Sammon, 1992).

It has been seen that there is a broad coincidence between the areas where maize is the staple and areas in eastern South Africa, Zimbabwe, in south-eastern Zambia, southern Malawi and in central Kenya where OC is common. In West Africa where OC is virtually unknown, the traditional staples are yams, cassava, bananas and rice in the wetter areas and millet and sorghum in the drier zones towards the Sahara and Kalahari deserts. In western Kenya, staples such as millet and sorghum have now been replaced by maize which spread widely in the area from the 1930s (Wagner, 1956; Ominde, 1968). In Kenya as a whole, maize is now planted on half the cultivated land (O'Connor, 1966). It represents over 30% of the total food crop and in districts of western Kenya the proportion is as high as 60%. In South Africa maize began to spread widely as a foodcrop toward the beginning of the nineteenth century. In central Kenya the change to maize seems to have taken place between 1890 and 1910 (Morgan, 1967). In both areas however, the change was too early for the spread of maize as a food to be directly associated with the recent rise in the frequency of OC.

According to Sammon (1992) maize, beans, pumpkin, bread and margarine are the main caloric providers of the diet, which are supplemented by meat, eaten occasionally, garden vegetables and wild vegetables. The staple diet of maize,

pumpkin and beans may cause an underlying predisposition to carcinogenesis by tobacco or other plant carcinogens. The involvement of specific vitamin (vitamins A, B_{12}, E, folic acid) and trace element deficiencies (selenium) in the high-risk populations with a very poor socioeconomic status has also been considered an important factor (Jaskiewicz *et al.*, 1988a; Jaskiewicz *et al.*, 1988b). Table 2.1 indicates some of the biochemical parameters measured for populations in the former Transkei region.

Table 1.1. Some biochemical parameters measured in the population of the former Transkei compared to normal values. (Data from Jasklewicz *et al.*, 1988a,b)

Blood biochemical parameters	Former Transkei Region	Normal Values[a]
Folic acid (ng/ml)	242 – 307	210 – 980
Vitamin B_{12} (µg/dl)	227 – 366	220 – 750
Vitamin E (µg/dl)	3.7 – 5.3	> 6.0
Vitamin A (µg/dl)	25 – 40	20 – 70
Selenium (ng/ml)	58 – 69	112 – 210

[a]Nutrition Information Centre of the University of Stellenbosch (NICUS) (www.sun.ac.za/nicus) accessed November 2006.

In the hospital-based case-control study of Sammon (1992), cases reported more frequent use of traditional dietary staples than controls. In the case-control study in Durban, Natal (van Rensburg *et al.*, 1985), consumption of maize meal was a risk factor for OC (OR for daily consumers 5.7, weekly consumers 2.4, compared with those who consumed it less often). It has been suggested that chronic oesophagitis is a precursor of OC, but it is unclear under what circumstances acute oesophagitis develops into chronic oesophagitis.

Van Rensburg *et al.* (1985) also found that daily or week-end use of butter had a 49% (95% CI 0.37-0.69) and 29% (95% CI 0.52-0.97) reduced risk reduced risk of developing OC. The results of this study appear to lend support to the existence of a nutritional predisposition to OC associated with a dietary staple low in vitamins and minerals (Van Rensburg, 1981). Relevant to this is the identification of a set of micronutrients that tends to be deficient in high risk populations and that also significantly protect against experimentally produced oesophageal carcinogenesis in rats (Van Rensburg, 1982, Van Rensburg *et al.*, 1983). Daily use of commercial maize meal, which is low in magnesium, zinc, nicotinic acid and riboflavin was observed to have an almost 6-fold increased risk (OR=5.73, 95% CI 3.09-10.63).

Daily use of butter and magarine was suggested to contribute to the intake of fat soluble vitamins, particularly vitamin A. It was also suggested that as the African diets are exceedingly low in oils, any fatty acids could enhance immunological responses as well as absorption of other nutrients, including minerals.
Levels of zinc in distilled spirit from Zambia far above the recommended limit of safety for drinking water was found by McGlashan (1969), but evidence from animal experiments suggested that a deficiency rather than an excess of zinc causes damage to oesophageal tissue which might predispose to malignant change.

Various other sources of contamination have also been suggested, in particular lead, zinc, copper, or polyaromatic hydrocarbons from the old oil or asphalt drums sometimes used as containers during backyard fermentation or distillation, or from the discarded exhaust pipes which have been used as part of the distilling apparatus (Oettlé, 1964; Reilly and McGlashan, 1969; Rose, 1968, & McGlashan, 1969). Exposure to polyaromatic hydrocarbons have also been implicated as a possible risk factor in northeastern Iran (Kamangar *et al.*, 2005). Attention has also been drawn to additives such as metal polish, apparently included in some distilled liquors in South Africa to give extra flavour and strength (Burrell, 1957). Many of these however are features of the preparation of drinks in urban areas and cannot explain the very high frequencies of OC in rural areas such as the Transkei (Oettlé, 1964). A survey of the methods of preparation of alcoholic drinks in a range of areas of differing frequency in Kenya and Uganda showed marked regional variation in the use of containers made of clay or metal and in the use of copper piping, but the distribution patterns showed no association with the distribution of OC (Cook *et al.*, 1971). There was also no evidence for the use of exhaust pipes as part of the distilling apparatus or for the inclusion of exotic additives in the drinks consumed.

The discussions above lend support to the existence of a nutritional predisposition to OC associated with a dietary staple low in vitamins and minerals. Relevant to this is the identification of a set of micronutrients that tend to be deficient in high risk populations.

1.4.2.2 Mycotoxin Contamination

A number of ecological studies have addressed the relationship between exposure to *Fusarium* toxins and oesophageal cancer. Marasas *et al.* (1981) studied the prevalence of three *Fusarium* species and other fungi in home-grown maize

harvested in 1985 in the high OC incidence area of Centane and the low-incidence area of Bizana, both districts of the former Transkei region. The mean proportions of maize kernels infected with the fungus *F. verticillioides* in both healthy and mouldy maize samples from households in the high OC incidence area were significantly higher (42% in healthy and 68% in mouldy maize) than those in the low-incidence area (8% and 35%, respectively). Sydenham *et al.* (1990a) found high concentrations of various *Fusarium* mycotoxins in the same samples of mouldy home-grown maize collected during 1985. Significantly higher mean numbers of kernels infected with *F. moniliforme* and correspondingly higher levels of the mycotoxins fumonisin B_1 and fumonisin B_2 were also found in mouldy maize samples in the high-risk OC area than in the low-risk area ($p < 0.01$). Fumonisin B_1 and B_2 levels in healthy maize samples from the low-risk area were approximately 20 times lower than those in healthy samples from the high-risk area (Sydenham *et al.*, 1990b). However, because of the methodological difficulties encountered in these studies, the IARC working group concluded that there was inadequate evidence in humans for the carcinogenicity of toxins derived from *F. moniliforme* and that toxins derived from *F. moniliforme* were classified as "possibly carcinogenic to humans" (Group 2B) (IARC, 1993a). Recently fumonisin B_1 was also classified as a group 2B carcinogen by the International Agency for Research on Cancer (IARC, 2002b).

1.4.2.3 Use of Wild Plants

In the study conducted by Sammon (1992), it was observed that those reporting consumption of the plant *Solanum nigrum* had an increased association with OC. Those consuming this plant had a 3.6-fold increased risk of developing OC (OR=3.62, 95% CI 1.40 – 9.33). As an adjunct to this study, a further 30 cases and controls were interviewed and an analysis of the combined data still showed an increased risk of OC following intake of *S. nigrum* (OR=2.55, 95% CI 1.17-5.54). The possibility of a spurious association due to geographic distribution of the plant coinciding with the maximum incidence of the disease or by its better growth on soils deficient in molybdenum could not be ruled out (Schutte, 1966) although the chemical compound, solanide, an alkaloid contained in this plant has been shown to produce fetal malformations in hamsters. A study conducted by Purchase *et al.* (1975) has also shown that rats fed a diet consisting of maize, beans salt, *S. nigrum* and *Sonchus oleraceus* had, among other disorders, severe epithelial dysplasia of the oesophagus. The use of Xhosa medicines which are taken for a variety of ailments, some of which are used as emetics was also found to increase the risk of

developing OC (OR=2.08, 95% CI 0.92 – 4.66). The plant *Pteridium aquilinum* (L.) Kuhn, commonly known as the bracken fern, has also been associated with an increased risk of oesophageal and stomach cancer in humans. In Japan a 2.1 and 3.7-fold increased risk of developing oesophageal cancer was estimated for males and females respectively (Kamon and Hirayama, 1975). An association between bracken consumption (as food) and cancer of the oesophagus and stomach has also been noted in Brazil (Marliere *et al.*, 1995). In South Africa, this plant is used in rural regions of KwaZulu-Natal and the Eastern Cape Province to cure stomach ache, diarrhoea and as an antihelminthic (Hutchings, 1996). A correlation was also observed between the intake of plants and OC in the island of Curacao, the Netherlands (Morton, 1968). The indigenous dish, cadushi soup, made from the powdered flesh of the wild cactus, *Cereus repandus*, was consumed by 44 of the 49 oesophageal cancer patients. These studies lend support to further investigations especially in areas of the former Transkei where the consumption of plants or the medicinal use thereof is widespread.

1.4.3 Human Papillomavirus

Apart from the environmental and dietary factors discussed above, the role of viruses cannot be excluded. Oncogenic types of the human papillomavirus (HPV), most notably HPV 16 and HPV 18, are recognized as the most significant risk factors for cervical cancer (Bosch *et al.*, 2002), and are also important risk factors for cancers of the vulva, anus, penis, and oropharynx (Syrjanen, 2002 & 1987). This virus has also been implicated as one of the risk factors of oesophageal squamous cell carcinoma (ESCC) since the observation of condyloma-like lesions in ESCCs some 20 years ago (Syrjänen, 1982). Since the oral mucosa is in direct continuity with the oesophagus, it is not surprising that earlier reports indicated that this virus might be involved in the development of both benign (Syrjänen *et al.*, 1982) and malignant (Syrjänen, 1982) squamous cell lesions of the oesophagus as well. Morris and Price (1987) hypothesized that papillomavirus was an essential part of the carcinogenic process, causing a 'glycogenic acanthosis', a process favoured by a reduction in immune competence in malnutrition. To date approximately 73 different HPV genotypes have been described (Zur Hausen and De Villiers, 1994) of which the predominant type detected in ESCC is HPV 16 and 18.

Further evidence came to light when morphological studies showed similarities between HPV induced lesions in the genital tract and squamous cell papillomas and

carcinomas of the oesophageal mucosa (Syrjänen, 1982; Kashima and Mounts, 1987), which was subsequently confirmed by detecting the HPV antigens in squamous cell papillomas using immunohistochemical techniques. The advancement of technology such as DNA hybridisation and polymerase chain reaction (PCR) techniques also aided in identifying HPV DNA sequences in benign and malignant oesophageal lesions (Kulski *et al.*, 1986; Chang, 1990; Benamouzig *et al.*, 1992). Epidemiologic studies using type-restricted serologic assays have also showed inconsistent results (Kamangar *et al.*, 2006). While some serologic studies have found a positive association between ESCC and HPV16 (Dillner *et al.*, 1995; Bjorge *et al.*, 1997; Han *et al.*, 1996), others have found no association (Kamangar *et al.*, 2006; Lagergren *et al.*, 1999; van Doornum *et al.*, 2003). It can be seen that unlike cervical carcinoma in which an almost 100% association with HPV is reached, the causal role of HPV infection in the development of oesophageal SCC remains controversial partly due to the wide variations (0 – 60%) in reported infection rates among different studies (Syrjanen, 2002). The incidence of infection differs markedly depending on the geographic location of the patient population being studied.

The highest prevalence rates of HPV infections have been reported from China and South Africa (Sur & Cooper, 1998) both of which also have the highest rates of OC. Prevalence rates of 26-71% have been detected from South African patients with OC (Cooper *et al.*, 1995; Lavergne & de Villiers, 1999). An association between the HPV infection and OC was also observed by Hale *et al.* (1989) in South African blacks. It was found that 13/20 specimens from patients in Johannesburg who had undergone invasive squamous carcinoma of the oesophagus tested positive for morphological evidence of HPV infection. Upon further investigations using immunoperoxidase and modified Feulgen staining to detect the viral antigen, 15 specimens tested positive. Williamson *et al.* (1991) also reported that 10/14 patients with OC tested positive for HPV DNA either in tumour biopsies or in adjacent tissue. The wide range of HPV positivity has been suggested to the differences in the sensitivity of the various detection methods (Hendricks & Parker, 2002). The relationship between anti-HPV 16 IgG seropositivity and cancer of the oesophagus was also investigated by Sitas *et al.* (2007) as part of an ongoing cancer case-control study run in the two major tertiary hospitals in Johannesburg, South Africa. Sera from 369 OC cases were analysed and compared to 2055 controls. A statistically significant association between OC and increased anti-HPV 16 IgG antibody (Ab) levels was observed (OR for medium Ab levels 1.3; OR for high Ab levels 1.6, $p = 0.002$). Despite these South African studies, the results obtained from a large prospective study in Linxian, China

(Kamangar *et al.* 2006) show that HPV 16, HPV 18 and HPV 73 do not play a major role in the aetiology of OC.

In Iran, the prevalence of HPV 16 and 18 in tumour tissues from 38 OC cases and 38 biopsied tissues from endoscopically normal individuals were compared (Farhadi *et al.*, 2005). Five of the cases (13.2%) and none of the controls tested positive for the HPV 16 E6/E7 gene whilst only three of the cases (7.9%) and 5 controls (13.2%) tested positive for the HPV 18 E6/E7 gene. No sample was positive for both HPV16 and HPV18. A case-control study conducted in Linxian, China (Kamangar *et al.*, 2006) using prediagnostic serum from 99 cases of OC and 381 age- and sex-matched controls revealed that fewer than 15% OC cases tested positive for HPV16 or HPV18. The adjusted odds ratio for HPV16 seropositivity was 1.6 (95% CI 0.8 – 3.3) whilst the OR for HPV18 seropositivity was 1.0 (0.4 – 2.1) and hence did not support the major role of HPV16 or HPV18 in the aetiology of OC in Linxian, China. In addition to this study, a cross-sectional study was also conducted using cytologic and biopsy specimens from 702 adult inhabitiants of Linxian (Gao *et al.*, 2006). A multivalent HPV hybridization probe was used to determine the HPV infection status of the cytologic specimens and the endoscopic biopsies were used to classify each subject's oesohageal disease. HPV positivity was identified in 13% (61/475) of subjects without squamous dysplasia, 8% (8/102) with mild dysplasia, 7% (6/83) with moderate dysplasia, 16% (6/38) with severe dysplasia and zero (0/4) with invasive ESCC thus suggesting that HPV infection is not a major risk factor for ESCC in this high-risk population. The results from these various studies indicate that further exploration is required in this regard as the evidence still remains circumstantial.

1.4.4 Nitrosamines

Many nitrosamines have been shown to be highly carcinogenic, and several are specific to the oesophagus causing tumours at this site by whichever route they had been administered (Magee and Barnes, 1967). An ecological association was reported from the Transkei between the place of residence of OC patients and the occurrence of plants affected with a disease caused by molybdenum deficiency (Burrel *et al.*, 1966). This particular deficiency leads to an accumulation of nitrates in plants and these could combine with naturally occurring secondary amines to produce nitrosamines in foodstuffs. In a high OC risk area of Zambia nitosamine compounds seemed to occur in spirits distilled from sugar and maize husks (McGlashan, 1969). However subsequent analysis of spirit samples from areas of

high and low frequency in East Africa by gas-liquid chromatography and mass spectrometry showed no evidence for the presence of nitrosamines down to a level of 0.1 ppm (Cook *et al.*, 1971). In addition to this, a systematic review undertaken by Jakszyn and Gonzáles (2006) of the epidemiologic evidence from 11 cohort and 50 case-control studies from the period 1985 – 2005 revealed that there was insufficient evidence to associate the intake of nitrite and nitrosamines with the development of OC.

1.4.5 Mechanical Trauma

Other aetiological factors commonly invoked are those which cause mechanical trauma; excessively hot food and liquids swallowed down an oesophagus partially anaesthetized by home-distilled spirit (Burrel, 1957) and silica particles from the grinding stone used to prepare flour (Rose, 1968). However, whereas many of these may seem convincing, none of them account for the geographical distribution of OC throughout Africa nor for the distinctive features of the sex ratio and the changing frequency over time. Self-induced vomiting with or without the use of traditional emetics and a history of previous pellagra did not confer a risk (Sammon, 1992). A previous study by Van Rensburg *et al.* (1985) also exonerated the use of emetics or purgatives as a risk factor.

In other endemic areas such as China and Iran, chronic oesophagitis was shown to be quite widespread (Muñoz *et al.*,1982 and Crespi *et al.*, 1979). The study of Muñoz *et al.* (1982) has also shown the progression of 4 of 20 patients with dysplasia to OC suggesting that oesophagitis is a precursor to OC. According to Sammon (1992) a high prevalence of oesophagitis has not been shown in the former Transkei region although it cannot be ignored as high levels of oesophagitis have been shown to occur in other areas with South Africa where there is a high incidence of OC (Oettle *et al.*, 1976). Self-induced vomiting can result in regular assault on the oesophageal mucosa, bathing it in gastric and possibly duodenal juices and causing oesophagitis, however in Sammon's study (1992), no significant association was found. Gastrooesophageal reflux disease (GERD) has also been hypothesised as a risk factor but more so for adenocarcinoma of the lower part of the oesophagus and hence does not form part of this review.

1.5 Conclusion

The occurrence of such a wide range of incidence rates of OC in different populations worldwide suggests the influence of an environmental factor or cluster of factors that is region specific, but remains unclear as to which factors are solely responsible. The role of tobacco usage in OC development is highly evident and is concistent with other studies conducted in South Africa, although the role of alcohol as a potential risk factor still needs further exploration as evidenced by the conflicting results of the various studies discussed. The role of nutritional deficiencies also needs further epidemiological exploration especially in a population that is highly dependant on maize which lacks other essential elements. Deficiencies in micronutrients such as zinc, molybdenum, selenium, magnesium, riboflavin, nicotinic acid and vitamin A have been put forward as risk factors, based on experimental data and biochemical evaluation studies. The evidence from ecological studies for the role of mycotoxins in OC development is also poor. In addition, non-human primates (vervet monkeys; *Cercopithecus aethiops*) fed a diet containing culture material of the fungus *Fusarium verticillioides* over a period of 13 years have not shown evidence of OC development. An association between the use of certain wild plants in the diet and OC was found however there is no mechanisitic evidence to indicate that certain phytochemicals are carcinogenic. The theory that certain plant-based chemicals may be carcinogenic and contribute to the high incidence of OC also needs to be further explored through epidemiological and mechanistic (laboratory-based) studies. The role of nitrosamines and HPV infection as a risk factor for OC within the South African population also needs futher exploration due to the conflicting strengths of the evidence both locally and internationally. This review served to highlight that regional variations do exist and the plethora of sufficient and component causes can vary between populations.

CHAPTER 2: UNDERLYING PRINCIPLES AND OBJECTIVES

The incidence of OC is subject to considerable regional variation not just in South Africa but throughout the world making it an enigma for scientists. The several risk factors discussed in Chapter 1 have been associated with SCC of the oesophagus, however it is unlikely that the high rates observed in the former Transkei region are solely due to these factors, as they are also reported in many other low risk areas of the world. It has been noted that a relationship has always been found with smoking and to a lesser extent with alcohol consumption, and that this holds whether the incidence is low, as in England, Sweden and the United States; moderate as in India and France, or high, as in the former Transkei region (Parkin *et al.*, 2002). However these factors alone do not produce much disease (because of the low rates of OC – compared with, say, lung cancer – in regions where smoking and drinking are common and that they certainly cannot account for the very high incidence recorded in some parts of the world. Such is the case in parts of Iran where alcohol and probably cigarette smoking have negligible role as risk factors.

The results of epidemiologic studies have indicated that it is difficult to attribute OC to one cause rendering OC as an intriguing disease with an obscure aetiology. At present, based on the evidence from the studies discussed, it would seem that cancer of the oesophagus develops as a result of exposure to a number of factors, some of which may be universally common and some of which are only locally significant. From the South African studies, the role of tobacco can be regarded as a component cause however the wide geographical variation has given rise to a search for relevant local complements of other component causes. The relative contributions of these risk factors are likely to vary from one geographical location to another and is expected if the causal component is different.

The role of alcohol in OC risk has been conflicting with some studies showing moderate risk whilst others showing no significant risk within the high-risk population of the former Transkei. The mere quantity of alcohol consumed has not been sufficient to explain the enormous geographical variation in frequency locally and throughout the world. In addition the role of two wild plants on OC risk have been evaluated in two smaller studies but no mechanistic evidence has been provided as to their carcinogenic properties and whether other wild plants that are consumed and used as traditional medicines also possess carcinogenic phytochemicals that increase the risk of developing OC. This is an area that requires further exploration

since the use of wild plants as dietary supplements or for medicinal purposes occurs frequently where it is said to play a vital role, as it is the first and sometimes the only resource of people living in rural areas where modern health care facilities are not easily accessible. Furthermore, the services and advice given by indigenous practitioners are valued because they are given in terms that patients can understand and in the context of cultural values and practices that are shared by both patients and healers alike.

The link between OC and mouldy maize consumption and mycotoxin exposure has been made from ecological correlation studies and needs to be studied further using a more rigorous study design. The low intake of fresh fruit and vegetables has been shown to be strong risk factors (Sammon, 1992), however the geographical patterns also imply that other factors play an important role. The involvement of specific vitamin (vitamins A, B_{12}, E, folic acid) and trace element deficiencies (Se, Mo) in the high-risk populations with a very poor socioeconomic status has also been considered important factors (Jaskiewicz *et al.*, 1988a, 1988b, Marasas, 1994) however the picture is not entirely clear and there is need to clarify, confirm and disentangle these effects. Furthermore there needs to be further investigations to measure the relative importance of different alcoholic drinks within the former Transkei region. The role of potential contaminants within these beverages also needs further exploration.

Consequently one important objective (Part 1) of this work is to conduct a more systematic and larger study which is designed to:

1. Confirm the local component causes at work for OC risk and,
2. To investigate other (new or suspected) possible component causes of OC specifically related to the use of local dietary and medicinal plants.

The literature shows that while some studies were properly conducted epidemiologic studies, many others were a combination of ecological studies, mechanistic studies elucidating causal mechanisms, anecdotal evidence and the like.

Consequently a second important objective (Part 2) of this work is to conduct various substudies to elucidate potential causal mechanisms and in this way attempt to provide a biological basis for the statistical associations that might arise from the epidemiologic study in Part 1. These substudies will investigate dietary and medicinal

plant exposures in their ability to cause cancer in general and by implication to cause OC.

Hence the objectives of this study are:

1. To conduct an epidemiological study to investigate some of the likely component causes of OC to either confirm known or suspected causes (eg. tobacco smoking and alcohol consumption) and to investigate new causes in particular reported dietary and medicinal wild plant usage and,

2. To enhance the epidemiological findings with systematic mechanistic (i.e. biological) evidence of dietary and medicinal plant exposure in relation to carcinogenicity.

This project combined the disciplines of epidemiology, chemistry and biochemistry to bring about an understanding in the relationship between traditional habits and OC, as well as the burden of OC in the Eastern Cape Province. This multidisciplinary approach is consistent with international trends in public health research as it makes possible the establishment of biological plausibility through laboratory-based investigations, and throws light on public health interventions.

Part 1 of this study begins with Chapter 3 which describes the epidemiological methods and study design used to undertake the study. Selection criteria, data analysis methodologies and ethical considerations have been described. Chapter 4 discusses the demographic information obtained from the epidemiological data analysis while chapter 5 discusses the role of tobacco smoking and alcohol consumption on OC risk. Chapter 6 provides evidence for the role of diet on OC risk whilst chapter 7 discusses the the association between wild plant intake and OC.

Part 2 of the study begins with Chapter 8 which discusses the *in-vitro* evidence of mutagenicity for the various plants evaluated in Chapter 7. Chapters 9 and 10 discuss the role of trace elements and mycotoxin contamination in these plants. Chapters 8 – 10 attempts to provide biological plausibility for the epidemiological evidence provided for wild plant intake on OC risk as discussed in Chapter 7.

Chapter 11 provides a short overview on the key findings as well as the strengths and limitations of this study.

PART 1

CHAPTER 3: SUBJECTS AND METHODS

3.1 Study Design

This study was a multi-centre hospital-based case-control study with incidence density sampling, a schematic of which is provided in Figure 3.1.

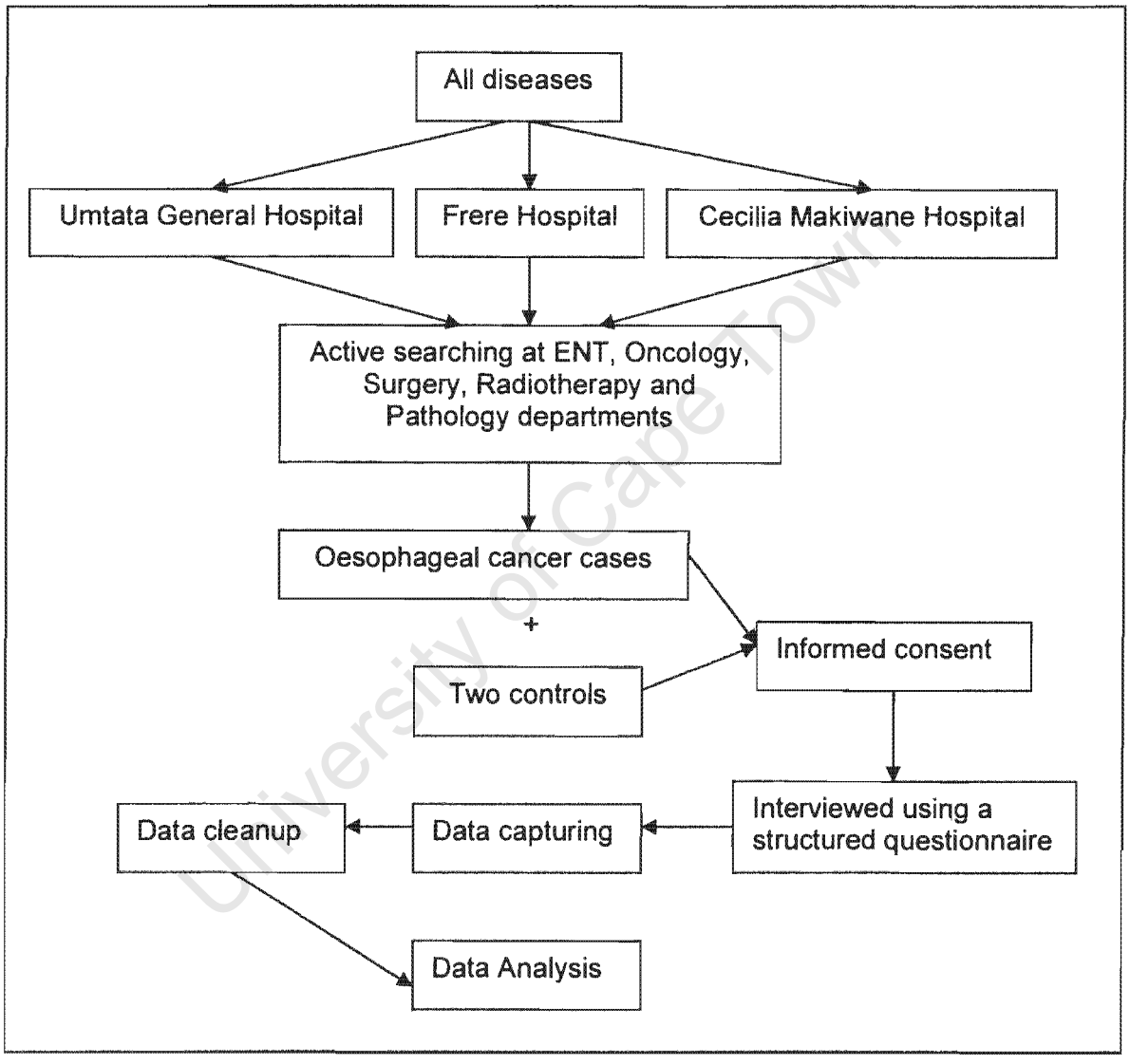


Figure 3.1: Patient recruitment and data collection procedure for this study

Incident OC cases were preferred for several reasons in comparison to prevalent cases. Firstly the time of disease onset is closer to the time of aetiological exposure. Thus an incident case is likely to recall prior exosures better than a prevalent case the experience or exposures under evaluation. Similarly, recent medical records were more likely to be available than older records. Furthermore, a series of incident OC cases may not have been acted upon by the determinants of survival whereas a

series of prevalent cases will. That is, the OC cases prevalent at any point in time are the survivors of a larger series of incident OC cases diagnosed in a preceding period. If the incident and prevalent cases differ with respect to risk factors, the use of prevalent cases would give an erroneous result. A third advantage of incident cases is that the effects of OC are less likely to appear as causes. If a case has been prevalent for several years he/she may have changed their environment or lifestyle in a number of ways. Hence, unless care is taken to restrict inquiry to the pre-morbid circumstances, a false exposure characterisation will occur. A final advantage of incident cases is that they relate more directly to the usual objective of this aetiological investigation; i.e., with incident cases it will be possible to evaluate the way in which the exposures relate to the underlying incidence rate of OC and not to its prevalence.

3.2 Setting

This study was undertaken at three of the major hospitals in the Eastern Cape Province of South Africa. Much of the Province consists of rolling grasslands and agricultural practices, mainly the cultivation of corn, serve as the major source of food for survival. The majority of the residents are black Xhosa-speaking Africans and recent statistics have confirmed a high incidence of OC among the population. Data from the CENSUS 2001 (StatsSA 2001) indicate that this Province has a total population of 6 436 764 of which 5 635 079 (87.5%) are South African black. The participating hospitals were Umtata General hospital, Frere hospital and Cecilia Makiwane hospital and these hospitals were the sources of patients and it was expected that in excess of 80% of all OC patients diagnosed with OC and attending public hospital facilities would be captured in either one of these three hospitals.

3.3 Study Population

The study population was patients attending any of the three referral hospitals stated above. These hospitals have a catchment area that cumulatively covers approximately 80% of the population of this Province.

3.4 Selection of Interviewers

The interviewers selected for this study were appropriately qualified with either nursing or social science degrees, and familiar with qualitative research methodology. The interviewers were also fluent in the Xhosa language.

3.5 Sample Size Calculation

Sample size was calculated according to the method by Lwanga and Lemeshow (1991) as follows:

Anticipated probability of "exposure" for people with the disease (P1*):	36.65%
Anticipated probability of "exposure" for people without disease (P2*):	30%
Anticipated odds ratio:	1.35
Level of significance (α):	5%
Power of the test (1-β):	80%
Ratio of case-to-control	1:2

For a 2-sided test :

$$n = \{ Z_{1-\alpha/2} [2 P2^* (1 - P2^*)]1/2 + Z_{1-\beta} [P1^* (1 - P1^*) + P2^* (1 - P2^*)]1/2 \}2/(P1^* - P2^*)2$$

Therefore:

Sample size in case group:	609
Sample size in control group:	1218

3.6 Selection of Cases

In the time period between November 2001 and February 2003, all patients with incident cases of histopathologically, radiologically or endoscopically confirmed squamous cell carcinoma of the oesophagus were considered eligible for this study. In the presence of a strong clinical suspicion, a case was interviewed before a diagnosis was established, pending subsequent confirmation. Early identification was possible through active searching by means of periodic visits to the hospital departments where cases were diagnosed or treated (i.e., ENT, oncology, surgery, radiotherapy and pathology). The frequency of the visits (daily, weekly etc.) depended on the cancer burden of each department. During data collection, patients were required to be of sufficiently good physical and mental health to give reliable answers to the questionnaire. Recruitment into this study further required that patients lived in the Eastern Cape Province for at least 5 years prior to diagnosis. Only subjects directly interviewed by the interviewers were included in the study. A total of 670 cases (334 males and 336 females, ratio 1:1) were identified. During this period, the response rate was 98%.

3.7 Selection of Controls

Controls were identified from in-patients (medical and/or surgical wards) and out-patients of the same hospitals as cases and were frequency matched to the expected

sex and age distribution of the cases. Controls were admitted to the same hospital and during the same period as the cases for diseases or conditions not related to upper gastro-intestinal diseases and diet. Hence a combination of incidence density sampling with frequency matching (with respect to sex and age group) was undertaken. For inclusion, controls also had to have resided in the Eastern Cape Province for a minimum period of 5 years. For each eligible case, the target was to recruit 2 controls. The controls also had to be in sufficiently good physical and mental health to give reasonably reliable answers to the questionnaires. If an eligible control refused to participate, a replacement with the same matching criteria given above was used. A total of 1188 controls (621 males and 567 females) were recruited into the study. The main diagnostic categories among controls are listed in Table 3.1. The response rate for controls was 96%.

Table 3.1. Diagnostic categories among control patients

ICD 10 code	ICD 10 group	Male	Female	Total	%
A05-B91	Certain infectious and parasitic diseases	52	39	91	7.7
C08-C64	Malignant neoplasms in areas other than the oesophagus	0	4	4	0.3
D10-D36	Benign neoplasms	13	20	33	2.8
D39-D48	Neoplasms of uncertain or unknown behaviour	8	13	21	1.8
E03-E66	Endocrine, nutritional and metabolic diseases	6	17	23	1.9
G03-G93	Diseases of the nervous system	20	14	34	2.9
H10-H57	Diseases of the eye and adnexa	34	27	61	5.1
H61-H93	Diseases of the ear and mastoid process	1	6	7	0.6
I05-I85	Diseases of the circulatory system	24	25	49	4.1
J01-J93	Diseases of the respiratory system	64	48	112	9.4
K00-K91	Diseases of the digestive system	81	85	166	14.0
L02-L98	Diseases of the skin and subcutaneous tissue	48	66	114	9.6
M00-M99	Diseases of the musculoskeletal system and connective tissue	14	19	33	2.8
N05-N95	Diseases of the genitourinary system	79	39	118	9.9
Q66	Congenital deformities of feet	0	1	1	0.1
R00-R69	Symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified	91	72	163	13.7
S02-T91	Injury, poisoning and certain other consequences of external causes	78	63	141	11.9
X20-Y87	External causes of morbidity and mortality	3	8	11	0.9
Z30-Z61	Factors influencing health status and contact with health services	5	1	6	0.5
	Total	621	567	1188	100

3.8 Interviews and Data Collection

Study subjects were interviewed using a structured questionnaire designed to elicit self-reported information retrospectively on the following:

(1) demographic and socioeconomic characteristics (residence and education),

(2) history of smoking (duration, number of cigarettes per day, type of cigarette),

(3) history of tobacco chewing (duration) and snuff use,

(4) history of alcohol drinking (type of alcoholic beverage and amount of each consumed and frequency of alcohol consumption),

(5) a food-frequency questionnaire which assessed the dietary patterns of 10 food items representative of the usual diet of the local Xhosa population. The food items were:

i. Maize
ii. Sorghum
iii. Wheat-based products
iv. Green leafy vegetables
v. Green podded vegetables
vi. Imifino (a collective name for dietary wild plants)
vii. all fruits
viii. Beans
ix. Meat
x. Pickled food

This part of the questionnaire was modified from that used by Pacella-Norman *et al.* (2002) to allow for national comparability.

The second part of the questionnaire focused on each of the wild plants used as dietary supplements and traditional medicines. Participants were asked to identify the plants used from a list and to indicate how often per week, they consumed the plants they had identified. From the list of the plants, they were asked to identify those plants eaten consistently over the last five years and to subsequently include ones that have been eaten but were not currently consumed. Information on the medicinal plants also included the reason for use, where obtained, and the form in which they were used. The same interviewer interviewed each case and the corresponding two controls and was unblind as to the case-control status. The interviewers were familiar with the local customs and traditions and conducted the interviews in the local African language (isiXhosa). Tables 3.2 and 3.3 provide the traditional and botanical names of the plants used for dietary and medicinal purposes respectively. The Xhosa and

Zulu names were indicated on the questionnaire so as to prevent any misclassification since the same plant can be known by various ethnic names. All the names by which each plant was known within the Eastern Cape Province was indicated on the questionnaire. The questionnaire was initially piloted amongst residence of the Eastern Cape Province and certain questions rephrased.

Table 3.2. Plants commonly used as dietary supplements

Common name	Botanical name	Family
idolo lenkonyana (X)	*Rumex lanceolatus* Thunb.	Polygonaceae
idumbe (lomfula), idumbi (Z)	*Colocasia esculenta*	Araceae
iGuzu (X)	*Physalis viscosa* L.	Solanaceae
imbabazane, uralijane (X)	*Urtica urens* L.	Urticaceae
imbilikikane (X), iMbicane	*Chenopodium album* L.	Chenopodiaceae
inlaba (X)	*Sonchus oleraceus* L.	Asteraceae
intebe (X, Z)	*Zantedeschia aethiopica* (L.) Sg	Araceae
isinama (X)	*Amaranthus asper* L.	Amaranthaceae
isiqwashumbe (X)	*Raphanus raphinastrum*	
lwatane (X)	*Raphanus nasturtio aquatica* L.	Brassicaceae
isithate (Z)	*Oxalis corniculata* L.	Oxalidaceae
umdiza wethafa (Z)	*Sida dregei* Burtt. Davy	Brassicaceae
umhlabangubo (X), uqadolo (Z)	*Bidens pilosa* L.	Asteraceae
umsobo wehlati (X)	*Solanum nodiflorum*	Solanaceae
umsobosobo, umsobo wenja (X), umsobo (Z)	*Solanum nigrum*	Solanaceae
Umtyutyu, unodlomboyi (X), umbhido (Z)	*Amaranthus hybridus* L.	Amaranthaceae
uNongotyozana, nonyongwana, inyongo (X), icukudwane (Z)	*Centella asiatica* L.	Umbelliferae
uqupose (X), imbuya (Z)	*Amaranthus thunbergii* Moq.	Amaranthaceae
uselwa-iwenyoka (Z)	*Coccinia rehmanii* Cogn.	Cucurbitaceae
uvevane (X, Z)	*Sida rhombifolia* L.	Malvaceae

X = isiXhosa name
Z = isiZulu Name

Table 3.3. Plants used as traditional medicines

Common name	Botanical name	Family
iGqwaka (X), umhlawazizi, umhlawazi (Z)	*Catha edulis* (Vahl.) Forsk ex Endl.	Celastraceae
ikhwane (X)	*Mariscus congestus* (Vahl.) C.B.Cl.	Cyperaceae
Imbilikikane (X), iMbicane	*Chenopodium album* L.	Chenopodiaceae
imPepha (X), impepho	*Helichrysum cymosum* (L.) D.Don	Asteraceae
iNdlebe ye-bokwe (X), amanzemnyama, ishwaga (Z)	*Pelargonium sp. cf. inqinans* (L.) L'Herit	Geraniaceae
iNxinene (X), inhlungunyembe (Z)	*Acokanthera oppositifolia* (Lam.) Codd	Apocynaceae
inzininiba (X), umsuzwane (Z)	*Lippia javanica* (Burm. F.) Spreng.	Verbenaceae
itolofiya (X)	*Opuntia vulgaris* Mill.	Cactaceae
uBobo (X)	*Dalbergia obovata* E. Mey.	Fabaceae
Ubuhlungu bethafa (X), isihlungu (Z)	*Teucrium reparium* Hochst.	Labiatae
Ubuvima (X), ibuvimba (Z)	*Withania somnifera* (L.) Dun	Solanaceae
uhlololwane (X), idolo-lenkonyane-elimhlophe, uhladlwana olukhulu (Z)	*Hypoestes aristata* (Vahl) Soland. Ex Roem. & Schult. Var. alba Balkwill	Acanthaceae
umAsibele (X), umBangabanga, uAasibele, idololenkawu, umaSibe, umAsibe, uMasibele (Z)	*Deinbollia oblongifolia* (E. Mey. ex Arn.) Radlk.	Sapidaceae
umathunga (X)	*Eucomis autumnalis* (Mill.) Chitt.subsp. autumnalis	Amaryllidaceae
umayime (X)	*Brunsvigia sp.*	Amaryllidaceae
umbhanga bhanga (X), uboqo, ugwanya (Z)	*Solanum mauritianum* Scop.	Solanaceae
umbhewe, umhlashosana (Z)	*Pteridium aquilinum* subsp. *aquilinum*	Dennstaedtiaceae
umHlaba (X), uNomaweni, iKhala (Z)	*Aloe Ferox* Mill.	Aloeaceae
umHlonyane (X)	*Artemisia afra* Jacq. Ex Wild	Asteraceae
umnyamanzi (X), umtoli, umNgamanzi (Z)	*Acacia caffra* (Thumb.) Willd	Mimosaceae
umTentekwane (X), iNtenendkiwane, inTentekiwane, isiThende, isidhende (Z)	*Maesa lanceolate* Forsk. var. rufescens	Myrsinaceae
umThombe (X), inTendekwane (Z)	*Ficus craterostoma* Warb. Ex Mild Br. & Burr.	Moraceae
umuNga (X)	*Acacia karroo*	Mimosaceae
umvumbangwe (X)	*Datura stramonium* L.	Solanaceae
umyama (X), umnama, umZungulwa (Z)	*Maytenus acuminata* (L.f) Loes	Celastraceae
uNongotyozana, nonyongwana, inyongo (X), icukudwane (Z)	*Centella asiatica* L.	Umbelliferae
uSingalamaxegwazana (X), umkhokha wehlathi, umkoka (Z)	*Convolvulus farinosus* L.	Convolvulaceae
uTywala bentaka (X), ubukhwezane, uguguvama, umphema (Z)	*Lantana rugosa Thunb.*	Verbenaceae
u-vuma (X), umlahlama, umhlatholan (Z)	*Turraea floribunda* Hochst	Meliaceae
uxobo (X, Z), uklenya (Z)	*Gunnera perpensa* L.	Gunneraceae

X = isiXhosa
Z = isiZulu

3.9 Data Cleaning

Records were removed from the database if they did not comply with the selection criteria for the study. Each of the variables under study was checked for range and consistency in order to identify possible inadmissible values. For example, males were coded "1" and females "2", therefore for the variable "sex", values other than 1 or 2 were flagged as errors and re-checked against original data. The data was checked using the "tab" and "summarize" commands in STATA.

3.10 Data Analysis

The data was first analysed using descriptive statistics. Sociodemographic variables such as age and years of education were converted to categorical variables. The study characteristics and distribution of age and sex for cases and controls at each hospital were tabulated. Categorical variables were summarized using frequency tables. None of the exposure-related variables were analysed as continuous variables but either as dichotomous or polytomous variables. The rationale for this categorisation was that analysis of exposures as continuous variables assumes a linear trend due to the nature of the model. However certain groups of individuals within certain exposure categories may be more susceptible to developing OC.

Potential risk factors for OC were divided into groups as listed in Table 3.4. Smokers were classified into three groups, i.e. Never, past and current smokers. The quantity of tobacco smoked was estimated in pipes, commercial and hand-rolled cigarettes assuming weights of 1g for commercial (Pacella-Norman *et al.*, 2002) and hand-rolled cigarettes and 3.5 g for pipes (Appel *et al.*, 1990). The total tobacco usage (in grams per day) was subsequently calculated. For alcohol-related variables, the quantities of each of the alcoholic beverages, i.e. Maize, sorghum, and commercial beers, home-made and commercial spirits as well as wine, consumed per week was calculated. The quantity of ethanol (grams per week) for each beverage type and total ethanol consumption was then calculated using the approximate label amounts for each of the beverages. Drinking patterns among alcohol drinkers were evaluated.

The frequency of consumption of each food item representing a typical isiXhosa diet was tabulated for the cases and controls. These comprised maize, sorghum, wheat, green leafy vegetables, green podded vegetables, wild greens (traditionally called imifino), fruit, beans, meat and pickled food.

The measures of the strength of association between the different exposure variables and OC were thereafter obtained by calculation of odds ratios (ORs) with 95% confidence intervals as a direct estimation of the relative risk (RR) from unconditional multiple logistic-regression models (Breslow and Day, 1980) using STATA Statistical Software package (Stata Corp., College Station, Texas). The key results were subsequently repeated on a restricted series of cases with histological confirmation.

3.11 Modelling Approach

The following modelling approach was used to examine the adjusted effects of variables of interest controlling for the other relevant variables. The tobacco and alcohol-related variables were reduced to two new variables viz. total tobacco used (grams/day) and total ethanol consumption (grams/week). Firstly all models were adjusted for age, hospital of admission, urban/rural status and years of education. Each of the tobacco-related variables (Table 3.4) were thereafter further adjusted for total grams alcohol consumed per week whilst the alcohol-related variables were further adjusted for total grams tobacco smoked per day. With respect to diet, each of the variables listed in Table 3.4 were furtheer adjusted for total grams tobacco smoked per day and total grams alcohol consumed per week. Adjusted odds ratios were also obtained for each of the medicinal and dietary wild plants being investigated after further adjustment for total tobacco usage and total alcohol consumption.

3.12 Principal Component Factor Analysis

Principal component factor analysis was subsequently undertaken on the dietary variables in an attempt to explain the dietary patterns in terms of a smaller number of factors and hence elucidate the relationship between these patterns and the development of OC. Nine food items were retained for factor analysis. The analysis was conducted using the factor procedure in STATA (STATA version 8, Stata Corp., College Station, Texas). Nine principal components were derived from the analysis of which only three were selected based on their variance. The three principal components cumulatively accounted for 68% of the variation in the data. Factor loadings were subsequently determined for the three principal components. Factors were rotated by the varimax method to obtain a simpler structure with greater interpretability (Jacobson & Stanton, 1986). In determining the number of factors to retain, we considered eigenvalues equal to or closest to 1. Factor 1 had a high positive loading for sorghum, green leafy vegetables, green podded vegetables, fruit and meat. Factor 2 had high positive loadings for maize, imifino and beans while

factor 3 had a high loading for wheat-based products. These 3 dietary patterns all contained elements of an isiXhosa diet common in the population studied and were labeled as dietary pattern 1 (sorghum, fruit, grean leafy and podded vegetables, meat), dietary pattern 2 (maize, imifino, dry beans) and dietary pattern 3 (wheat-based products). The last stage of the analysis involved calculating factor scores for each of the individuals which were then used as indices of dietary patterns among the population. Each dietary pattern was subsequently grouped into tertiles resulting in three categorical variables viz. low, moderate and high intake according to the distribution among controls. ORs for these patterns were fitted using the same model described earlier. This is discussed further in Chapter 6.

Table 3.4. Risk factors investigated in this study

TOBACCO SMOKING	ALCOHOL DRINKING	DIET	DIETARY SUPPLEMENTS	TRADITIONAL MEDICINES
1. Smoking status 2. No. of cigarettes smoked per day 3. No. of hand rolled cigarettes smoked per day 4. No. of pipes smoked per day 5. Total tobacco used (grams per day) (all smokers) 6. Total tobacco used (grams per day) (current smokers) 7. Total tobacco used (grams per day) (past smokers)	1. Alcohol consumption status 2. Frequency of consumption (per week) of: ▪ Maize beer ▪ Sorghum beer ▪ Commercial beer ▪ Commercial spirits ▪ Wine 3. Quantity consumed per week: ▪ Maize beer ▪ Sorghum beer ▪ Commercial beer ▪ Commercial spirits ▪ Wine 4. Grams alcohol per week 5. Total alcohol consumption (grams per week)	1. Maize 2. Sorghum 3. Wheat 4. Green leafy vegetables 5. Green podded vegetables 6. Imifino (wild greens) 7. Fruit 8. Beans 9. Meat 10. Pickled food 11. Source of: ▪ Maize ▪ Sorghum ▪ Wheat 12. Mouldy Maize consumption	User status of each plant as per Table 3.2.	User status of each plant as per Table 3.3.

For polytomous exposure variables, the likelihood ratio test was undertaken to determine whether there was a significant association between exposure and OC. This test for a multi-level exposure variable (with m levels) will have (m-1) degrees of freedom and is recommended since it has the property that it is unaffected by transformations of the parameter of interest. Tests for linear trend were performed by using the midpoint of each category of the variable in the model as a continuous variable so as to obtain the P-value for linear trend. An α-value of 0.05 was used as the indicator of statistical significance. All P-values were derived from 2-sided statistical tests.

3.13. Determination of Effect Modification

The combined effect of smoking and alcohol consumption was investigated. An interaction term, with 9 levels, was created between each category of tobacco usage (grams/day) and each category of alcohol consumption (grams/week) as shown in Table 3.5. Never-smokers who were also never-drinkers were used as the reference category. Effect modification was determined by calculating the likelihood ratio test statistic comparing the models containing the main effects and the main effects plus interaction terms.

Table 3.5. Cells created to show the joint effects of tobacco and alcohol ***(Each cell contained the OR and 95% CI for a combination of tobacco and alcohol exposures between the categories)***

Tobacco use (g/day)	Alcohol consumption (g/week)		
	0 Cases/Controls	0.5-370 Cases/Controls	371+ Cases/Controls
0	1 (reference)	2	3
1-14	4	5	6
14.1+	7	8	9

3.14. Population attributable proportion

Population attributable proportions due to smoking and alcohol consumption were calculated to determine the relative importance of these two risk factors for the population. This estimate is the percentage of cases that would not occur if tobacco or alcohol were removed from the population. Firstly, the prevalence of exposure to each of the two risk factors was calculated in the controls, on the assumption that these would give a reasonable distribution of the prevalence of a given exposure. This was done using the following equation:

$$Pe = \frac{number\ exposed}{total\ number} x100 \qquad (1)$$

Using the RR obtained from the logistic regression model, the following equation was used:

$$Population\ attributable\ proportion = \frac{Pe(RR-1)}{Pe(RR-1)+1} \qquad (2)$$

3.15 Ethical considerations

The study was approved by the Ethics Committee of the Medical Research Council, the Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town and Bisho Ethics Committee of the Eastern Cape Department of Health. Patients were informed of the purpose and nature of the study, the procedures involved, the benefits intended to result from the study and their right to refuse or withdraw at any time, without compromising the quality of care that they would receive at the hospital. Informed consent was obtained from each patient eligible for the study by having them sign or place their thumb print on the patient consent form. For patients that were illiterate, in addition to the thumbprint, a witness unrelated to the study was required to sign. Research staff were also be required to sign a confidentiality agreement prior to working with hospital records.

CHAPTER 4: EPIDEMIOLOGY SUMMARY STATISTICS

4.1 Summary Statistics

Data from 670 incident cases of squamous cell carcinoma of the oesophagus and 1188 controls were analysed in this study. The control-to-case ratio was 1.9 for males and 1.7 for females. 32%, 35% and 33% of the patients were recruited from Umtata General, Frere and Cecilia Makhiwane hospitals, respectively. Of all the eligible cases, 36% were diagnosed by histology, 60% by radiology and 4% by endoscopy. The middle third region of the oesophagus was the most frequent tumour location with 56% of the patients presenting, whilst 20% and 16% presented with tumours in the lower and upper third of the oesophagus respectively. The information with regard to tumour location on the remaining patients was either missing or unspecified. The distribution of the cases and controls according to sociodemographic variables were summarised using frequency tables, the results of which are presented in Table 4.1. The male to female ratio for the cases is 1:1, similar to that reported by Islami *et al.* (2005) in Golestan Province, Iran.

Table 4.1 Distribution of cases and controls according to sociodemographic variables.

Variable	Categories	All hospitals	
		Cases (670)	Controls (1188)
		No. (%)	No. (%)
Age (years)	≤44	52 (8)	107 (8)
	45-54	125 (19)	225 (19)
	55-64	209 (31)	400 (33)
	64-74	197 (29)	318 (28)
	75+	87 (13)	138 (12)
Sex	Male	334 (50)	621 (52)
	Female	336 (50)	567 (48)
Residence	Urban	416 (62)	826 (70)
	Rural	254 (38)	362 (30)
Education (years)	None	208 (31)	260 (22)
	1 to 7	260 (39)	430 (36)
	8 and more	160 (24)	427 (36)
	Missing	42 (6)	71 (6)

Age and years of education were converted into categorical variables. The table indicates that most of the cases were in the 55 – 64 year age-group and a larger proportion of cases (38%) than controls (30%) were from rural areas.

Table 4.2 summarises the odds ratios of OC (adjusted for hospital only) associated with the sociodemographic variables. It can be seen that with increasing years of education, a protective effect was observed. Males and females having 8 or more years of education had a 50% and 57% reduced risk of developing OC respectively (males OR = 0.5, 95% CI 0.34-0.73; females OR = 0.43, 95% CI 0.30-0.62). In addition, rural residents were more likely to develop OC than urban residents, with a significant association being observed only among males (OR = 1.56, 95% CI 1.18-2.07).

Tale 4.2 Odds ratios of oesophageal cancer associated with sociodemographic variables.

Variable	Categories	[a]Odds ratio (95% CI)	
		Males	**Females**
Age (years)	≤44	1.00	1.00
	45-54	1.39 (0.76-2.52)	0.97 (0.57-1.67)
	55-64	1.08 (0.61-1.93)	1.10 (0.68-1.80)
	64-74	1.45 (0.81-2.60)	1.18 (0.71-1.95)
	75+	1.47 (0.77-2.79)	1.23 (0.69-2.21)
		[*]p=0.34	p=0.86
Residence	Urban	1.00	1.00
	Rural	1.56 (1.18-2.07)	1.22 (0.92-1.64)
		p=0.0005	p=0.17
Education (years)	None	1.00	1.00
	1 to 7	0.90 (0.63-1.27)	0.63 (0.45-0.88)
	8 and more	0.50 (0.34-0.73)	0.43 (0.30-0.62)
		p=0.0004	P=0.0001

[a]Odds ratio adjusted for hospital only
[*]p-value obtained from likelihood-ratio test, P≤0.05 indicates a significant association between sociodemographic variables and oesophageal cancer.

CHAPTER 5: TOBACCO AND ALCOHOL EXPOSURE

5.1 Effect of tobacco-related variables on OC risk

5.1.1 Smoking status

Tables 5.1 and 5.2 show the estimated effects of tobacco-related variables on OC risk in males and females respectively. Two risk estimates have been presented, one after adjustment for age, hospital, residence and years of education and the second after further adjustment for total grams ethanol per week. All aspects of tobacco exposure have been found to be significantly related to risk and a monotonic dose-response was observed across the categories in all of the tobacco-related variables. Amongst males, current and past smokers were observed to have a 2.4 and 5-fold increase in risk compared with non-smokers. Current and past smokers accounted for 82% of males. It must be acknowledged that the number of males reporting as current smokers was low and a possible reason for this may be that many of them may have stopped smoking due to symptoms of OC.

In females, current and past smokers were observed to have a 2.2 and an almost 4-fold increase in risk compared with non-smokers. A similar effect was observed with regards to the possibility of misclassification between current and past smokers, where the OR for current smokers was lower (current OR = 2.23, 95% CI 1.26-3.95) than for past smokers (OR = 3.80, 95% CI 2.67-5.39). In comparison to males, only 38% of females indicated that they were smokers at some point in their lives.

5.1.2 Different forms of tobacco

Amongst the male smokers, 76% indicated smoking commercial cigarettes with both current and past smokers presenting with a significant increase in risk of developing OC (current OR = 1.89, 95% CI 1.08-3.29; past OR = 5.03, 95% CI 3.04-8.34). Similar trends in risk were observed for males smoking hand-rolled cigarettes and pipe smoking, which amongst smokers accounted for 51% and 38% of the smokers respectively. The proportions given however are not mutually exclusive as the same individuals may smoke various other forms of tobacco. A monotonic dose-response was nevertheless observed across all the categories of number of hand-rolled cigarettes and number of pipes smoked per day. Those reporting having smoked 7 or more hand-rolled cigarettes per day had a 4.4-times greater risk of developing OC (OR = 4.40, 95% CI 2.35-8.24), whilst those smoking 7 or more pipes per day had an

almost 8-fold increase in risk compared to non-smokers (OR = 7.72, 95% CI 3.99-14.92).

Amongst the female smokers, 43% indicated having smoked commercial cigarettes. Most popular was the use of the pipe with 48% having indicated using tobacco in this form. The risk estimates found in Table 5.2 reveal slightly lower risks compared to males. Once again it is important to note that the proportions are not mutually exclusive as the same individuals may smoke various forms of tobacco. However an increased risk of developing OC was observed across all the categories of number of hand-rolled cigarettes and number of pipes smoked per day. Those reporting having smoked 7 or more hand-rolled cigarettes per day had a 3-times greater risk of developing OC (OR = 3.14, 95% CI 1.09-9.07), whilst those smoking 7 or more pipes per day had an almost 6-fold increase in risk compared to non-smokers (OR = 5.63, 95% CI 2.05-15.43).

5.1.3 Total tobacco use

Based on total tobacco use, males currently smoking more than 14.5 grams of tobacco per day were observed to have approximately 4.5-times a greater risk of developing OC compared to non-smokers (OR = 4.36, 95% CI 2.24-8.48). A re-evaluation of the data on a restricted series of histologically confirmed cases also revealed similar trends in the exposure categories with those in the highest category having a 3-fold increased risk of developing OC (OR = 3.10, 95% CI 1.18 – 8.15). Although tobacco chewing and snuff use data were obtained, 96% of the respondents indicated that they did not chew tobacco or use snuff. The results also showed that all males who chewed tobacco also smoked.

In females, some of the tobacco-related categories indicated lower risk estimates compared to males. However, based on total tobacco use, females similarly to males currently smoking more than 14.5 grams of tobacco per day had a 4.5-times a greater risk of developing OC compared to non-smokers (OR = 4.56, 95% CI 1.46-14.30). Upon re-analyses of histologically confirmed cases, the risk estimate for the highest exposure category amongst current smokers was 15-fold (OR 15.22, 95% CI 2.47 – 93.77). Whilst a dose-response was observed across the categories, only 3% of the cases smoked more than 14.5 grams of tobacco. Hence the sample size in this cell was extremely small hence the large confidence interval.

With regards to tobacco chewing 92% of the respondents indicated that they did not chew tobacco. The odds ratio for snuff use among female non-smokers was 2.01 (95% CI 1.17-3.46).

5.2 Population attributable proportion due to tobacco smoking

The prevalence of exposure due to smoking amongst the controls was calculated as 52% using Equation 1 (See section 3.12). Based on a relative risk of 3.65 (95% CI 2.82 – 4.73), for ever smoking, a population attributable risk of 58% was obtained using equation 2. Thus assuming a causal relationship exists, 58% of the oesophageal cancers in the population would have been prevented if this exposure had been removed.

Table 5.1 Odds Ratios of oesophageal cancer associated with tobacco-related variables in males.

Exposure variables	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Smoking status	Non-smokers	23	145	1.00	1.00
	Current	65	176	2.47 (144-4.23)	2.43 (1.42-4.17)
	Past	246	300	5.35 (3.29-8.69)	5.17 (3.17-8.43)
Commercial cigarettes	Non-smokers	23	145	1.00	1.00
	Current	51	174	1.92 (1.10-3.36)	1.89 (1.08-3.29)
	Past	164	206	5.27 (3.20-8.68)	5.03 (3.04-8.34)
No. of cigarettes (per day)	Non-smokers	23	145	1.00	1.00
	1-4	71	123	3.55 (2.06-6.13)	3.48 (2.01-6.00)
	5-9	50	120	2.68 (1.52-4.73)	2.57 (1.45-4.55)
	10+	96	135	5.29 (3.11-9.02)	4.93 (2.87-8.49)
Hand-rolled cigarettes	Non-smokers	23	145	1.00	1.00
	Current	51	106	3.15 (1.77-5.58)	3.05 (1.72-5.44)
	Past	118	127	5.71 (3.39-9.60)	5.42 (3.19-9.19)
No. of hand rolled cigarettes per day	Non-smokers	23	145	1.00	1.00
	1-3	54	80	4.40 (2.48-7.83)	4.23 (2.37-7.54)
	4-6	69	93	4.53 (2.60-7.90)	4.29 (2.45-7.53)
	7+	41	57	4.74 (2.56-8.79)	4.40 (2.35-8.24)
Pipe	Non-smokers	23	145	1.00	1.00
	Current	30	35	5.82 (2.95-11.48)	5.53 (2.79-10.99)
	Past	112	125	5.92 (3.47-10.08)	5.65 (3.29-9.69)
No. of pipes per day	Non-smokers	23	145	1.00	1.00
	1-3	50	69	4.69 (2.60-8.47)	4.58 (2.53-8.29)
	4-6	43	51	5.49 (2.93-10.30)	5.23 (2.77-9.88)
	7+	44	37	8.18 (4.28-15.63)	7.72 (3.99-14.92)
Total Tobacco (grams per day) (All smokers)	Non smokers	23	145	1.00	1.00
	1-7	58	153	2.39 (1.38-4.14)	2.37 (1.37-4.12)
	7.1-14	93	157	3.92 (2.32-6.61)	3.89 (2.30-6.57)
	14.5+	156	162	6.43 (3.87-10.68)	6.27 (3.74-10.52)
Total Tobacco (grams per day) (Current Smokers)	Non smokers	23	145	1.00	1.00
	1-7	11	58	1.25 (0.57-2.78)	1.25 (0.57-2.77)
	7.1-14	25	73	2.26 (1.18-4.32)	2.23 (1.17-4.27)
	14.5+	28	43	4.46 (2.29-8.66)	4.36 (2.24-8.48)
Total Tobacco (grams per day) (Past Smokers)	Non smokers	23	145	1.00	1.00
	1-7	47	95	3.04 (1.70-5.42)	3.01 (1.70-5.39)
	7.1-14	68	84	5.28 (3.02-9.21)	5.24 (3.00-9.16)
	14.5+	128	119	7.20 (4.27-12.14)	7.05 (4.14-12.02)

[a]OR_1, adjusted for age, hospital, residence and years of education;
[b]OR_2, further adjusted for total grams ethanol per week; *P-value obtained from likelihood-ratio test, All *p*-values ≤0.0001 indicating a significant association between exposure and oesophageal cancer.

Table 5.2 Odds Ratios of oesophageal cancer associated with tobacco-related variables in females.

Exposure variables	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Smoking status	Non-smokers	140	422	1.00	1.00
	Current	32	33	2.92 (1.69-5.04)	2.23 (1.26-3.95)
	Past	164	112	4.53 (3.24-6.35)	3.80 (2.67-5.39)
Commercial cigarettes	Non-smokers	140	422	1.00	1.00
	Current	16	17	3.00 (1.45-6.22)	2.23 (1.04-4.77)
	Past	66	48	4.64 (2.99-7.22)	3.94 (2.50-6.19)
No. of cigarettes (per day)	Non-smokers	140	422	1.00	1.00
	1-4	29	37	2.42 (1.40-4.17)	2.17 (1.25-3.78)
	5-9	28	17	5.85 (3.04-11.26)	4.64 (2.36-9.13)
	10+	24	12	7.03 (3.37-14.66)	5.66 (2.66-12.05)
Hand-rolled cigarettes	Non-smokers	140	422	1.00	1.00
	Current	21	16	4.11 (2.05-8.25)	2.99 (1.44-6.20)
	Past	71	48	4.72 (3.03-7.35)	4.02 (2.55-6.34)
No. of hand rolled cigarettes per day	Non-smokers	140	422	1.00	1.00
	1-3	36	29	3.95 (2.29-6.81)	3.47 (1.99-6.04)
	4-6	46	26	5.58 (3.24-9.63)	4.53 (2.58-7.96)
	7+	10	7	4.67 (1.71-12.75)	3.14 (1.09-9.07)
Pipe	Non-smokers	140	422	1.00	1.00
	Current	21	13	5.10 (2.41-10.81)	3.67 (1.68-7.99)
	Past	85	45	5.80 (3.69-9.10)	4.85 (3.05-7.71)
No. of pipes per day	Non-smokers	140	422	1.00	1.00
	1-3	44	33	4.22 (2.48-7.17)	3.78 (2.20-6.48)
	4-6	41	16	7.76 (4.09-14.73)	6.07 (3.14-11.75)
	7+	18	6	8.54 (3.21-22.71)	5.63 (2.05-15.43)
Total Tobacco (grams per day) (All smokers)	Non smokers	140	422	1.00	1.00
	1-7	59	81	2.20 (1.46-3.33)	2.02 (1.33-3.07)
	7.1-14	71	35	6.53 (4.06-10.48)	5.68 (3.49-9.24)
	14.5+	64	26	7.39 (4.39-12.42)	5.60 (3.23-9.73)
Total Tobacco (grams per day) (Current Smokers)	Non smokers	140	422	1.00	1.00
	1-7	13	20	1.94 (0.93-4.08)	1.75 (0.83-3.70)
	7.1-14	7	7	3.02 (1.03-8.89)	2.22 (0.73-6.80)
	14.5+	12	5	7.56 (2.53-22.55)	4.56 (1.46-14.30)
Total Tobacco (grams per day) (Past Smokers)	Non smokers	140	422	1.00	1.00
	1-7	46	61	2.28 (1.45-3.59)	2.07 (1.30-3.27)
	7.1-14	64	28	7.48 (4.49-12.48)	6.50 (3.85-10.96)
	14.5+	52	21	7.30 (4.16-12.80)	5.57 (3.10-9.99)

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams ethanol per week, *p*-value obtained from likelihood-ratio test, All *p*-values ≤0.0001 indicating a significant association between exposure and oesophageal cancer.

5.3 Effect of alcohol-related variables on OC risk

The adjusted risk estimates for alcohol-related variables in males and females are presented in Tables 5.3 and 5.4 respectively.

5.3.1 Alcohol consumption

Males and females were categorised into 'never' and 'ever' drinkers. After further adjustment for total grams tobacco smoked per day, male drinkers had an almost 3.5-fold increase in OC risk (OR=3.48, 95% CI 1.99-6.06) and females with a 2-fold increased risk (OR=2.23, OR=1.60-3.11) compared to non-drinkers.

5.3.2 Types of alcoholic beverages and consumption patterns

The effects of reported consumption of maize, sorghum and commercial beers as well as home-made spirits, commercial spirits and wine were evaluated. Increased frequency of exposure to maize beer was positively associated with OC risk and a monotonic dose-response was observed across all the consumption categories in males, whilst a plateau effect was observed in females. Males and females consuming maize beer between 2 – 4 days per week had a 4-fold increase in OC risk compared to non-drinkers (Males OR = 4.04, 95% CI 2.19 – 7.46; Females OR = 4.29, 95% CI 2.49-7.37). A similar pattern was observed for sorghum beer although much less of the participants consumed sorghum beer than maize beer as a result of its availability and cultural link in the Eastern Cape Province of South Africa. Amongst alcohol consumers, maize beer was consumed by 88% of males and 93% of females, whilst sorghum was only consumed by 58% and 51% of males and females respectively. Sorghum beer consumption was associated with an almost 6-fold greater risk of developing OC when consumed for 2 or more days per week. An increased association was observed for all other categories of commercial beer, commercial spirits and wine. In addition to sorghum beer, traditional maize beer was also the preferred choice even when compared to the number of alcohol consumers who consumed commercial beer (74% males and 53% females), commercial spirits (57% males and 45% females) and wine (31% males and 23% females). The risk estimates for the various alcoholic beverages were slightly lower in females when compared to males.

In males, it can be noted that although the risk from consuming sorghum beer is slightly higher, the prevalence of exposure is lower than that of maize beer consumption. Reported consumption of commercial beer was also associated with a 6-fold and 4-fold increased risk in males and females respectively (male OR = 6.28, 95%CI 2.85-13.81; female OR = 4.12, 95% CI 1.64-10.33). A small proportion of the alcohol consumers (31% males and 23% females) did consume wine and an increased risk was observed in both sexes (Tables 5.3 and 5.4). The low prevalence of wine consumption is expected since much of the residents are poor and wine is generally the social drink amongst those who can afford it.

An attempt was also made to evaluate drinking patterns amongst alcoholic drinkers on a weekly basis. The rationale for this was to elucidate any possible binge drinking where large amounts of certain beverages were consumed at specific intervals. No distinct reported drinking pattern was observed in either males or females.

5.3.3 Quantity of each alcoholic beverage type

Similar to the consumption patterns for each alcoholic beverage discussed in section 5.3.2., the quantity of each beverage type also had positive associations. The risk estimates for most beverages ranged between 4.00 – 5.50 in the highest quantity category, the exception being females consuming more than 1 litre of wine per week who had a 7 times greater risk of developing OC (OR = 7.10, 95% CI 3.39-14.87).

5.3.4 Amount of Ethanol

Since ethanol was the common ingredient in all of the alcoholic beverages investigated, it was decided that the risk estimates be calculated for ethanol intake from each beverage. The alcohol content was calculated using the following data:

Alcoholic beverage	% ethanol
Maize beer	3
Sorghum beer	3
Commercial beer	5
Commercial spirits	40
Home-made spirits	30
wine	12

Using the density of ethanol and the weekly amount of alcoholic beverage consumed, it was possible to calculate the weekly amount of ethanol each consumer was exposed to. The amount variable was then converted to a categorical variable and the results presented in Tables 5.3 and 5.4. It was evident that a positive association was observed for OC risk irrespective of beverage type. The risk estimates were also consistent and this led to the conclusion that alcohol was the main ingredient responsible for the observed trend in both males and females.

Total ethanol consumption (sum of averages of gram ethanol from beers, spirits and wine) was positively associated with OC risk with male drinkers of more than 371 grams per week being almost 5-times more likely to develop OC (OR = 4.72, 95 % CI 2.64 – 8.41) than non-drinkers. Amongst the histologically confirmed cases, those drinking more than 370 grams of total ethanol per week were 5-times more likely to develop OC (OR = 5.23, 95% CI 2.50 – 10.99).

In female drinkers, a 5-fold increased risk was observed for those consuming more than 371 grams ethanol per week (OR = 5.24, 95% CI 3.34-8.23). As expected, lower alcohol consumption categories presented lower risk estimates. Furthermore, in comparison to tobacco exposure, alcohol consumption was associated with lower risk estimates. A re-analysis of this variable in a restricted series of histologically confirmed cases revealed a dose-reponse amongst the exposure categories with those drinking more than 370 grams of total ethanol per week presenting with a 15-fold increase in risk (OR = 15.58, 95% CI 7.52 – 32.26). Once again an extremely limited sample size was observed in the highest category hence the large confidence interval.

It must be emphasised that maize beer was the most consumed alcoholic beverage amongst the drinkers. With regards to commercially attainable beverages including wine, the prevalence of consumption is much lower although all these beverages were associated with increased risks. Hence in this population, the amount of maize beer consumption would be a better indicator of risk associated with ethanol exposure than a measure of total ethanol consumption.

Table 5.3 Odds Ratios of oesophageal cancer associated with alcohol-related variables in males.

Alcoholic beverages	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Alcohol consumption	Never	16	100	1.00	1.00
	Ever	313	510	3.49 (2.00-6.09)	3.48 (1.99-6.06)
Maize beer (consumption per week)	Non drinkers	16	100	1.00	1.0
	<=1 day	154	263	3.31 (1.85-5.91)	3.29 (1.84-5.87)
	2-4 days	90	131	4.04 (2.19-7.45)	4.04 (2.19-7.46)
	5-7 days	41	50	4.70 (2.33-9.48)	4.68 (2.32-9.44)
Quantity of maize beer per week (Litres)	Non drinkers	16	100	1.00	1.00
	<=1	34	81	2.45 (1.24-4.86)	2.41 (1.21-4.77)
	1.01-3	76	133	3.10 (1.68-5.70)	3.10 (1.69-5.71)
	3.01+	153	201	4.28 (2.40-7.65)	4.26 (2.39-7.62)
Sorghum beer	Non drinkers	16	100	1.00	1.00
	<=1 day	83	164	3.33 (1.77-6.26)	3.31 (1.76-6.22)
	2-4 days	79	85	5.82 (3.09-10.97)	5.84 (3.10-11.00)
	5-7 days	34	36	5.90 (2.77-12.54)	5.80 (2.72-12.34)
Quantity sorghum beer per week (Litres)	Non drinkers	16	100	1.00	1.00
	<=1	35	66	3.52 (1.76-7.06)	3.45 (1.72-6.93)
	1.1-3	45	80	3.70 (1.89-7.24)	3.72 (1.90-7.27)
	>3	106	127	5.42 (2.92-10.04)	5.43 (2.93-10.07)
Commercial beer	Non drinkers	16	100	1.00	1.00
	<=1 day	148	242	3.51 (1.97-6.24)	3.49 (1.96-6.22)
	2-4 days	69	101	3.89 (2.09-7.23)	3.88 (2.08-7.21)
	5-7 days	27	26	6.34 (2.88-13.94)	6.28 (2.85-13.81)
Quantity of commercial beer consumed per week (Litres)	Non drinkers	16	100	1.00	1.00
	<=1	35	66	3.55 (1.77-7.12)	3.49 (1.73-6.99)
	1.01-2	16	32	3.28 (1.44-7.46)	3.29 (1.45-7.47)
	>2	135	175	5.08 (2.77-9.30)	5.10 (2.78-9.33)
Home-made spirits	Never	16	100	1.00	1.00
	Ever	11	10	5.55 (1.98-15.56)	5.56 (1.98-15.59)
Commercial spirits	Non drinkers	16	100	1.00	1.00
	<=1 day	136	211	3.75 (2.09-6.76)	3.75 (2.08-6.75)
	2-4 days	33	45	4.37 (2.16-8.84)	4.34 (2.14-8.78)
	5-7 days	21	23	5.30 (2.30-12.20)	5.31 (2.31-12.24)
Quantity commercial spirits consumed per week (Litres)	Non drinkers	16	100	1.00	1.00
	0.025-0.1	75	127	3.23 (1.73-6.03)	3.23 (1.73-6.03)
	0.11+	103	136	4.54 (2.50-8.24)	4.53 (2.49-8.23)
Wine	Never	16	100	1.00	1.00
	<=1 day	64	103	3.69 (1.98-6.91)	3.66 (1.96-6.85)
	2+ days	36	52	4.30 (2.15-8.62)	4.32 (2.15-8.65)

Quantity of wine consumed per week (Litres)	Non drinkers	16	100	1.00	1.00
	0.1-1	46	73	3.64 (1.89-6.99)	3.58 (1.86-6.90)
	>1	45	67	4.30 (2.21-8.38)	4.31 (2.21-8.41)
Grams Alcohol per week					
Maize beer	Non drinkers	16	100	1.00	1.00
	0.75-60	77	161	2.62 (1.43-4.79)	2.60 (1.42-4.76)
	61-225	89	142	3.65 (1.99-6.70)	3.66 (1.99-6.73)
	226+	97	112	4.93 (2.69-9.04)	4.90 (2.67-8.99)
Sorghum beer	Non drinkers	16	100	1.00	1.00
	0.1-30	35	66	3.50 (1.75-7.01)	3.43 (1.71-6.89)
	31-120	61	98	4.16 (2.17-7.98)	4.18 (2.18-8.02)
	121+	90	109	5.23 (2.80-9.76)	5.24 (2.81-9.77)
Commercial beer	Non drinkers	16	100	1.00	1.00
	1-50	90	144	3.41 (1.87-6.23)	3.40 (1.86-6.20)
	51-150	65	91	4.27 (2.29-7.97)	4.27 (2.29-7.97)
	151+	69	113	3.49 (1.88-6.49)	3.46 (1.86-6.44)
Commercial spirits	Non drinkers	16	100	1.00	1.00
	1-40	75	127	3.23 (1.73-6.03)	3.23 (1.73-6.03)
	41+	103	136	4.54 (2.50-8.24)	4.53 (2.49-8.23)
Wine	Non-drinkers	16	100	1.00	1.00
	1-150	51	76	3.87 (2.03-7.39)	3.80 (1.99-7.27)
	151+	44	65	4.34 (2.22-8.48)	4.35 (2.22-8.50)
Total alcohol consumption (grams per week)	Non drinkers	16	100	1.00	1.00
	0.5-115	42	100	2.42 (1.26-4.64)	2.38 (1.24-4.57)
	116-370	86	176	2.72 (1.50-4.95)	2.71 (1.49-4.93)
	371+	164	205	4.73 (2.65-8.43)	4.72 (2.64-8.41)

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day, *p*-value obtained from likelihood-ratio test, All *p*-values ≤0.0001 indicating a significant association between exposure and oesophageal cancer.

Table 5.4 Odds Ratios of oesophageal cancer associated with alcohol-related variables in females.

Alcoholic beverages	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Alcohol consumption	Never	87	240	1.00	1.00
	Ever	243	299	2.23 (1.60-3.11)	2.23 (1.60-3.11)
Maize beer (consumption per week)	Non drinkers	87	240	1.00	1.00
	<=1 day	142	208	1.89 (1.31-2.71)	1.88 (1.31-2.71)
	2-4 days	53	29	4.28 (2.49-7.37)	4.29 (2.49-7.37)
	5-7 days	33	37	2.65 (1.49-4.72)	2.65 (1.49-4.71)
Quantity of maize beer per week (Litres)	Non drinkers	87	240	1.00	1.00
	<=1	41	110	1.03 (0.64-1.66)	1.03 (0.64-1.65)
	1.01-3	69	93	1.94 (1.26-3.00)	1.94 (1.26-3.00)
	3.01+	106	53	5.04 (3.25-7.80)	5.05 (3.26-7.82)
Sorghum beer	Non drinkers	87	240	1.00	1.00
	<=1 day	63	110	1.74 (1.09-2.80)	1.74 (1.09-2.79)
	2-4 days	37	17	5.20 (2.71-9.99)	5.21 (2.71-10.00)
	5-7 days	21	30	2.22 (1.11-4.43)	2.21 (1.11-4.42)
Quantity sorghum beer per week (Litres)	Non drinkers	87	240	1.00	1.00
	<=1	39	87	1.47 (0.89-2.46)	1.47 (0.89-2.45)
	1.1-3	32	38	2.46 (1.37-4.42)	2.46 (1.37-4.42)
	>3	44	24	4.84 (2.66-8.83)	4.85 (2.66-8.85)
Commercial beer	Non drinkers	87	240	1.00	1.00
	<=1 day	113	101	2.94 (1.99-4.35)	2.94 (1.99-4.35)
	2-4 days	34	18	4.51 (2.39-8.54)	4.52 (2.39-8.55)
	5-7 days	14	9	4.16 (1.66-10.41)	4.12 (1.64-10.33)
Quantity of commercial beer consumed per week (Litres)	Non drinkers	87	240	1.00	1.00
	<=1	39	87	1.48 (0.89-2.47)	1.48 (0.89-2.47)
	1.01-2	14	23	1.80 (0.85-3.83)	1.80 (0.85-3.83)
	>2	62	39	4.32 (2.56-7.29)	4.32 (2.56-7.29)
Home-made spirits	Never	87	240	1.00	1.00
	Ever	12	2	15.36 (3.31-71.17)	15.36 (3.31-71.18)
Commercial spirits	Non drinkers	87	240	1.00	1.00
	<=1 day	96	79	3.44 (2.24-5.30)	3.45 (2.24-5.30)
	2-4 days	24	11	5.94 (2.75-12.87)	5.96 (2.75-12.90)
	5-7 days	16	16	3.23 (1.46-7.13)	3.21 (1.45-7.08)
Quantity commercial spirits consumed per week (Litres)	Non drinkers	87	240	1.00	1.00
	0.025-0.1	61	56	3.00 (1.85-4.89)	3.00 (1.85-4.89)
	0.11+	66	41	4.67 (2.86-7.63)	4.67 (2.86-7.63)
Wine	Never	87	240	1.00	1.00
	<=1 day	51	48	2.94 (1.80-4.81)	2.94 (1.80-4.81)
	2+ days	14	11	3.65 (1.56-8.56)	3.66 (1.56-8.57)

Quantity of wine consumed per week (Litres)	Non drinkers	87	240	1.00	1.00
	0.1-1	29	39	1.98 (1.13-3.49)	1.99 (1.13-3.49)
	>1	29	12	7.09 (3.39-14.85)	7.10 (3.39-14.87)
Grams Alcohol per week					
Maize beer	Non drinkers	87	240	1.00	1.00
	0.75-60	95	185	1.39 (0.95-2.04)	1..39 (0.95-2.04)
	61-225	55	42	3.21 (1.95-5.30)	3.21 (1.95-5.29)
	226+	66	29	5.68 (3.38-9.53)	5.69 (3.39-9.54)
Sorghum beer	Non drinkers	87	240	1.00	1.00
	0.1-30	39	87	1.48 (0.89-2.46)	1.48 (0.89-2.46)
	31-120	34	39	2.53 (1.42-4.49)	2.53 (1.42-4.49)
	121+	42	23	4.86 (2.64-8.96)	4.87 (2.65-8.98)
Commercial beer	Non drinkers	87	240	1.00	1.00
	1-50	72	80	2.31 (1.50-3.56)	2.31 (1.50-3.56)
	51-150	48	21	5.99 (3.34-10.73)	5.97 (3.33-10.71)
	151+	38	19	4.93 (2.64-9.20)	4.94 (2.64-9.21)
Commercial spirits	Non drinkers	87	240	1.00	1.00
	1-40	61	56	3.00 (1.85-4.89)	3.00 (1.85 (4.89)
	41+	66	41	4.67 (2.86-7.63)	4.67 (2.86-7.63)
Wine	Non-drinkers	87	240	1.00	1.00
	1-150	32	40	2.14 (1.23-3.71)	2.14 (1.23-3.71)
	151+	27	12	6.59 (3.13-13.90)	6.60 (3.13-13.91)
Total alcohol consumption (grams per week)	Non drinkers	87	240	1.00	1.00
	0.5-115	56	156	1.01 (0.66-1.54)	1.01 (0.66-1.53)
	116-370	79	74	2.73 (1.78-4.19)	2.73 (1.78-4.19)
	371+	96	48	5.25 (3.35-8.23)	5.24 (3.34-8.23)

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day, *p*-value obtained from likelihood-ratio test, All All *p*-values ≤0.0001 indicating a significant association between exposure and oesophageal cancer.

5.4 Population attributable proportion due to alcohol consumption

The prevalence of exposure due to alcohol consumption amongst the controls was calculated as 68% using Equation 1 (See section 3.12). Based on a relative risk of 2.38 (95% CI 1.82 – 3.11), as seen below for ever having consumed alcohol, a population attributable risk of 48% was obtained using equation 2. Thus assuming a causal relationship exists, 48% of the oesophageal cancers in the population would have been prevented if this exposure had been removed.

5.5 Joint effects of tobacco smoking and alcohol consumption

The interaction between tobacco smoking and alcohol consumption was investigated by fitting appropriate logistic regression models with interaction terms for these variables to estimate the effect of alcohol consumption in each stratum of tobacco use and the effect of tobacco use in each stratum of alcohol amount (Table 4.7). The point estimate shows that higher risks were associated with increased alcohol consumption across all tobacco amount strata but no evidence of interaction on the multiplicative scale (P=0.37). Similarly increased tobacco use also increased the risk estimates across the alcohol amount strata. Hence those using more than 14 grams of tobacco/day and consuming more than 371 grams ethanol/week had a 8.5-fold increased risk of developing oesophageal cancer (OR = 8.45, 95% CI 5.51-12.96) compared to those who are both non-smokers and non-drinkers. The population attributable fraction for the highest exposure category was found to be 42%.

Table 5.5 Odds ratio of oesophageal cancer associated with the combined effects of smoking and alcohol consumption.

Tobacco use (g/day)	Alcohol consumption (g/week)		
	0 Cases/Controls	0.5-370 Cases/Controls	371+ Cases/Controls
0	1.00 80/287	1.21 (0.82-1.80) 61/197	1.62 (0.78-3.36) 13/37
1-14	2.57 (1.33-4.99) 17/39	2.84 (1.95-4.13) 127/242	5.70 (3.80-8.54) 113/110
14.1+	3.04 (1.07-8.65) 6/13	7.02 (4.38-11.26) 74/61	8.45 (5.51-12.96) 131/106

[a]OR_1, adjusted for age, sex, hospital, residence and years of education
p-value for the interaction between tobacco use and alcohol consumption = 0.37.

5.6 Discussion

The results of this study are well in agreement with previous studies that have addressed the risks associated with tobacco and alcohol exposure and also show clearly the independent effects of these two exposures.

5.6.1 Effect of Smoking

The risk associated with smoking was higher in males (current OR = 2.43; past OR = 5.17) with 82% having indicated being either a past or current smoker. A lower risk estimate (current OR = 2.23; past OR = 3.80) was observed for females with only 38% having indicated being a smoker. Smoking has been shown to be a major risk factor for OC in this population. It is important to note that processed tobacco contains over 3000 compounds including 30 carcinogens with polyaromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (TSNAs), and aromatic amines being the three major classes of carcinogens responsible for tobacco-associated cancers (Bartsch *et al.*, 2000). The predominant role of tobacco smoking in OC development has also been observed in several other hospital-based case-control studies in South Africa and abroad. The earliest (Bradshaw & Schonland, 1969) examined 98 cases and 341 hospital controls (unmatched for age) in Durban. This study was reanalyzed along with a later study (Bradshaw & Schonland, 1974) of 196 male cases aged >35 and 1064 age-matched controls in Baragwanath hospital, Johannesburg. In both studies there was a marked association with tobacco use, particularly the use of pipe tobacco in cigarettes (relative risks of 5.4 in Durban, and 7.8 in Johannesburg, relative to non-smokers). The hospital-based study by Sammon (1992) conducted in the rural population of the former Transkei, where 100 OC patients were compared with controls matched by age and education level, also found a positive association with smoking (RR = 2.6, 95% CI 1.5-5.0). The recent study by Pacella-Norman *et al.* (2002) carried out in the greater Johannesburg area also found that tobacco smoking, past or current, to be the major risk factor for OC. Ex-smokers and 'light' current smokers had about a 3-fold increased risk of OC with males at a higher risk than females.

Apart from smoking status, other parameters of tobacco smoking are also related to cancer risk in accordance with the basic principles of chemical carcinogenesis. Hence risk is also determined by the dose of the carcinogen, the duration of administration and the intensity of exposure (Stewart & Kleihuis, 2003). As seen in our study, increased number of cigarettes (commercial and hand-rolled) and pipes

smoked resulted in an increased exposure to the carcinogens present in tobacco and hence the increased risk. Females however had lower risk estimates. The risk presented in males from smoking 10 or more commercial cigarettes per day was similar to that observed in males from South America who smoked more than 25 cigarettes per day (Castellsagué, *et al.*, 1999). In comparing such risks, one must consider that the Transkeian population is also predisposed due to nutritional deficiencies which may increase their susceptibility to carcinogenesis. Hence lower doses of carcinogens can result in higher risks in comparison to other population groups.

In Bulawayo, Zimbabwe, a restrospective case-control study from the Bulawayo Cancer Registry data also revealed that smoking was a strong predictor of risk of carcinoma of the oesophagus (Vizcaino *et al.*, 1995). The risk was significantly elevated in all smoking categories compared with non-smokers with a marked geographical gradient in risk in both sexes, which remained after adjustment for lifestyle variables. Women who had ever smoked exhibited a significantly increased risk for OC, relative to those who had never used any tobacco products, after adjustment for alcohol consumption (OR = 4.0). There was also a clear dose-response effect in men, with the highest risk in the heaviest smokers (OR = 4.3 in smokers of ≥ 15 cigarettes daily, 5.7 in smokers of ≥ 15 g of tobacco) which was independent of the other factors such as alcohol consumption. In women, the prevalence of smoking was very low (13% and 2.2% among cases and controls respectively). Alcohol intake showed no independent effect on risk. Low socioeconomic status (OR = 1.5; 95% CI 1.0-2.1) and working as a miner (OR = 2.5; CI 1.5-4.2) conferred increased risks in comparison with men of high socioeconomic status.

In a population-based case-control study in high- and low-risk areas of Jiangsu Province, China, consistent smoking also elevated the risk of developing OC in both counties (Wu *et al.*, 2006). In the high risk area of Dafeng, former and current smokers had a 1.93- and 2.42-fold increased risk of developing OC in comparison to 'never-smokers', with the risks slightly lower in Ganyu county.

The European prospective investigation into Cancer and Nutrition (EPIC) study, which is a network of prospective studies involving about 500 000 subjects from 10 Western countries (France, Italy, Spain, Netherlands, the United Kingdom, Germany, Greece, Sweden, Denmark and Norway) also found that smoking was a risk factor

associated with cancer of the bladder, kidney, pancreas, upper aero-digestive tract and lung (Bjerregaard *et al.*, 2006). Compared to never smoking, current smoking showed a relative risk of 2.7 for 1-20 cigarettes, and of 9.0 for more than 20 cigarettes per day (Riboli & Lambert, 2002).

Tobacco smoking has been estimated to cause approximately 25% of all cancers in men and 4% in women, and, in both genders, approximately 16% of cancer in more developed countries and 10% in less developed countries (Parkin, D.M *et al.*, 1994), although some estimates are as high as 30% (Doll & Peto, 1981). Cancer causation by tobacco is not attributable to any one chemical component as discussed earlier, or to any one class of chemicals present, but to an overall effect of the complex mixture of carcinogenic chemicals in tobacco smoke. Mechanistic interferences which can be made from epidemiological studies, together with relevant experimental data, indicate a scenario compatible with "multistage carcinogenesis" as understood at the cellular and molecular level (Shields, 2000).

5.6.2 Type of Tobacco

In this study risk assessments were made from commercial and hand-rolled cigarettes as well as pipe smoking. Commercial cigarettes contain shreds of tobacco rolled in paper together with filters. Hand-rolled cigarettes however are used in which pipe tobacco is used wrapped in newspaper or brown paper. No filters are present. Such cigarettes are often preferred because of their flavour and their slow burning quality. They are also cheaper than commercial cigarettes. Among males, the risk related to tobacco type was of the following order: current pipe smokers>current hand-rolled cigarette smokers>current commercial cigarette smokers. These results are further strengthened by the 2002 IARC working Group where it was found that pipe smoking was strongly and causally related to cancers of the oral cavity, oropharynx, hypopharynx, larynx, lung and oesophagus (IARC, 2002a). The risk associated with the use of pipe tobacco was similar to that observed by Bradshaw & Schonland (1974) but was much higher than that observed by van Rensburg *et al.* (1985) (OR = 2.1). This study was undertaken in Durban, South Africa between the period 1978-1981 with 211 hospital cases (Zulu males) and compared with controls matched for age and residence (urban-rural) on 273 variables (socioeconomic, carcinogen exposure, food, tobacco and alcohol). Sixteen variables initially emerged as significant, including education (higher risk in the educated) and use of home-made spirits (increased risk). However in the final multivariate model cigarette and

pipe smoking remained as significant risks (cigarette smoking relative risk [RR] = 2.64 current, 1.62 past; pipe smoking RR = 2.08 current, 1.44 past).

Pipe smoking in the former Transkei is a traditional practice. Long stemmed pipes being commonly smoked by both men and women were also noted by Burrel (1957). The study by McGlashan *et al.* (1982) compared areas of high, medium and low incidence of OC in the Transkei region and suggested that the prevalence of tobacco smoking (particularly pipes) was correlated with OC risk. Despite the fact that hand-rolled cigarettes and pipes contain the same type of tobacco, the higher risk from pipe smoking may be due to other habits such as the swallowing of tobacco pyrolysis products from the pipe stem as observed by Rose (1978). Although in South Africa this is not the case, black-tobacco smoking in South America is associated with a twofold increased risk of OC as compared with the smoking of blond or mixed tobacco (Castellsagué *et al.*, 1999). Despite these varied effects on risk, the risk presented from total tobacco use was consistent with a dose-response effect on carcinogenicity and the study reveals that irrespective of tobacco type, increased exposure results in increased risk of developing OC.

5.6.3 Ingested Tobacco

Non-combustive use of tobacco, or smokeless tobacco use, comes in the form of chewing tobacco and snuff (ground or powdered tobacco, either moist or dry) which is inhaled nasally or placed in the mouth. The habit of tobacco chewing has been observed in the Transkeian population by Rose (1978), especially the swallowing of tobacco pyrolysis products from the pipe stem. This was thought to be associated with increased OC risk since a bacterial mutagenicity assay demonstrated a significant mutagenic activity in this pyrolysis product (Hewer *et al.*, 1978). However our study revealed that only 4% of the participants chewed tobacco or used snuff, a result similar to that of Bradshaw *et al.* (1983) where only 2-5% of the population claimed to do this. Tobacco-specific *N*-nitrosamines are plausible candidates for the carcinogenicity of smokeless tobacco products.

5.6.4 Effect of Alcohol

The prevalence of alcohol consumption in the male and female controls were 84% and 56% respectively and higher to those obtained in the Johannesburg study undertaken by Pacella-Noman *et al.* (2002) where the prevalence in males and

females was found to be 67% and 38% respectively indicating that the former Transkei is an area of very high consumption. In this study, reported alcohol consumption was associated with an almost 3.5-fold increased risk of developing OC, with the risk estimate being lower in females (OR = 2.23). These risk estimates were similar to those obtained for men and women in South America, respectively (Castellsagué *et al.*, 1999) but much lower in Sichuan Province, South Western China (OR = 2.49) (Yang *et al.*, 2005). Similarly, as for tobacco use, increasing alcohol consumption also associated with an increased risk in the Transkeian population, with males who consumed more than 370 g ethanol/day presenting with an almost 5-fold increased risk of developing OC. The risk estimate was similar for females. Through analytical epidemiological studies the causal association of drinking alcohol has definitely been established in terms of oral, oesophageal, liver and other cancers (IARC, 1988) . Alcohol drinking is estimated to be involved in the etiology of 3% of all cancers (that is, 4% in men, 2% in women) (Stewart, B.W and Kleinhues, P; 2003). It has long been suggested that alcohol may act as a solvent facilitating the transport of carcinogens through the oesophageal mucosa (Horie *et al.*, 1965), but it may also act as a chronic irritant, raising the susceptibility to carcinogens by accelerating cell turnover and thus favouring contact between the carcinogens and the dividing target cells (Day and Muñoz, 1982). Accrual of evidence however has led to the conclusions that ethanol is not a carcinogen itself, but may promote carcinogenesis via:

1. Generation of free radical products during its metabolism (Eskelson *et al.*, 1993);
2. Solvent effects on tobacco and other carcinogens;
3. Induction of microsomal enzymes involved in carcinogen metabolism (Yokoyama *et al.*, 2003);
4. Metabolism of acetaldehyde which has been proven to be a carcinogen in animal experiments (Woutersen *et al.*, 1986; Feron *et al.*, 1982).

In this context it is of interest that polymorphisms of ADH2 (ADH2 metabolises alcohol to acetaldehyde) and ALDH2 (ALDH2 metabolises acetaldehyde to acetate) have been shown to modify risk of OC through affecting the metabolism of acetaldehyde (Matsuo *et al.*, 2001; Yokoyama *et al.*, 2003; Hori *et al.*, 1997). Studies in Japan, exhibiting polymorphisms in genes have shown an increased risk of cancer in subjects with a genetic profile that is associated with higher acetaldehyde levels following alcohol consumption (Matsuo *et al.*, 2001). In a population-based prospective study conducted in Japan on cancer and cardiovascular disease to examine the interaction by cancer type (alcohol-related cancer versus other cancers)

using extended follow-up data, it was found that the risk of OC was closely associated with alcohol consumption (Hara *et al.*, 2002). However, alcohol consumption was also associated with the risk of other cancer deaths (pharynx, larynx and liver); the relative risk (RR) among the heaviest drinkers (ethanol intake > 450g/week) compared with occasional drinkers was significantly high after adjustment for possible confounders. Alcohol consumption in Linxian, China however has been shown to play a small role for risk of OC, adding to the worldwide enigma of this disease (Yu *et al.*, 1993). Whilst this study has found that alcohol consumption is a risk factor for OC in the Transkeian population, several previous studies did not find an association or the association was not significant. The studies of Bradshaw & Schonland (1969 and 1974) analysed together showed no significant effect of alcohol (traditional beer, western type liquors or 'concoctions') on OC risk. The study by Sammon (1992) in the former Transkei also did not find a significant relationship with the drinking of 'traditional' beer (OR=1.6, 95% CI 0.9-3.0). Similarly, in Bulawayo, Zimbabwe, Vizcaino *et al.* (1995) found no relation to alcohol drinking (OR = 0.9). In contrast to these studies however, Segal *et al.* (1988) found that alcohol had an independent effect in the population of Soweto, where the RR in relation to alcohol consumption (adjusted for tobacco) were 5.4 in those consuming 31-60 g ethanol/day, 10.5 in those consuming 61-90 g and 18.3 for the highest daily consumption of ethanol (>90 g/day). Similarly, Pacella-Norman *et al.* (2002) also found that daily alcohol consumption was a risk factor in the population of Johannesburg and Soweto where males and females had an almost 2 times greater risk (Male OR = 1.80, 95% CI 1.90-20.20; Female OR = 1.70, 95% CI 1.00-2.90).

The possible reason for the rather discrepant results between earlier studies and now, with respect to alcohol consumption and risk, may lie in the actual amounts of alcohol consumed. In the Transkei population studied, amounts larger than 370 g ethanol/week were recorded which was similar to 31-60 g/day recorded by Segal *et al.* (1988). The risk estimates between these two studies were also comparable and found to be similar to the risk estimates obtained in the European Prospective Investigation into Cancer and Nutrition (EPIC) study where the mean alcohol consumption of 30-60 g/day at different ages was found to present a significantly elevated risk (RR=3. 17) (Riboli & Lambert, 2002). In the two later South African studies the amounts of alcohol consumed was far greater than that reported in the studies from Bulawayo and rural South Africa, where the principal alcoholic beverage is maize beer, with a low alcohol content (2-4%). In the Bulawayo study it was estimated that individuals in the heaviest drinking category were consuming only

about 20-40 g ethanol/day, a quantity that is associated with only about a doubling of the OC risk in studies elsewhere. It is therefore possible that the early studies could not detect such low relative risks.

5.6.5 Types of Alcoholic Beverages

Various alcoholic beverages were evaluated to estimate the risk associated with each. The reported quantity of each beverage type consumed per week was evaluated and it was observed that commercial and sorghum beer (also produced commercially) was associated with similar risk estimates in males and females. Although the consumption of traditionally produced maize beer and the amount of commercial spirits consumed per week produced a similar risk estimate in the highest exposure category, focus must be directed to the varying amounts consumed. Males drinking more than 110 ml of commercial spirits had a similar risk to those drinking more than 3 litres of maize beer. This difference is plausible and related to the amount of ethanol present in these beverages. Whilst the exposure categories in terms of 'amount consumed' varied, the increased exposure was associated with an increased risk of developing OC. However, none of the beverages were associated with risk that made it distinct from the rest. Furthermore, upon estimating the risk for total alcohol consumption per week, it was once again observed that the risk estimates were somewhat similar leading to the assumption that alcohol was the predominant agent in these beverages that was responsible for the underlying effect. The risk estimates for the various beverages were slightly lower in females than in males although when presenting the risk associated with total alcohol consumption, females had slightly elevated risks than compared with men. It must be noted however that traditional maize beer was the most frequently consumed beverage in this population and would therefore be a better estimator of risk. Previous studies by Cook (1971) and van Rensburg (1981) also showed a geographical correlation between the incidence of OC and the consumption of beer brewed from maize, however several studies on the carcinogenicity of these maize-based beers have been conducted but none has shown, at the individual level, an association with OC. This indicates that these maize-based beers do not have underlying contaminants that are responsible for OC, but the alcohol itself, which as we know is not a carcinogen on its own but is thought to lead to a carcinogenic metabolite as a result of human biological processes as discussed earlier.

Drinking of specific alcoholic beverages has also been implicated in several clusters of elevated OC mortality rates around the world (Blot, 1994). In a population-based cohort study in Copenhagen, Denmark, Grønbæk *et al.* (1998) reported that a high proportion of beer and spirits intake implied a higher risk of upper digestive tract cancer than did a low proportion. In the same line, Tuyns *et al.* (1979) showed that the linear relationship between overall daily ethanol consumption and OC risk was more marked among drinkers of strong beverages than drinkers of lighter beverages, once again underlying the effect of ethanol. Whilst Launoy *et al.* (1997b) showed that the risk of OC depended on the type of alcoholic beverage, the current study did not distinctly show a similar effect, although commercial beer and spirits did present with a slightly elevated risk which may be due to the larger ethanol content than traditionally brewed beverages. Furthermore the alcohol content is also measured with less error than home brews. It is said that in the Transkei region, individuals usually use alcoholic beverages intermittently, at most over weekends, and then only when grain for brewing is plentiful. However no such correlations were observed in this study.

Traditional beer (either brewed at home, or brewed commercially using local recipes) is low on alcohol content (±3%) and the increase in risk associated with the use of maize meal as the major ingredient in beer accords with the finding by Cook (1971) of the rise in the frequency of OC and hence an association in Africa with the use of maize for beer making. In fact maize has been an ingredient of beer even before the turn of the century (Novellie, 1986) but the percentage of maize used in beer has subsequently increased considerably with sorghum retained only as the fermenting agent. A typical recipe given by Oxford (1926) contains maize meal (27.8%), sorghum meal (37.6%) and sorghum malt (34.6%). In 1964 however 57% of the content of traditional beer was derived from maize (Cook, 1971).

Since the repeal of prohibitive alcohol legislation for blacks in 1961 there has been a steady shift among black drinkers from the exclusive consumption of traditional alcoholic beverages to the consumption of Western style drinks as well (Segal *et al.*, 1988). In addition to urbanisation, the lifestyle changes for black Africans have also resulted in increased rates of alcohol consumption (Yach and Townsend, 1988; Segal *et al.*, 1988). The drinking of home-distilled spirits is said to be common in parts of Africa but there has been no geographical evidence for an association with OC development (Cook, 1971). Home-distilling of liquor still takes place although the

extent of which is not known. In our study, only 2.6% of both male and female drinkers indicated that they consumed home-made spirits.

Whilst studies in many parts of the world have indicated that the consumption of alcoholic drinks can be a factor in the development of OC, the mere quantity of alcohol consumed is not a sufficient factor to explain the enormous geographical variation in frequency of OC throughout the world (Doll, 1969), and, moreover, areas of moderate or high frequency of OC such as India or Iran exist where alcohol seems to play no part in the development of OC (Wynder and Bross, 1961). Furthermore the consumption of distilled spirits in areas such as the former Transkei is less than in the urban areas of South Africa (Burrel, 1962) and there are areas such as southern and north-western Uganda where the drinking of home-made spirits is common but where the frequency of OC is low (Cook and Burkitt, 1971). Therefore, based on these anomalies, it may be prudent to state that alcohol is a component cause, the extent of this causal factor may vary in different populations based on other risk factors that interplay to bring about OC.

With regards to wine intake, 31% of the male alcohol drinkers indicated having wine, the intake of which was associated with an increased risk of developing OC. Wine intake was also found to be a risk factor in other studies. In a pooled analysis of 5 studies in South America, Castellsagué *et. al.* (1999) also found that wine only intake was associated an almost 10-times greater risk. Bosetti, *et al.* (2000c) also investigated the separate and combined effect of wine-drinking and other alcoholic beverages on OC in a high-wine consuming population in the greater Milan area and in the province of Pordenone, in northern Italy. Two case-control studies were undertaken between 1984 and 1998 with 714 OC cases and 3137 controls. Multiple logistic regression models revealed that with reference to total alcohol drinking, as compared to non- or moderate drinkers (<3 drinks per day), the multivariate ORs were 1.98 for drinkers of 3 – 4 drinks per day, 4.22 for 5 – 7, 7.60 for 8 – 11, and 12.35 for ≥12 drinks per day. Higher risks were observed for wine-only drinkers and the corresponding values were 1.70, 4.21, 8.76 and 17.90. After allowance for wine intake, no association was observed between beer and spirit drinking and OC, in a population in which 80% of alcohol came from wine.

5.6.6 Combined Effects of Tobacco and Alcohol

In our study, tobacco use and alcohol drinking had independent effects on OC risk although higher risk estimates were obtained across the tobacco and alcohol exposure categories. No evidence of interaction was observed on the multiplicative scale. The joint effects of tobacco smoking and alcohol consumption were also explored in high- and low-risk areas of Jiangsu Province in China (Wu *et al.*, 2006). As with this study, no significant interaction was observed. Previous case-control studies among South African black populations have shown an elevated risk for the smoking of pipe tobacco and more recently for the smoking of cigarettes but no evidence for an independent risk associated with the consumption of alcohol (Bradshaw and Schonland, 1969, 1974; van Rensburg *et al.*, 1985). This could be because in these earlier studies the alcohol content may have been low. However a population survey conducted in areas of high and low incidence in the Transkei suggested that a combined effect of smoking pipe tobacco and of drinking may be of importance there (McGlashan *et al.*, 1982). In contrast, a more recent case-control study in the more urban population of Soweto, South Africa (Segal *et al.*, 1988) had found that both alcohol and tobacco smoking had independent (and multiplicative) effects for men who smoked more than 40 grams of tobacco and consumed more than 91 grams of alcohol daily presenting with a RR of 39 in comparison to those who smoked less than 19 grams of tobacco and consumed less than 30 grams of alcohol per day. Similarly, studies of western populations have also indicated a multiplicative effect of alcohol and tobacco in the development of OC with risk increasing more sharply with rising alcohol intake than with rising tobacco consumption (Tuyns *et al.*, 1977; Day, 1984). Castellsagué *et al.*, (1999) also found a statistically significant synergistic interaction between type of tobacco and amount of cigarettes smoked, and subjects simultaneously exposed to black-tobacco and heavy alcohol drinking represented the sub-group with the highest risk for esophageal cancer (OR>100). In an analysis of three case-control studies from Italy and Switzerland, it was found that for the combined exposure of heavy drinking with heavy smoking, the OR was 12.8 (Gallus *et al.*, 2001).

5.6.7 Population Attributable Risks

Based on a RR of 3.65 and the prevalence of exposure to tobacco smoking amongst the controls (52%), a population attributable risk of 58% was obtained. Thus assuming a causal relationship exists, 58% of the OCs in this population would have been prevented if this exposure had been removed. Similarly for alcohol consumption, where the prevalence of alcohol consumption amongst controls (males and females combined) was 68% and a RR of 2.38, a population attributable risk of 48% was obtained. The prevalence of alcohol consumption among males and females combined was higher than that calculated from the Johannesburg study (49%) (Pacella-Norman *et al.*, 2002). However, despite the alcohol prevalence being higher, the risk from such an exposure is lower than that observed from tobacco smoke. Hence from a public health perspective, tobacco would be the more important factor to target in cessation strategies.

5.6.8 Policy Implications of Study Findings

Oesophageal cancer is one of the most fatal forms of carcinoma. Because improvements in prognosis have been unimpressing (Farrow & Vaughan, 1996), primary prevention and intervention are important in the control of the disease. Our study shows that 58% and 48% of the aetiological fraction of OC were attributed to smoking and alcohol consumption respectively. These data indicate that a substantial health benefit to OC could be expected by efforts of reducing the prevalence of smoking and drinking.

Recent data suggest that only after at least 10 years of abstaining from drinking does the risk of esophageal cancer return within the upper risk levels for abstainers (Castellsagué *et al.*, 1999; Bosetti *et al.*, 2000a). In contrast, the quitting data for cigarette smoking indicate that, stopping for 5 years cut the risk by 50%. After 10 or more years since stopping both habits the RR is only about one-tenth of that of current smokers and drinkers (Castellsagué *et al.*, 1999). It has been suggested that tobacco has a strong role both in the early and in the late stages of carcinogenesis (cancer initiation and promotion), while alcohol appears to play a more important role in late stages of carcinogenesis (cancer promotion). Based on the findings of the Department of Health's South African Demographic and Health Survey (SADHS) conducted in 1998 by the MRC and Macro International Inc., just under half of men (45%) and one-sixth of women (17%) 15 years and older report that they currently consume alcohol (South African Health Review, 2000). Similarly, in a case-control

study from Italy and Switzerland, it was found that the risk of OC declined with time since cessation of smoking and drinking (Bosetti *et al.*, 2000b). The OR for having stopped drinking for 10 or more years, adjusted for time since smoking cessation, was 0.37 (95% CI 0.14- 0.99) and the OR was more strongly reduced with 10 or more years since cessation of both habits (OR= 0.11, 95% CI 0.01-0.90).

Today at least 15% of all cancers are estimated to be attributable to smoking, but this figure is expected to increase because of the uptake of tobacco use in low-income countries. Although this proportion is higher in men (25%) than in women (4%), and higher in high-income countries (16%) than low- income countries (10%) (Parkin *et al.*, 1994), the uptake of smoking by women and people in low-income countries may eventually eliminate these differences (Molarius *et al.*, 2001). In addition, the World Bank estimates that within the next year, tobacco is expected to kill approximately 4 million people worldwide. Already, it is responsible for one in 10 adult deaths; by 2030 the figure is expected to be one in six, or 10 million deaths each year-more than any other cause and more than the projected death tolls from pneumonia, diarrheal diseases, tuberculosis, and the complications of childbirth for that year combined. If current trends persist, about 500 million people alive today will eventually be killed by tobacco, half of them in productive middle age, losing 20 to 25 years of life (The World Bank, 1999). Of the 1.1 billion smokers worldwide, 800 million are found in developing countries, 700 million of these are men and 300 million are Chinese (WHO, 1996). China is the world's largest producer and consumer of tobacco, dwarfing countries like the USA (with only 4.4% of the world's population) and South Africa (with less than 1%). In the absence of widespread application of successful quitting progammes, it is predicted that by the 2020s there will be 10 million tobacco-related deaths annually, of which 70% will occur in developing countries (Peto *et al.*, 1994).

In South Africa, the male smoking rate was reported to be at 52% (Yach, 1995), the 16th highest in the world and was similar to those in Lithuania and Poland, whilst the rate of 17% among women ranked 45th, equivalent to Bulgaria's and above that of Portugal (Yach, 1995). The knowledge of the health effects of tobacco amongst a national representative sample of the South African population (2238 adults) and their attitudes towards tobacco control was assessed by Reddy *et al.* (1996). It was found that 34% of adults smoked (52% male, 17% female) and there were notable differences in gender smoking rates for Indians (61% of men and 7% of women smoked) and blacks (53% of men and 10% of women smoked) but not for coloureds (58% of men and 59% of women). The majority of the respondents (87%)

acknowledged the harmful effects of direct smoking. There was substantial support for a total ban of tobacco advertising on radio (61%), for local authorities to regulate smoking in public places (78%), for government assistance to farmers for tobacco crop replacement (53%) and for an increase in tobacco excise tax if the money was to be used for health purposes (50%). Over the past decade, prevalence rates for adult daily cigarette smoking have continuously declined. Adult (15+ years) daily smoking rates fell by a fifth, decreasing from 30.2% in 1995 to 24.1% in 2004. An estimated 2.5 million smokers stopped smoking during this period (Medical Research Council Technical Report, 2006). With regards to alcohol consumption, South Africa, which is considered as one of the Afro - region E countries has the 7th highest alcohol consumption rates of all 14 WHO regions, with an estimated 7.1 liters of absolute alcohol consumed per adult per year (Parry, 2005).

CHAPTER 6: DIETARY FACTORS AND OESOPHAGEAL CANCER

Many areas of high OC incidence are typified by poor diets that have specific nutrient deficiencies and some of these aspects are evaluated in this chapter. Ten "indicator" dietary items that are thought to represent a typical Xhosa diet were evaluated in this study to determine their association with the risk of developing OC. These food items were maize, sorghum, wheat-based products, green leafy vegetables, green podded vegetables, *imifino* (wild greens), fruit, dry beans, meat and pickled food. Tables 6.1 and 6.2 summarize the risk estimates of the "indicator" dietary items assessed in this study after adjusting for socio-demographic variables, total grams tobacco used per day and total grams ethanol consumed per week.

6.1 Effect of Maize, Sorghum, Wheat and Dry beans

In males, the reported frequency of consumption of maize was associated with a slightly elevated risk of developing OC although this was not statistically significant. In the highest consumption category (5-7 days), an OR of 1.21 was observed (95% CI 0.68-2.17). In females, the maize consumption frequency did not confer a risk in any of the three categories. Unlike the highest category in males, in females an OR of 1.07 (95% CI 0.61-1.87) was observed.

A decreased risk estimate, although not statistically significant, was observed for the consumption of sorghum in both males and females in the moderate consumption category. In males the risk dereased slightly from OR = 1.64 (95% CI 1.09-2.47) in the low consumption category to 1.41 (95% CI 0.87-2.27) for moderate consumers. Similarly, in females an inverse association was observed with higher frequencies of consumption. Hence those consuming sorghum ≤1 day/week had an almost 2-fold increased risk of developing OC (95% CI 1.29-3.03) whilst those consuming sorghum for 5-7 days/week had almost no risk of developing OC (OR = 1.07, 95% CI 0.51-2.24).

An inverse association was observed for wheat-based products in both males and females in comparison to the previous two food items, although these estimates were not statistically significant. In males a 34% reduction in risk was observed when consuming wheat for 5-7 days/week (OR = 0.66, 95% CI 0.40-1.10) whilst in females, a 22% reduction in OC risk was observed (OR = 0.78, 95% CI 0.45-1.34).

Dry bean consumption did not provide any effect on OC risk in both males and females. The risk estimates were not significant as observed in Tables 6.1 and 6.2.

Table 6.1 Odds Ratios of oesophageal cancer associated with selected food groups in males.

Dietary Variables	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Maize	<=1 day	22	59	1.00	1.00
	2-4 days	113	206	1.34 (0.76-2.36)	1.33 (0.75-2.35)
	5-7 days	196	353	1.23 (0.69-2.19)	1.21 (0.68-2.17)
				*p=0.84	p=0.88
Sorghum	Never	143	291	1.00	1.00
	<=1 day	84	143	1.55 (1.03-2.32)	1.64 (1.09-2.47)
	2-4 days	46	92	1.43 (0.89-2.30)	1.41 (0.87-2.27)
	5-7 days	29	42	1.91 (1.02-3.55)	1.54 (0.81-2.94)
				p=0.04	P=0.12
Wheat-based products	<=1 day	60	96	1.00	1.00
	2-4 days	118	200	0.92 (0.61-1.40)	1.00 (0.66-1.53)
	5-7 days	152	323	0.62 (0.37-1.02)	0.66 (0.40-1.10)
				p=0.05	P=0.07
Green leafy Vegetables	<=1 day	70	98	1.00	1.00
	2-4 days	172	310	0.91 (0.62-1.33)	0.92 (0.63-1.34)
	5-7 days	89	210	0.64 (0.40-1.03)	0.62 (0.38-0.99)
				p=0.06	p=0.04
Green podded vegetables	Never	38	87	1.00	1.00
	<=1 day	114	184	1.52 (0.96-2.41)	1.53 (0.96-2.42)
	2-4 days	124	267	1.33 (0.82-2.15)	1.29 (0.80-2.09)
	5-7 days	40	69	1.62 (0.89-2.95)	1.36 (0.73-2.53)
				p=0.26	P=0.55
Imifino (wild greens)	Never	64	179	1.00	1.00
	<=1 day	78	173	1.25 (0.81-1.92)	1.24 (0.80-1.91)
	2-4 days	119	165	1.84 (1.25-2.72)	1.76 (1.19-2.60)
	5-7 days	67	97	1.57 (1.00-2.47)	1.42 (0.89-2.24)
				p=0.006	P=0.03
Fruit	<=1 day	147	186	1.00	1.00
	2-4 days	128	297	0.60 (0.44-0.83)	0.59 (0.43-0.82)
	5-7 days	57	135	0.58 (0.37-0.91)	0.51 (0.32-0.81)
				p=0.005	P=0.001
Dry beans	<=1 day	23	42	1.00	1.00
	2-4 days	144	291	0.88 (0.50-1.53)	0.87 (0.50-1.52)
	5-7 days	166	287	0.93 (0.53-1.65)	0.91 (0.51-1.61)
				p=0.94	p=0.86
Meat	<=1 day	132	206	1.00	1.00
	2-4 days	137	286	0.86 (0.62-1.19)	0.82 (0.59-1.15)
	5-7 days	60	127	0.85 (0.56-1.31)	0.75 (0.48-1.17)
				p=0.41	P=0.17
Pickled food	Never	79	147	1.00	1.00
	<=1 day	108	173	1.36 (0.90-2.06)	1.33 (0.88-2.02)
	2-4 days	62	180	0.76 (0.47-1.21)	0.73 (0.46-1.17)
	5-7 days	23	29	1.73 (0.84-3.57)	1.34 (0.62-2.89)
				p=0.68	p=0.33

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day and total grams ethanol per week,
*p-value ≤0.05 indicates a significant trend between exposure and risk of developing oesophageal cancer.

Table 6.2 Odds Ratios of oesophageal cancer associated with selected food groups in females.

Dietary Variables	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Maize	<=1 day	26	54	1.00	1.00
	2-4 days	100	179	1.02 (0.59-1.77)	0.97 (0.55-1.70)
	5-7 days	207	332	1.04 (0.60-1.80)	1.07 (0.61-1.87)
				*p=0.88	P=0.67
Sorghum	Never	145	299	1.00	1.00
	<=1 day	86	122	2.07 (1.37-3.15)	1.98 (1.29-3.03)
	2-4 days	36	69	1.67 (0.99-2.80)	1.58 (0.93-2.70)
	5-7 days	27	42	2.28 (1.20-4.30)	1.07 (0.51-2.24)
				p=0.009	p=0.31
Wheat-based products	<=1 day	46	62	1.00	1.00
	2-4 days	113	171	0.94 (0.58-1.53)	1.05 (0.63-1.73)
	5-7 days	174	334	0.66 (0.39-1.13)	0.78 (0.45-1.34)
				p=0.06	p=0.20
Green leafy Vegetables	<=1 day	83	99	1.00	1.00
	2-4 days	160	260	0.90 (0.61-1.32)	0.86 (0.58-1.27)
	5-7 days	90	207	0.61 (0.37-1.00)	0.50 (0.30-0.84)
				p=0.05	p=0.007
Green podded vegetables	Never	37	47	1.00	1.00
	<=1 day	131	192	0.91 (0.55-1.50)	0.97 (0.58-1.62)
	2-4 days	122	256	0.71 (0.43-1.20)	0.73 (0.43-1.25)
	5-7 days	30	67	0.74 (0.39-1.43)	0.46 (0.22-0.94)
				p=0.16	p=0.02
Imifino (wild greens)	Never	22	79	1.00	1.00
	<=1 day	68	144	1.75 (0.97-3.18)	1.78 (0.98-3.25)
	2-4 days	143	183	2.63 (1.51-4.57)	2.40 (1.37-4.20)
	5-7 days	102	159	1.84 (1.04-3.27)	1.55 (0.87-2.77)
				p=0.05	p=0.26
Fruit	<=1 day	159	200	1.00	1.00
	2-4 days	126	240	0.75 (0.54-1.03)	0.76 (0.55-1.06)
	5-7 days	50	125	0.57 (0.36-0.92)	0.42 (0.25-0.70)
				p=0.02	p=0.001
Dry beans	<=1 day	24	45	1.00	1.00
	2-4 days	135	237	1.03 (0.59-1.78)	1.04 (0.59-1.81)
	5-7 days	177	284	1.03 (0.59-1.80)	1.06 (0.60-1.88)
				p=0.91	P=0.82
Meat	<=1 day	156	189	1.00	1.00
	2-4 days	138	255	0.74 (0.54-1.02)	0.70 (0.51-0.97)
	5-7 days	42	122	0.46 (0.29-0.74)	0.30 (0.18-0.51)
				p=0.001	P<0.0001
Pickled food	Never	100	149	1.00	1.00
	<=1 day	108	179	1.21 (0.82-1.79)	1.16 (0.78-1.72)
	2-4 days	54	137	0.8 (0.53-1.38)	0.86 (0.53-1.39)
	5-7 days	32	42	1.85 (0.95-3.60)	0.94 (0.45-1.98)
				p=0.70	P=0.57

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day and total grams ethanol per week,
*p-value ≤0.05 indicates a significant trend between exposure and risk of developing oesophageal cancer.

6.2 Effects of Green Vegetables and Fruit

Increased frequency of reported consumption of green leafy vegetables was associated with a protective effect. Males consuming green leafy vegetables for five or more days per week had a 38% reduced risk of developing OC (OR = 0.62, 95% CI 0.38-0.99), whilst in females the risk was reduced to 50% in the highest frequency category (5-7 days/week, OR = 0.50, 95% CI 0.30-0.84). In both sexes, a monotonic dose response was observed in relation to increased consumption levels and the trends were significant (p=0.04 for males and 0.007 for females). A re-analysis of the data on histologically confirmed cases also revealed protective effects with a 33% reduction in risk in males (OR = 0.67, 95% 0.32 – 1.39) and a 32% reduction in risk in females (OR = 0.68, 95% 0.26 – 1.77) for the highest consumption category.

Green podded vegetables (legumes) seem to elevate the risk of OC in males although this effect is not statistically significant. In females, however, a significant decrease in risk was observed (p=0.02). Hence females eating green podded vegetables for five or more days per week had a 54% reduced risk of developing OC (OR = 0.46, 95% CI 0.22-0.94).

Imifino, which is an isiXhosa term for wild green herbs, was positively associated with OC risk in both males and females although the risk was greater in females. After adjusting of age, hospital, residence, years of education, total grams tobacco smoked per day and total grams ethanol consumed per week, males consuming *imifino* for 2 – 4 days/week had an almost 2 times greater risk (OR = 1.76, 95% CI 1.19-2.60). A further increase in the exposure category resulted in a plateau effect. In females a similar trend was observed with females having a 2.4-times greater risk of developing OC if consuming *imifino* for 2 or more days per week. A re-analysis of the data on histologically confirmed cases revealed similar risk estimates. Males presented with an almost 2-fold greater risk when consuming imifino 2-4 days per week (OR = 1.70, 95% CI 1.02 – 2.83), whilst females presented with a 2-fold increased risk in the similar exposure category (OR = 2.09, 95% CI 1.09 – 4.02).

The relationship between reported fruit consumption and OC risk was evident in both males and females with both sexes presenting with a reduced risk. Males consuming fruit on five or more days had a 49% reduced risk of developing OC (OR=0.51, 95% CI 0.32-0.81). The inverse trend observed between the consumption categories was significant (p=0.001). Females also had a 58% reduction in OC risk when consuming fruits for five or more days (OR=0.42, 95% CI 0.25-0.70). A re-analysis of the

histologically confirmed data revealed similar risk estimates (Male OR = 0.39, 95% CI 0.15 – 1.03; Female OR = 0.40, 95% CI 0.10 – 1.64).

6.3 Effects of Meat Intake and Pickled Food

Meat intake was also associated with a reduced risk of developing OC although the trend was not significant. Males consuming meat on five or more days per week had a 25% reduced risk in comparison to those who ate meat at most once per week (OR = 0.75, 95% CI 0.48-1.17). In females, however the protective effects of meat intake were significant, with those eating meat at least five times per week have a 70% reduction in OC risk (OR = 0.30, 95% CI 0.18-0.51). A significant trend was observed in this case ($p<0.0001$). There was no association between OC risk and the consumption of pickled foods in both males and females as seen in Tables 6.1 and 6.2.

6.4 Principal Component Factor Analysis

Principal component factor analysis with varimax rotation was undertaken on the dietary items in an attempt to explain the dietary patterns in terms of a smaller number of factors, and hence elucidate the relationship between these variables. Combinations of the different variables are intended to produce indices that are uncorrelated. The lack of correlation is a useful property because it means that the indices are measuring different 'dimensions' in the data. The principal components will be of interest as measures of underlying 'dimensions' in the data.

Nine principal components were derived from the analysis (Figure 6.1). The first principal component had a variance of 3.31 whereas the second and third each had a variance of 1.96 and 0.88 respectively. This indicates that the first principal component is by far the most important of all the principal components for representing the variation in the measurement of dietary intake. The variances of the principal components are the eigenvalues and they account for all of the variation in the original data with respect to diet. The eigenvalues add up to the sum of the diagonal terms in the correlation matrix. The eigenvectors provide the coefficients on the principal components. The eigenvalue for a principal component indicates the variance that it accounts for out of the total variances of 9. Thus the first principal component accounts for 3.31/9x100%=37% of the variance and so fourth. Of the 9 principal components 2 principal components had eigenvalues greater than 1 (Figure 6.2). The third component was however also selected as it was close to 1. The three principal components cumulatively accounted for 68% of the variation in the data.

The others were discarded since they only accounted for a small proportion of the variation in the data (3-10%).

Figure 6.1 STATA output showing the 9 principal components

```
. factor eat_maizeref eat_sorgh eat_wht eat_glvegref eat_gpveg
eat_imif eat_fruitref eat_beanref eat_meatref,pc
(obs=1634)

            (principal components; 9 components retained)
Component     Eigenvalue     Difference     Proportion     Cumulative
-------------------------------------------------------------------------
     1         3.31213         1.35293       0.3680          0.3680
     2         1.95920         1.07456       0.2177          0.5857
     3         0.88465         0.24012       0.0983          0.6840
     4         0.64453         0.03081       0.0716          0.7556
     5         0.61372         0.15719       0.0682          0.8238
     6         0.45653         0.02615       0.0507          0.8745
     7         0.43038         0.03621       0.0478          0.9223
     8         0.39417         0.08947       0.0438          0.9661
     9         0.30470               .       0.0339          1.0000
```

Figure 6.2 STATA output showing the eigenvalues and factor loadings

```
. factor eat_maizeref eat_sorgh eat_wht eat_glvegref eat_gpveg
eat_imif eat_fruitref eat_beanref eat_meatref,pcf mineigen (0.8)
(obs=1634)

            (principal component factors; 3 factors retained)
  Factor      Eigenvalue     Difference     Proportion     Cumulative
-------------------------------------------------------------------------
     1         3.31213         1.35293       0.3680          0.3680
     2         1.95920         1.07456       0.2177          0.5857
     3         0.88465         0.24012       0.0983          0.6840
     4         0.64453         0.03081       0.0716          0.7556
     5         0.61372         0.15719       0.0682          0.8238
     6         0.45653         0.02615       0.0507          0.8745
     7         0.43038         0.03621       0.0478          0.9223
     8         0.39417         0.08947       0.0438          0.9661
     9         0.30470               .       0.0339          1.0000

                Factor Loadings
    Variable |       1           2           3      Uniqueness
-------------+------------------------------------------------
eat_maizeref |    0.22222     0.80256    -0.09636     0.29723
   eat_sorgh |    0.65226     0.25291    -0.37284     0.37158
     eat_wht |    0.45345     0.20234     0.79211     0.12600
eat_glvegref |    0.78122    -0.16695    -0.20550     0.31959
   eat_gpveg |    0.76710    -0.13018     0.02725     0.39387
    eat_imif |   -0.08533     0.74530    -0.19391     0.39965
eat_fruitref |    0.84288    -0.17050    -0.01393     0.26030
 eat_beanref |    0.28506     0.71853     0.16750     0.37440
 eat_meatref |    0.79622    -0.25407    -0.00942     0.30139
```

Factor loadings were then determined for the three principal components. The second stage of the analysis known as factor rotation was undertaken. Thus the provisional factors were transformed in order to find new factors that were easier to interpret. If this variance is large then the values tend to be either close to zero or close to unity. Varimax rotation therefore maximizes the sum of these variances for all the factors. It is desirable that the factor loadings for the new (rotated) factors are close to zero or very different from zero.

Hence as can be seen from Figure 6.3, factor 1 had a high positive loading for sorghum, green leafy veg, green podded veg, fruit and meat. Factor 2 had high positive loadings for maize, imifino and dry beans while factor three has a high loading for wheat-based products. The uniqueness is the percentage of the variance for the variable that is not explained by the factors. The higher the uniqueness, the more likely that it is more than just measurement error. Values over 0.6 are considered high. If the uniqueness is high then the variable is not well explained by the factor.

Figure 6.3 STATA output showing the rotated factor loadings

```
. rotate
            (varimax rotation)
              Rotated Factor Loadings
    Variable |       1          2          3     Uniqueness
-------------+------------------------------------------------
eat_maizeref |   0.05776    0.83040    0.09938     0.29723
   eat_sorgh |   0.65517    0.43068   -0.11697     0.37158
     eat_wht |   0.17358    0.10368    0.91275     0.12600
eat_glvegref |   0.82465    0.01246    0.01452     0.31959
   eat_gpveg |   0.74218   -0.00346    0.23514     0.39387
    eat_imif |  -0.19300    0.74457   -0.09333     0.39965
eat_fruitref |   0.83305   -0.02102    0.21282     0.26030
 eat_beanref |   0.06597    0.70437    0.35370     0.37440
 eat_meatref |   0.80639   -0.11024    0.19023     0.30139
```

The last stage of the analysis involved calculating factor scores creating a set of new variables (f1, f2, f3) that were estimates of the factors produced by the rotate command. These variables were then used as indices of dietary patterns among the population. The variable f1 was renamed "Dietary pattern 1" and comprised a diet including sorghum, green leafy vegetaables, green podded vegetables, fruits and meat. The variable f2 was renamed "Dietary pattern 2" and comprised a diet including maize, imifino (wild spinach) and dry beans. The variable f3 was renamed "Dietary pattern 3" and comprised a diet exclusively of wheat-based products.

The three dietary patterns (continuous variables) were each converted to three categories based on their factor score into low, moderate and high intake. Logistic regression analysis was subsequently conducted on each dietary pattern and the results presented in Table 6.3. It was observed that dietary pattern 1 conferred protective effects in both males and females. Males with a high level of adherence to this dietary pattern had a 21% reduced risk of developing OC (OR = 0.79, 95% CI 0.49-1.25). In females, both moderate and high levels of adherence to dietary pattern 1 produced protective effects with a 46% reduction in OC risk being observed in the high adherence category (OR = 0.54, 95% CI 0.34-0.89). Similarly, dietary pattern 3 also produced a 35% reduction in OC risk in both males (OR = 0.65, 95% CI 0.40-1.06) and females (OR = 0.63, 95% CI 0.37-1.07) when conforming to a high level of adherence. Dietary pattern 2 however reflects an increased risk in OC in both the moderate and high categories. In males and females, a 62% greater risk was observed for those in the 'moderate' category.

Box and Whisker (b-w) plots were drawn to depict the summary statistics of the three dietary patterns, by the level of education (Figure 6.4). It can be observed from the b-w plots for dietary pattern 1 that the data is positively skewed for "0" and "1-7" years of education while there is slight negative skewing of the data for "8+" years of education. Using the medians and the interquartile range, it is observed that there is an increase in the level of adherence to dietary pattern 1 as the level of education increases. The inverse is observed with regards to dietary pattern 2 (maize+beans+wild spinach). Dietary pattern 2 is more common amongst the poorer group of people. Dietary pattern 3 (exclusive to wheat-based products) increases moderately with an increase in education.

Figure 6.4 Box and Whisker plots showing the effect of level of education on dietary patterns

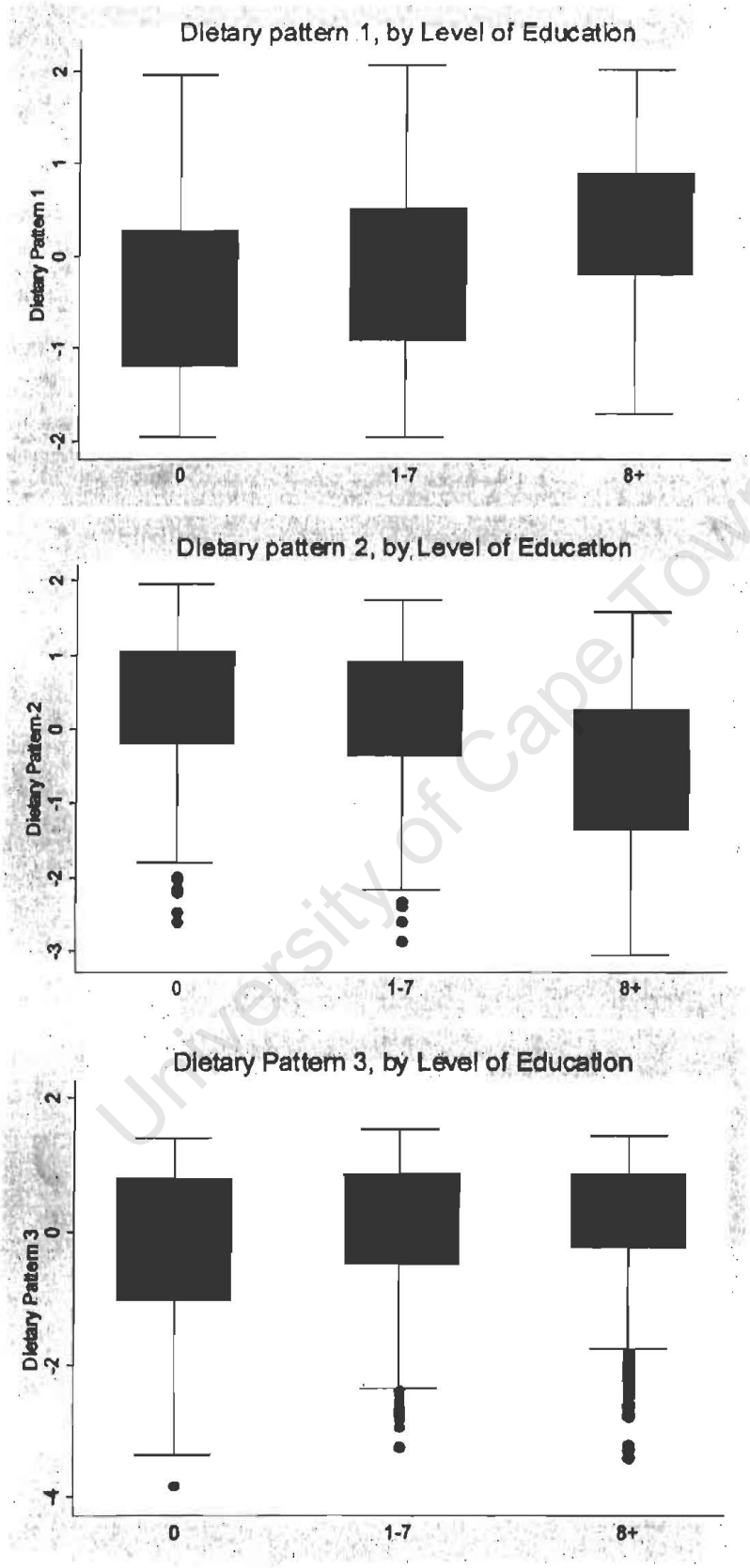


Table 6.3 Odds Ratios of oesophageal cancer associated with selected dietary patterns.

Variables	Categories	Males				Females			
		Cases (No.)	Controls (No.)	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)	Cases (No.)	Controls (No.)	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Dietary pattern 1 *(Sorghum, green leafy veg, green podded veg, fruit and meat)*	Low	141	258	1.00	1.00	177	251	1.00	1.00
	Moderate	76	145	1.08 (0.75-1.58)	1.06 (0.73-1.54)	47	136	0.52 (0.35-0.78)	0.52 (0.34-0.78)
	High	62	146	0.87 (0.55-1.37)	0.79 (0.49-1.25)	60	135	0.71 (0.45-1.12)	0.54 (0.34-0.89)
				p=0.22	p=0.15			p=0.0001	$p\leq$0.0001
Dietary pattern 2 *(Maize, imifino and dry beans)*	Low	57	171	1.00	1.00	46	135	1.00	1.00
	Moderate	160	283	1.63 (1.09-2.44)	1.62 (1.08-2.42)	137	244	1.65 (1.08-2.52)	1.62 (1.05-2.50)
	High	62	95	1.64 (1.02-2.65)	1.50 (0.92-2.44)	101	143	1.88 (1.19-2.97)	1.67 (1.04-2.67)
				p=0.02	p=0.03			p=0.0004	p=0.0002
Dietary pattern 3 *(Wheat-based products)*	Low	89	133	1.00	1.00	76	113	1.00	1.00
	Moderate	126	277	0.65 (0.44-0.96)	0.66 (0.45-0.98)	146	266	0.79 (0.52-1.22)	0.83 (0.53-1.29)
	High	64	139	0.61 (0.37-0.99)	0.65 (0.40-1.06)	62	143	0.50 (0.30-0.84)	0.63 (0.37-1.07)
				p=0.03	p=0.04			p=0.0004	p=0.0006

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day and total grams ethanol per week
*p-value obtained from likelihood-ratio test, p-values ≤0.05 indicates a significant association between exposure and oesophageal cancer.

6.5 Effect of Mouldy Maize Consumption

The effect of reported mouldy maize consumption was also explored and Table 6.4 presents the adjusted risk estimates for OC development following exposure to mouldy maize. It has often been observed that home-grown maize in the Eastern Cape Province of South Africa becomes mouldy through poor storage conditions. Maize source was stratified into commercial, home-grown and both. In our study, the participants were asked to indicate the source of the maize they consumed. Of the respondents, 16% males and 13% females indicated having exclusively home grown maize while 73% and 70% of males and females respectively indicated eating both home-grown and commercial maize. The use of home-grown maize was found to be associated with a 2.7-fold increased risk of developing OC in males (OR = 2.71, 95% CI 1.51 – 4.85) and a 1.5 times greater risk in females (OR = 1.52, 95% CI 0.90-2.57) while those eating maize from both sources (home-grown and commercial) had a slightly lower risk, as expected. When shown a photograph of mouldy maize and asked whether maize of this nature was consumed, of the maize eaters, 80% males and 78% females indicated that they consumed such maize. In male and females consumers, the adjusted risk estimates showed that there was no significant effect of mouldy maize consumption (Males OR = 0.98, 95% CI 0.61 – 1.57; Female OR = 0.86, 95% CI 0.55 – 1.34). The duration of mouldy maize consumption also did not show any statistically significant risk of OC development across any of duration strata. It is also known that mouldy maize is used in the preparation of traditional maize beer. The results also indicate that the consumption of traditional beer made from mouldy maize does not present with an increased risk of developing OC in both males and females (after adjustement for alcohol) strengthening the evidence that mouldy maize itself is not a risk factor for the development of OC. This observation is consistent with a previous finding of Sammon (1992). Hence while maize intake may be a risk factor due to the lack of essential vitamins and minerals, the mould contamination on the maize itself does not seem to pose as a risk factor for the development of OC.

Table 6.4 Odds ratio of oesophageal cancer associated with mouldy maize consumption.

Variables	Categories	Males			Females		
		Cases (No.)	Controls (No.)	OR (95% CI)	Cases (No.)	Controls (No.)	OR (95% CI)
Maize source	Commercial	23	88	1.00	44	101	1.00
	Home-grown	59	90	2.71 (1.51-4.85)	50	71	1.52 (0.90-2.57)
	Both	252	437	2.64 (1.50-4.65)	242	392	1.43 (0.89-2.29)
				p=0.0001			*p*=0.06
Mouldy maize consumption	No	62	130	1.00	72	121	1.00
	Yes	272	490	0.98 (0.61-1.57)	264	445	0.86 (0.55-1.34)
				p=0.27			*p*=0.30
Durations of mouldy maize consumption (years)	0	62	130	1.00	72	121	1.00
	<=54	98	171	1.16 (0.68-2.00)	80	131	1.12 (0.66-1.90)
	55-64	75	156	0.86 (0.51-1.47)	85	142	0.78 (0.47-1.32)
	65+	82	131	0.97 (0.55-1.71)	83	138	0.68 (0.39-1.20)
				p=0.70			*p*=0.31
Consumption of traditional beer from mouldy maize	No	49	131	1.00	61	118	1.00
	Yes	260	466	1.18 (0.75 – 1.85)	263	435	1.03 (0.66 - 1.61)
				p=0.47			*p*=0.89

OR, adjusted for age, hospital, residence and years of education, total grams tobacco per day and total grams ethanol per week
p-value obtained from likelihood-ratio test, P≤0.05 indicates a significant association between exposure and oesophageal cancer.

6.6 DISCUSSION

There is substantial evidence from different areas of the world indicating that dietary factors may play a major role in the pathogenesis of OC (Launoy *et al.*, 1998; Cheng and Day, 1996). Our study has confirmed the important role of diet in the development of OC in the Eastern Cape Province of South Africa and is discussed further in following paragraphs.

6.6.1 Effect of Grains

In relation to current published evidence on diet and OC risk, maize intake has once again been shown to increase the risk of developing OC more so in males than in females (male OR 1.21; female OR 1.07, highest consumption category), however non-significant. This can be attributed to not enough contrast in exposures between cases and controls. The consumption of sorghum was also associated with an increased risk in males (OR=1.54, non significant) whilst in females a distinct inverse trend was observed indicating a protective effect with increasing sorghum consumption. A halving of the risk was observed in females when comparing the lowest (≤1 day) to the highest (5-7 days) consumption category. Wheat-based products were observed to confer protective effects in both males and females with an approximate 30% reduction in risk in both males and females. Maize is the staple diet consumed by all in the former Transkei, hence this lack of contrast between cases and controls is not surprising although Sammon (1992) did report on varied frequencies of intake between the cases and controls. The study of van Rensburg *et al.*, (1985) also found that consumption of maize meal was a risk factor for OC, where daily and weekly consumers had an almost 6 and 2.4 times greater risk of developing OC respectively in comparison to those who consumed maize less often.

An association between an increased risk of cancer of the oesophagus and the consumption of cereal-based diets (maize, wheat, millet) has been observed in at least nine case-control studies in various parts of the world (including China, India, South Africa and Italy) (De Carli *et al.*, 1987; Brown *et al.*, 1988; Li *et al.*, 1989; Yu *et al.*, 1988; Wang, 1992; De Jong *et al.*, 1974; Wahrendorf *et al.*, 1989; Van Rensburg, 1981; Franceschi *et al.*, 1990). Oesophageal SCC has followed some decades after the adoption of maize as the dietary staple in east Africa, mirroring its geographical spread. Indeed by far the strongest evidence for a role of maize in OC development comes from surveys in Africa. Maize is an introduced crop in Africa, and its spread as a staple food and an ingredient in traditional beer seems to coincide, after allowing

for latency, with the rise in the frequency of OC (Cook, 1971, van Rensburg *et al.*, 1981, 1983). Maize is easier to grow and more resistant to attacks by birds than other grains like sorghum. Now, throughout the world, all areas of high risk for OC use maize as staples (Van Rensburg, 1981). The risk rises with increasing consumption of maize by the individual (Van Resnburg *et al.*, 1985, Rossi *et al.*, 1982).

The elevation in risk resulting from frequent consumption of maize could not be easily explained in terms of the confounding effect of age, education, occupation, tobacco use, or alcohol intake since all examined studies made allowances for such factors including ours. Van Rensburg (1981) described the association in Africa as fitting with prolonged exposure to a predominantly maize diet dating from childhood. There are possibly two explanations for such findings. Firstly, a specific deficiency in certain nutrients predisposes maize-eating populations to the processes of oesophageal carcinogenesis (Franceschi *et al.*, 1990), compared to populations whose staple foods are rice, cassava, peanuts, etc. (van Rensburg *et al.*, 1981). Secondly, a relatively high intake of maize may be an indicator of a less affluent diet, potentially poor in various nutrients which can prevent OC. Maize also has low levels of niacin, vitamin C, zinc, calcium and magnesium as well as inadequate riboflavin the content of which is further reduced by any refinement. Riboflavin deficiency has been implicated in the early stages of oesophageal cell proliferation and dysplasia (Muñoz *et al.*, 1987). Intervention experiments have been carried out among selected groups of people in high risk areas in China, in which the effect of supplementation of the diet with riboflavin on the incidence of precancerous lesions in the oesophagus was studied. No supplementation-associated improvement in the condition was detected (Muñoz *et al.*, 1985), but this was probably due to an improvement in the diet which occurred in the control group as well as in the treated populations (Wahrendorf *et al.*, 1988). When this was taken into account, a decrease in precancerous lesions correlated with an improvement in riboflavin status. In an integrated series of case-control studies conducted in Vaud, Switzerland on the risk of OC, the RRs were inversely and strongly related to the intake of whole grains, but directly related to refined grains (Levi *et al.*, 2000b) once again highlighting the role of essential micro- and macronutrients which are important in OC prevention. Initially African grains such as millet and sorghum were largely cultivated but these were gradually supplanted by high-yielding maize, a crop that, when supplemented with animal products, wild fruits and vegetables, probably constituted a well-balanced diet.

The protective effect of wheat is consistent with studies elsewhere. The study by Soler *et al.* (2001) also showed that whole grain cereals/wheat may have a protective role on OC. Wheat and wheat-based products are known to contain high amounts of insoluble fiber which may help protect against OC and a variety of other cancers. Reddy and Cohen (1986) however suggested that specific components of fiber, rather than total fiber, are more likely to be responsible for any protective effect against cancer. However both black and white men in the USA showed significant reductions in risks for OC when comparing the highest versus lowest quartile of consumption for total fibre (OR= 0.3, 95% CI = 0.1 – 0.8) (Brown *et al.*, 1998). In a study conducted to investigate the possible differential role of refined and whole grain cereals on the risk of upper and respiratory tract neoplasms in Switzerland, it was found that refined cereals are unfavourable, while whole grains are a favourable indicator of the risk of upper digestive and respiratory tract neoplasms (Levi *et al.*, 2000a).

6.6.2 Effect of Fruit and Vegetables

Our findings indicated that high consumption (5 or more days) of fruit was observed to lower the risk of developing OC by 49% in males and 58% in females and a significant trend was observed in both sexes. A similar associaton was observed for green leafy vegetables, while green podded vegetables like peas and green beans were only found to be protective in females with no significant association in males. This could be the result of males not eating adequate amounts in comparison to females or perhaps recall bias may have influenced the answers provided by males. Furthermore, the questionnaire focused on frequency of intake rather than portion sizes. In a comparison of areas of moderate and high risk of OC in South Africa, persons living in areas of high risk had lower levels of vitamins A and C, magnesium and riboflavin (van Rensburg *et al.*, 1983). Fruits and vegetables are excellent sources of such micronutrients and deficiencies can lead to tissue predisposition to carcinogenesis. The low intake of fresh fruit and vegetables has also been shown to be a strong risk factor in the former Transkei (Sammon, 1992). The involvement of specific vitamin (vitamins A, B_{12}, E, folic acid) and trace element deficiencies (selenium) in the high-risk populations with a very poor socioeconomic status has also been considered an important factor (Jaskiewicz *et al.*, 1988a; Jaskiewicz *et al.*, 1988b).

The results of our study concer with the wealth of information on the protective effects of fruit and vegetables on cancer risk and are consistent with the recommendations in the 'National 5 A Day for Better Health Program' (Heimendinger *et al.*, 1996) of the consumption of 5 or more servings of fruits and vegetables per day for cancer prevention. Similarly, a retrospective cohort study in Linxian, China, as reported by Yu *et al.* (1993) also found that frequent consumption of fresh vegetables was associated with decreased risk of developing OC. Subsequently the protective effect of fruits on OC was also observed in a study conducted to investigate the role of common foods in the etiology of OC in China (Li *et al.*, 2003) where the risk decreased (*p* for trend less than 0.001) as frequencies of fresh fruit intake increased. Whilst similar results were also observed in Uruguay (De Stefani, *et al.*, 2000) and Golestan province, Iran (Pourshams *et al.*, 2005) where fruit and vegetable intake was associated with a reduction in OC risk (fruit OR for high intake = 0.18; Vegetable OR = 0.64), these results concurred with our findings in that fruit intake was more protective than vegetable intake. Apart from the high OC incidence areas Vainio *et al.* (2003) also reported on three case-control studies in the USA that found significant inverse associations for fruit consumption.

During the last thirty years, over 300 epidemiological studies (case-control, cohort or ecological correlations) have been conducted around the world to investigate the relationship between fruit and vegetable consumption and cancer risk. About 80% of these studies found a significant protective effect on overall consumption of vegetables and/or fruit, or at least of some types of vegetables and fruits (WCRF/AICR, 1997). Preliminary results from the large European Prospective Investigation into Cancer and Nutrition (EPIC) study confirm these results, suggesting, for example, that a daily consumption of 500g of fruit and vegetables can decrease incidence of cancers of the digestive tract by as much as 2.5% (Bueno *et al.*, 2002). In a recent meta-analysis of fruit and vegetable intake and risk of developing cancer including OC from 13 studies (1 cohort and 12 case-control), all were supportive of a protective effect of green vegetables and fruit (Riboli & Norat, 2006). On average, there was a significant protective effect of fruit and vegetables which seemed to be more important for fruit than for vegetables. In Europe, it has been postulated that the decreasing trends in OC mortality in some countries despite the increases in the consumption of tobacco and alcohol, might be due to the protection conferred by the increasing trends in the intake of fruit (Cheng *et al.*, 1992).

Fruit and vegetables are rich in antioxidant substances and micronutrients that are thought to neutralize the harmful effects of DNA-damaging free radicals such as those produced by metabolism and by external factors such as pollution and smoking (Shklar, 1998) and so play a protective role in carcinoma of the oesophagus. An inverse association with OC has been reported with various micronutrients, most notably vitamins C and E and β-carotene (Decarli *et al.*, 1987; Nomura *et al.*, 1997). In a recently published case-control study conducted in three areas of Northern Italy, Franceschi *et al.* (2000) showed that the majority of examined micronutrients, most notably carotene, lutein+zeaxathin, vitamins C and niacin, were inversely related to the risk of OC. Fruit and vegetables are also the primary source of many other substances with hypothesized antioxidative (and other anticarcinogenic) properties, such as carotenoids, dithiolthiones, glucosinolates, indoles, isothiocyanates, flavonoids, phenols, protease inhibitors, plant sterols, allium compounds and limonene (Steinmetz & Potter, 1991). In Uruguay, De Stefani *et al.*, (2000) examined the role of antioxidants in OC development and found 12 of 15 dietary antioxidants to display significant inverse associations on OC risk. The strongest effect was observed for high intake of β-cryptoxanthin (OR = 0.16). α-Carotene, lycopene, and β-sitosterol were also associated with significant reductions in risk. Flavonoids in fruits and vegetables also have a wide variety of biological effects, and those of particular interest in relation to cancer prevention include their antimutagenic and antiproliferative capability, strong antioxidant capacity, and involvement in cell signaling, cell cycle regulation, and angiogenesis (Mayne *et al.*, 1994).

When associations between antioxidant intake and cancer are stronger in the presence of a third factor, one that is presumed to be a marker for increased free radical exposure (such as smoking), this may be seen as support for the neutralizing role antioxidants are thought to play in the pathway between fruit and vegetable consumption and disease. From this perspective, one can speculate that antioxidant mechanisms will be more important in the presence of higher levels of oxidizing free radicals. Indeed, stronger associations with fruit and vegetable consumption (and antioxidant vitamins) have been seen amongst smokers (Barone *et al.*, 1992, Terry *et al.*, 2000) and heavy drinkers (Launoy *et al.*, 1998; Bosetti *et al.*, 2000) when such analyses were performed for oesophageal squamous-cell carcinoma. Folic acid (folate), also found in several fruits and vegetables, has attracted increasing attention in recent years. Folate is crucial for normal DNA synthesis and repair, and deficiency

of this nutrient is hypothesized to lead to cancer through consequent faults in these processes (Duthie, 1999).

6.6.3 Effect of Dry Beans

While no effect of bean consumption on OC risk was observed in females a protective effect was found in males with those presenting with a 10% reduced risk, although not significant. A protective effect of bean consumption was also observed in Linzhou City, China where a 63% reduction in risk was observed amongst those eating more than 80 g/week (Xibin, S., 2003). The lack of any significant effect in our study may have been due to our inability to determine the portion sizes consumed as the questionnaire used in this study only took into account the frequency of consumption resulting in effect dilution due to non-differential misclassifcation.

In contrast, Sammon's study (1998) in the former Transkei found a significant association between OC and the consumption of beans, particular Lima beans. Sammon (1998) proposed that since beans contain very high trypsin inhibitory action (Bradbury *et al.*, 1985; Aletor & Ojo, 1989), these protease inhibitors allow for the stong proliferative drive on oesophageal mucosa by epidermal growth factor and transforming growth factor-α (Playford *et al.*, 1993, 1995) which can be controlled in the presence of protease action.

6.6.4 Effect of *Imifino*

As a source of food, the leaves of various wild plants are cooked traditionally with other pot herbs to form a relish that is served with maize meal. The collective name for these plants is *imifino* and it was found to be a risk factor for OC in both males and females (male OR 1.76, female OR 2.40; 2-4 day consumption, significant). The results concur with those found by Sammon (1992), where it was observed that those consuming the plant *Solanum nigrum* had a 3.6-fold increased risk of developing OC Another study conducted by Purchase *et al.* (1975) also showed that rats fed a diet consisting of maize, beans, salt, and the wild plants *Solanum nigrum* and *Sonchus oleraceus* had, among other disorders, severe epithelial dysplasia of the oesophagus. A correlation was also observed between the intake of plants and OC in the island of Curacao, the Netherlands (Morton, 1968). The indigenous dish, cadushi soup, made from the powdered flesh of the wild cactus, *Cereus repandus*, was consumed by 44 of the 49 oesophageal cancer patients.

However it is important to note that the *imifino* dish may vary depending on geographic and seasonal availability of plants. The nutritional importance of traditional leafy vegetables was also stressed by Gockowski *et al.* (2003), where it was found that the physical quantities consumed of these vegetables and the method by which they are prepared influence the extent to which they are beneficial to one's health. Hence the risk associated with dietary wild plant intake is investigated further in Chapter 7 where each plant is explored individually.

6.6.5 Effect of Meat Intake

Meat intake displayed protective effects which were more pronounced in females (70% reduction) than in males (25% reduction). A cross-sectional survey in Southern Africa (Jaskiewicz, 1989) also found that higher intakes of animal protein were associated with decreased incidence of mild cytological lesions. A survey conducted by Bradshaw *et al.* (1983) also revealed that there was a low intake of meat in the Transkei, which may be a risk for OC. In Uruguay (De Stefani *et al.*, 1990), increased fresh meat intake was associated with a statistically non-significant decrease (OR = 0.6) in the risk of OC. In Calvados, France (Tuyns *et al.*, 1987), meat was also protective; with risk decreasing across quartiles and the reduction being greatest in the highest quartile of intake (OR = 0.2). Meat is a good source of protein and the study of Pourshams *et al.* (2005) also found that the protein intake among Turkmen women was lower than that of Persian women and that Turkmen women were of higher risk of developing OC. However, although the above studies indicate protective effects of meat, the results of epidemiological studies on macro-nutrients such as protein have thus far been much less consistent in establishing an associated risk of OC (Stewart *et al.*, 2003). The latest reviews (Cheng and Day, 1996; World Cancer Research Fund, 1997) reflect the difficulties in making a judgement about the relationship between red meat consumption and OC. Hence no clear risk patterns have emerged from international studies for consumption of red meat. Conflicting results have been documented (Ziegler *et al.*, 1981; Gao *et al.*, 1994; Launoy *et al.*, 1998; Yu *et al.*, 1994).

It is important to note that meat intake in the population studied is mainly through curries rather than salted or barbequed. The latter two forms of meat intake has been considered as a source of exogenous nitrosamines, like nitrodimethylamine (De Stefani *et al.*, 1994) and has been considered as related to nitrosamine exposure (Eicholzer and Gutzwiller, 1998).

6.6.6 Effect of Pickled Foods

Although an elevated risk was observed in the highest exposure category for males, the risk estimate was not significant. Females however did not present with an increased risk of developing OC. International studies also indicate some merit in the association of pickled foods with OC risk (Yang *et al.*, 1980; Chang-Claude *et al.*, 2002) although no relationship was found in the Linxian study (Guo et al., 1994). A case-control study conducted in Hong Kong reported a direct association between pickled vegetable consumption and OC risk. The OR for consumption of pickled food daily or more versus < once/year was 13.1 (95% CI = 2.60-66.9) (Cheng *et al.*, 1992). Similar results were reported by Wang *et al.* (1992) in their research in two areas of Shanxi, North Central China. The varied risks including those in this study can be attributed to the methods of preparation/processing of the pickled foods/vegetables and the period of storage which can affect the contamination of nitrosamines.

6.6.7 Principal component factor analysis

The complexity of diet as an environmental exposure creates certain difficulties in nutritional epidemiology since both nutrients and non-nutrients are present in the same food, thus making it almost impossible to evaluate the effect of individual nutrients separately among humans. Principal component factor analysis (PCFA) thus provided a means of highlighting groups of foods that reflect commonalities in dietary patterns within the population thereby differentially predicting OC risk. This enhances the analysis based on individual foods as it became possible to establish food group consumption patterns in this population.

Stratified by level of education, it was observed that highly educated individuals (>8 years of education) had a greater level of adherence to dietary pattern 1 which can be labeled as a healthy diet, whilst individuals with liitle or no education adhered more to dietary pattern 2 which does result in deficiencies in micro- and macro-nutrients, hence presenting them with a greater risk of OC. If differences in nutrient intakes did occur, it would be more a function of different amounts of foods rather than the types of foods consumed.

It was evident from the data that dietary pattern 1 was protective in both males and females, whilst dietary pattern 2 was associated with an increased risk of developing OC. Similarly to dietary pattern 2 in our study, Sammon (1998) observed a significant

association with the consumption of a combined diet consisting of maize, pumpkin and beans. Dietary pattern 3 which consisted exclusively of wheat-based products and was more representative of a wealthier group of people was found to be protective in both males and females. Based on these results it was possible to evaluate the diets that are high and low risk for OC and as such intervention strategies can be tailored for this population group. It is important to note that these dietary change strategies may need to focus on the females, who in many settings are the food "gatekeepers" in the home.

6.6.8 Effect of Mouldy Maize Consumption

The seed-borne fungus *Fusarium verticillioides* (Sacc.) Nirenberg, which is a ubiquitous contaminant of corn (*Zea mays* L.) worldwide (Marasas, 1996) is responsible for mould growth on maize and the consumption of this maize has been correlated with OC risk in the former Transkei, where corn is a dietary staple (Marasas *et al.* 1981; 1988). The fumonisins are toxic carcinogenic secondary metabolites, produced by this fungus and while there are to-date 28 structural fumonisin analogues known (Rheeder, 2002), most research has focused on the most commonly and widely occurring natural form, fumonisin B_1. Numerous surveys have been conducted in high and low OC incidence areas worldwide to show the relation of fumonisin exposure with OC. However these studies demonstrated only inconclusive associations between fumonisins and human cancer. Investigators in South Africa suggested an association between high levels of fumonisin-producing moulds on corn used to make alcoholic beverages and OC in human subgroups (Rheeder *et al.*, 1992). However, those studies were limited by the lack of controlled conditions, particularly for established confounding risk factors (e.g., Tobacco smoking, alcohol consumption, and dietary factors), and therefore do not allow any definitive conclusions to be made about cancer causation in humans. Other studies associating high levels of fumonisin-producing moulds on corn with OC lacked similar controls (Chu and Li, 1994; Shephard *et al.*, 2000), or did not measure fumonisin levels (Franceschi *et al.*, 1990). Further, a controlled study with corn pancake from 16 households in an area of China with high incidence of gastric cancer, Groves *et al.* (1999) observed a lack of association between consumption of fumonisin contaminated corn with gastric or any other human cancer. Similarly studies performed in Italy did not establish any correlation between the intake of FB_1 and the oesophageal cancer incidence (Pascale *et al.*, 1995; Logrieco *et al.*, 1995; EHC, 2000).

The results of our study indicate that mouldy maize consumption itself is not a risk factor for OC in male and females, and exposure to FB_1 is not a risk for the following reasons:

1. After having adjusted for tobacco and alcohol exposures, if the toxins in the mouldy maize were associated with OC risk, then a residual effect should have at least been observed, which was not the case in this study.
2. Furthermore, the duration of exposure did not show a dose response. Once again if the toxins were associated with OC development, there would have been a dose-response effect observed in this study. This is one of the parameters necessary for a causal association according to the Bradford-Hill criteria.
3. In the rural high and low incidence areas of South Africa, including this population, inhabitants of these areas also separate maize into 'visibly good' and 'mouldy' and use the latter for beer brewing. Studies on yeast fermentation of maize have shown that fumonisins are not destroyed during fermentation (Bothast *et al.*, 1992). High levels of fumonisins are present in mouldy maize in comparison to 'visibly good maize'. Therefore consumption of traditional maize beer should have presented as a risk factor for OC. However after adjustment for sociodemographic variables, tobacco and alcohol exposures, no increased risk was observed for either males and females. Once again, an underlying effect would have been observed if the toxins in mouldy maize were responsible for increase OC risk.
4. A chronic feeding study undertaken by Gelderblom *et al.* (2001) where varying levels of culture material of *Fusarium verticillioides* Strain MRC826 was mixed into the diet of vervet monkeys and fed for over 13.5 years did not show any evidence of OC.

Furthermore the data provided in Table 6.5 also indicates that the levels of fumonisin B_1 are variable and no correlation can be made exclusively on the basis of FB_1 levels and OC risk. Levels found in Bizana, a low OC incidence area were twice that found in Mazandaran Province and in the 1994 harvest from Linxian, both areas of high OC incidence. Furthermore, the levels found in Mazandaran Province are comparable to the levels found in the low OC area of Shangqui.

Table 6.5 Comparison of FB_1 levels from high and low oesophageal cancer incidence areas.

Country	Region	Sample type	Year	FB_1 levels (mg/g)	Reference
South Africa	Bizana (low OC)	mouldy corn	1989	4.05	Rheeder *et al.*, 1992
	Centane (high OC)	mouldy corn	1989	53.74	Rheeder *et al.*, 1993
Iran	Mazandaran (high OC)	Corn	1998	2.27	Shephard *et al.*, 2000
	Isfahan (low OC)	Corn	1998	0.17	Shephard *et al.*, 2001
China	Linxian (high OC)	mouldy corn	1991	49.3	Chu & Li, 1994
	Cixian (high OC)	mouldy corn	1991	93.8	Chu & Li, 1995
	Shangqui (low OC)	Corn	1994	2.7	Gao & Yoshizawa, 1997
	Linxian (high OC)	Corn	1994	2.73	Gao & Yoshizawa, 1998

Based on the inconclusive evidence from published studies and the evidence obtained from this study, it is apparent that FB_1 exposure through mouldy maize consumption is not a risk factor for OC, and this lends further support to nutritional deficiencies within this population that result from high maize intake. Another avenue that might be worth exploring is the role of silica in OC (Yu *et al.*, 2005) since the consumption of home-grown maize did present with a significant increase in OC risk. This measure could be a surrogate for silica exposure as a result of the grinding of the maize in grinstones.

The role of imifino and other wild plants used for traditional medicines is explored further in the next Chapter.

CHAPTER 7: THE EFFECT OF DIETARY AND MEDICINAL WILD PLANT USE ON OESOPHAGEAL CANCER

There are close to 30 000 species of flowering plants, accounting for almost 10% of the world's higher plants, many of which, through cultural diversity, are used daily either as food or phytomedicines (Van Wyk & Gericke, 2000). Indigenous edible plants or veld foods also still play a significant role and are mainstays in the diets of people living in the rural areas of the former Transkei (Rose, 1974). Although they are seasonal, the variety of plants used ensures that some will be available throughout the year. The leaves of certain plants such as *Solanum nigrum* can be dried and kept for use during winter months. The leaves of most of the wild plants are chopped or crushed and mixed with maize meal and is traditionally known as *isigwampa.* In addition to dietary wild plants, South Africa also has a rich diversity of medicinal plants and their use amongst the population is widespread with the majority of South Africans consulting traditional healers and using African Traditional Medicines (ATMs) to alleviate symptoms of illness or to prevent illness (Evans, W.C., 1989). Such a resource plays a vital role, as it is the first resource of people living in rural areas. Although health care facilities are present, the services and advice given by indigenous practitioners are valued because they are given in terms that patients can understand and in the context of cultural values and practices that are shared by both patients and healers alike.

Recent publications have however expressed suspicions that certain wild vegetables may contribute to squamous cell carcinoma of the oesophagus (Sammon & Alderson, 1998; Sammon, 1992). Plants such as *Solanum nigrum* L. (garden nightshade) and *Pteridium aquilinum* (L.) Kuhn (bracken fern) are used by the Zulu and Xhosa people for a wide range of medicinal purposes and cooked as vegetables (Van Wyk *et al.*, 1997; Hutchings *et al.*, 1996; Watt & Breyer-Brandwijk,1962; Robets, 1990) *S. nigrum* has been found to induce hepatotoxicity in sheep and cause fetal malformations in hamsters (Watt & Breyer-Brandwijk,1962). Extracts of *P. aquilinum* demonstrated mutagenicity for *Salmonella typhimurium* strain TA 100 (Pamukcu *et al.*, 1980) and have been found to induce ileal and bladder tumours in rats (Evans & Osman, 1974; Hirono *et al.*, 1987). This plant is consumed as a food in Japan and has been associated with an increase in OC and stomach tumours (Pamukcu *et al.*, 1980). A variety of dietary and medicinal wild plants used by this study population have thus been evaluated for their risk in the development of OC. In addition to the

results presented in this Chapter, various mechanistic studies were undertaken to bolster the epidemiologic evidence and are discussed in the subsequent Chapters.

7.1 Effect of Detary Wild Plant Intake

The effect of dietary wild plant consumption on OC risk was explored in males and females and the adjusted risk estimates are presented in Tables 7.1 and 7.2 respectively. Of the 19 dietary wild plants investigated, eight were observed to produce a significant increase in OC risk amongst males and nine amongst females. The plant *Rumex lanceolatus* was associated with an almost 2-fold increased risk in males (OR=1.86, 95% CI 1.31- 2.63) whilst in females the risk estimate was slightly lower (OR=1.68, 95% CI 1.18-2.41). This plant was consumed by 21% males and females. A re-analysis of a restricted series of histologically confirmed cases also revealed a postive association although the risk estimates were slightly lower (Male OR = 1.60, 95% CI 0.86 - 2.98; Female OR =1.62, 95% CI 0.80 - 3.26). The plant *Colocasia esculenta* was also associated with an increased risk in both males and females although the risk estimate was much higher in females (males OR=1.78, 95% CI 1.06-2.97; females OR=2.03, 95% CI 1.19-3.45). Unlike *R. lanceolatus*, the plant *C. esculenta* was only consumed by 7-8% of the population. It was startling to observe however that consumption of the plants *Raphanus raphinastrum*, *Raphanus nasturtio aquatica* and *Sida dregei*, all of which belong to the Brassicaceae family, was associated with signicant increases in OC risks in both males and females. The order of the risk estimates in males and females were as follows: *S. dregei* > *R. nasturtio aquatica* > *R. raphinastrum*. The risk estimate for *S. dregei* however was higher in males (OR=3.80, 95% CI 1.66-8.70) than in females (OR=2.06, 95% CI 1.06-4.00). Of these three plants, *R. raphinastrum* was the most popular plant eaten (53% males, 75% females), whist the remaining two plants were consumed by less than 10% of the population. The plants *Urtica urens* and *Chenopodium album* were also two of the most popular plants consumed (Tables 7.1 and 7.2) and whilst the risk estimates were indicative of a slightly elevated risk of developing OC, these estimates were not significant in males and females. Males consuming the plant *Solanum nigrum* had a 32% increased risk of developing OC (OR=1.32, 95% CI 0.99-1.75) whilst females had a 27% increased risk (OR=1.27, 95% CI 0.91-1.77). The plant *Coccinia rehmanii*, although only used by 3% males and 6% females, was also associated with an increased risk (males OR=4.12, 95% CI 1.67-10.19; females OR=2.53, 95% CI 1.39-4.60). The plant *Sida rhombifolia* was also associated with an elevated risk in both males and females although the confidence intervals were extremely wide due to the low prevalence of consumption. These plants are cooked

fresh although some of them are sun-dried and stored for winter use. They are usually boiled with other pot herbs to form a relish that is served with maize meal.

Table 7.1. Odds Ratios of oesophageal cancer associated with the consumption of wild plants as dietary supplements amongst males (N=955)

Botanical name	Family	% Users	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Urtica urens L.	Urticaceae	53	No	140	312		
			Yes	194	309	1.28 (0.96-1.70)	1.26 (0.94-1.68)
Raphanus raphinastrum	Brassicaceae	53	No	133	316		
			Yes	201	305	1.45 (1.09-1.93)	1.40 (1.05-1.87)
Chenopodium album L.	Chenopodiaceae	52	No	144	318		
			Yes	190	303	1.26 (0.94-1.68)	1.21 (0.90-1.61)
Bidens pilosa L.	Asteraceae	48	No	164	337		
			Yes	170	284	1.10 (0.83-1.45)	1.07 (0.81-1.43)
Solanum nigrum complex	Solanaceae	48	No	153	347		
			Yes	181	274	1.39 (1.04-1.84)	1.32 (0.99-1.75)
Physalis viscosa L.	Solanaceae	47	No	163	343		
			Yes	171	278	1.17 (0.88-1.56)	1.14 (0.85-1.52)
Sonchus oleraceus L.	Asteraceae	38	No	193	398		
			Yes	141	223	1.29 (0.92-1.80)	1.22 (0.86-1.71)
Amaranthus hybridus L.	Amaranthaceae	36	No	206	408		
			Yes	128	213	1.11 (0.81-1.52)	1.07 (0.78-1.47)
Centella asiatica L.	Umbelliferae	26	No	234	471		
			Yes	100	150	1.17 (0.86-1.60)	1.14 (0.84-1.56)
Rumex lanceolatus Thunb.	Polygonaceae	21	No	240	514		
			Yes	94	107	1.93 (1.37-2.72)	1.86 (1.31-2.63)
Amaranthus thunbergii Moq.	Amaranthaceae	17	No	267	529		
			Yes	67	92	1.28 (0.88-1.84)	1.24 (0.86-1.80)
Zantedeschia aethiopica (L.) Sg	Araceae	10	No	288	567		
			Yes	46	54	1.53 (0.99-2.37)	1.45 (0.93-2.25)

Colocasia esculenta	Araceae	7	No	300	587		
			Yes	34	34	1.78 (1.06-2.98)	1.78 (1.06-2.97)
Raphanus nasturtio aquatica L.	Brassicaceae	6	No	306	595		
			Yes	28	26	1.92 (1.07-3.43)	1.93 (1.07-3.47)
Gunnera perpensa L.	Gunneraceae	5	Never	317	594		
			Ever	17	27	1.28 (0.66-2.50)	1.32 (0.68-2.57)
Sida dregei Burtt. Davy	Brassicaceae	3	No	314	612		
			Yes	20	9	4.01 (1.76-9.15)	3.80 (1.66-8.70)
Coccinia rehmanii Cogn.	Cucurbitaceae	3	No	316	614		
			Yes	18	7	4.30 (1.74-10.63)	4.12 (1.67-10.19
Oxalis corniculata L.	Oxalidaceae	2	No	324	615		
			Yes	10	6	2.86 (0.98-8.36)	2.60 (0.89-7.60)
Sida rhombifolia L.	Malvaceae	1	No	325	618		
			Yes	9	3	4.91 (1.26-19.11)	4.54 (1.17-17.60)

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day and total grams ethanol per week

Table 7.2. Odds Ratios of oesophageal cancer associated with the consumption of wild plants as dietary supplements amongst Females (N=903)

Botanical name	Family	% Users	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Raphanus raphinastrum	Brassicaceae	76	No	62	158		
			Yes	274	409	1.59 (1.12-2.26)	1.49 (1.04-2.12)
Urtica urens L.	Urticaceae	75	No	75	155		
			Yes	261	412	1.22 (0.87-1.71)	1.16 (0.82-1.64)
Chenopodium album L.	Chenopodiaceae	75	No	67	161		
			Yes	269	406	1.46 (1.03-2.08)	1.39 (0.97-2.00)
Solanum nigrum complex	Solanaceae	70	No	85	184		
			Yes	251	383	1.32 (0.95-1.83)	1.27 (0.91-1.77)
Bidens pilosa L.	Asteraceae	68	No	98	187		
			Yes	238	380	1.03 (0.75-1.42)	1.04 (0.75-1.44)
Physalis viscosa L.	Solanaceae	64	No	112	210		
			Yes	224	357	1.20 (0.82-1.53)	1.05 (0.77-1.45)
Sonchus oleraceus L.	Asteraceae	56	No	124	277		
			Yes	212	290	2.03 (1.42-2.91)	1.84 (1.28-2.65)
Amaranthus hybridus L.	Amaranthaceae	56	No	135	266		
			Yes	201	301	1.33 (0.95-1.85)	1.25 (0.89-1.76)
Centella asiatica L.	Umbelliferae	40	No	197	346		
			Yes	139	221	0.97 (0.73-1.29)	0.95 (0.71-1.27)
Amaranthus thunbergii Moq.	Amaranthaceae	24	No	252	437		
			Yes	84	130	0.99 (0.71-1.40)	1.01 (0.72-1.43)
Rumex lanceolatus Thunb.	Polygonaceae	21	No	245	468		
			Yes	91	99	1.74 (1.23-2.46)	1.68 (1.18-2.41)
Zantedeschia aethiopica (L.) Sg	Araceae	13	No	288	502		
			Yes	48	113	1.25 (0.82-1.89)	1.11 (0.72-1.71)
Colocasia esculenta	Araceae	8	No	297	535		

			Yes	39	32	2.20 (1.31-3.68)	2.03 (1.19-3.45)
Raphanus nasturtio aquatica L.	Brassicaceae	8	No	301	531		
			Yes	35	36	1.86 (1.11-3.13)	1.80 (1.06-3.07)
Coccinia rehmanii Cogn.	Cucurbitaceae	6	No	304	546		
			Yes	32	21	2.67 (1.49-4.79)	2.53 (1.39-4.60)
Sida dregei Burtt. Davy	Brassicaceae	5	No	312	549		
			Yes	24	18	2.27 (1.19-4.31)	2.06 (1.06-4.00)
Oxalis corniculata L.	Oxalidaceae	3	No	324	551		
			Yes	12	16	1.24 (0.57-2.71)	0.94 (0.41-2.16)
Gunnera perpensa L.	Gunneraceae	3	Never	319	555		
			Ever	17	12	2.80 (1.28-6.12)	2.26 (1.00-5.10)
Sida rhombifolia L.	Malvaceae	2	No	324	565		
			Yes	12	2	11.02 (2.37-51.37)	6.52 (1.30-32.73)

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day and total grams ethanol per week

7.2 Effect of Medicinal Plant Intake

The risk estimates for the medicinal plants investigated are presented in Tables 7.3 and 7.4 for males and females respectively. Amongst males, four medicinal plants showed elevated and significant risks of developing OC similarly as in females where four plants were observed to increase the risk of developing OC. The plant *Acokanthera oppositifloia* was used by 10% males and 8% females and was associated with an almost 2-fold increased risk in both sexes (males OR=1.91, 95% CI 1.21-3.01; females OR=1.90, 95% CI 1.12-3.23). The plant *Teucrium reparium* was also associated with an increase risk and was used by 16% males and 13% females (males OR=1.78, 95% CI 1.23-2.60; females OR=1.57, 95% CI 1.02-2.40). An equal proportion of males and females (5%) used the plant *Withania somnifera*, however the risk estimate was only significant in females (OR= 2.22, 95% CI 1.14-4.33). In contrast, the use of the bulb of the plant *Eucomis autumnalis*, belonging to the Amaryllidaceae family, was only associated with an increased risk in males (OR=1.48, 95% CI 1.10-2.00). Use of this medicinal plant is also common amongst males (37%) than amongst females (24%). Similarly the bulb of the plant *Brunsvigia sp.* also belonging to the Amaryllidaceae family was also associated with an increased risk in males (OR=1.80, 95% CI 1.12-2.88) whilst in females a 51% increased risk was observed although not statistically significant (95% CI 0.70-3.29). In comparison to *E. autumnalis* however, *Brunsvigia* bulb is only used by 9% males and 3% females. The plant *Artemisia afra* which is used by an equal proportion of males and females (65% and 67% respectively) however was only associated with an increased risk in females (OR=1.41, 95% CI 1.02-1.95). The plant *Helichrysum cymosum* which is quite commonly used (~55% males and females) did not present with any association to OC development.

Table 7.3. Odds Ratios of oesophageal cancer associated with the use of wild plants as traditional medicines amongst males (N=955)

Botanical name	Family	% Users	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Artemisia afra Jacq. Ex Wild	Asteraceae	65	Never	111	227		
			Ever	223	394	1.11 (0.83-1.49)	1.09 (0.81-1.46)
Helichrysum cymosum (L.) D.Don	Asteraceae	55	Never	139	287		
			Ever	195	334	1.08 (0.82-1.43)	1.06 (0.80-1.40)
Eucomis autumnalis (Mill.) Chitt.subsp. autumnalis	Amaryllidaceae	37	Never	190	412		
			Ever	144	209	1.57 (1.17-2.11)	1.48 (1.10-2.00)
Aloe Ferox Mill.	Aloeaceae	30	Never	229	438		
			Ever	105	183	1.05 (0.78-1.42)	1.07 (0.79-1.45)
Opuntia vulgaris Mill.	Cactaceae	28	Never	246	445		
			Ever	88	176	0.82 (0.59-1.14)	0.83 (0.60-1.16)
Lippia javanica (Burm. F.) Spreng.	Verbenaceae	25	Never	244	477		
			Ever	90	144	1.21 (0.87-1.69)	1.16 (0.83-1.62)
Pelargonium sp. cf. inqinans (L.) L'Herit	Geraniaceae	16	Never	277	524		
			Ever	57	97	1.11 (0.75-1.64)	1.06 (0.71-1.57)
Teucrium reparium Hochst.	Labiatae	16	Never	261	537		
			Ever	73	84	1.87 (1.30-2.70)	1.78 (1.23-2.60)
Acacia karroo	Mimosaceae	11	Never	291	555		
			Ever	43	66	1.22 (0.80-1.86)	1.18 (0.77-1.80)
Acokanthera oppositifolia (Lam.) Codd	Apocynaceae	10	Never	287	572		
			Ever	47	49	1.89 (1.20-2.97)	1.91 (1.21-3.01)
Lantana rugosa Thunb.	Verbenaceae	9	Never	304	569		
			Ever	30	52	1.10 (0.67-1.80)	0.99 (0.60-1.64)
Mariscus congestus (Vahl.) C.B.Cl.	Cyperaceae	9	Never	308	560		
			Ever	26	61	0.70 (0.42-1.19)	0.73 (0.43-1.23)
Brunsvigia sp.	Amaryllidaceae	9	Never	294	578		

			Ever	40	43	1.84 (1.15-2.94)	1.80 (1.12-2.88)
Hypoestes aristata (Vahl) Soland. Ex Roem. & Schult. Var. alba Balkwill	Acanthaceae	8	Never	309	566		
			Ever	25	55	0.87 (0.53-1.45)	0.90 (0.54-1.49)
Turraea floribunda Hochst	Meliaceae	7	Never	312	575		
			Ever	22	46	0.81 (0.47-1.39)	0.73 (0.42-1.28)
Withania somnifera (L.) Dun	Solanaceae	5	Never	316	595		
			Ever	18	26	1.22 (0.64-2.30)	1.24 (0.66-2.34)
Solanum mauritianum Scop.	Solanaceae	5	Never	314	594		
			Ever	20	27	1.33 (0.72-2.46)	1.23 (0.66-2.30)
Dalbergia obovata E. Mey.	Fabaceae	4	Never	321	595		
			Ever	13	26	0.82 (0.41-1.64)	0.85 (0.42-1.71)
Acacia caffra (Thumb.) Willd	Mimosaceae	4	Never	322	598		
			Ever	112	23	1.01 (0.28-2.10)	0.94 (0.45-1.97)
Solanum nigrum complex	Solanaceae	3	Never	319	607		
			Ever	15	14	2.10 (0.98-4.47)	1.85 (0.85-4.02)
Ficus craterostoma Warb. Ex Mild Br. & Burr.	Moraceae	2	Never	324	610		
			Ever	10	11	1.46 (0.60-3.52)	1.38 (0.57-3.35)
Catha edulis (Vahl.) Forsk ex Endl.	Celastraceae	2	Never	328	610		
			Ever	6	11	0.91 (0.33-2.51)	0.83 (0.29-2.34)
Centella asiatica L.	Umbelliferae	2	Never	322	611		
			Ever	12	10	1.88 (0.79-4.48)	1.70 (0.70-4.13)
Deinbollia oblongifolia (E. Mey. ex Arn.) Radlk.	Sapidaceae	1	Never	332	611		
			Ever	2	10	0.37 (0.08-1.72)	0.37 (0.08-1.72)
Pteridium aquilinum subsp. aquilinum	Dennstaedtiaceae	1	Never	330	616		
			Ever	4	5	1.21 (0.31-4.63)	1.17 (0.30-4.54)
Maesa lanceolate Forsk. var. rufescens	Myrsinaceae	1	Never	325	616		
			Ever	9	5	2.92 (0.95-8.97)	2.89 (0.94-8.91)
Datura stramonium L.	Solanaceae	1	Never	327	615		

			Ever	7	6	2.04 (0.66-6.27)	2.12 (0.69-6.55)
Maytenus acuminata (L.f) Loes	Celastraceae	0.5	Never	332	618		
			Ever	2	3	1.22 (0.20-7.44)	0.85 (0.12-5.74)
Convolvulus farinosus L.	Convolvulaceae	1	Never	330	612		
			Ever	4	9	0.74 (0.22-2.48)	0.77 (0.23-2.57)

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day and total grams ethanol per week

Table 7.4. Odds Ratios of oesophageal cancer associated with the use of wild plants as traditional medicines amongst Females (N=903)

Botanical name	Family	% Users	Categories	Cases	Controls	[a]OR_1 (95% CI)	[b]OR_2 (95% CI)
Artemisia afra Jacq. Ex Wild	Asteraceae	67	Never	90	208		
			Ever	246	359	1.41 (1.03-1.93)	1.41 (1.02-1.95)
Helichrysum cymosum (L.) D.Don	Asteraceae	56	Never	133	263		
			Ever	203	304	1.13 (0.85-1.51)	1.03 (0.76-1.38)
Opuntia vulgaris Mill.	Cactaceae	34	Never	230	366		
			Ever	106	201	0.69 (0.50-0.95)	0.75 (0.54-1.04)
Aloe Ferox Mill.	Aloeaceae	27	Never	240	415		
			Ever	96	152	0.96 (0.70-1.31)	1.03 (0.75-1.42)
Lippia javanica (Burm. F.) Spreng.	Verbenaceae	26	Never	243	424		
			Ever	93	143	0.97 (0.70-1.36)	0.87 (0.62-1.23)
Eucomis autumnalis (Mill.) Chitt.subsp. autumnalis	Amaryllidaceae	24	Never	244	441		
			Ever	92	126	1.31 (0.94-1.83)	1.19 (0.84-1.68)
Teucrium reparium Hochst.	Labiatae	13	Never	280	506		
			Ever	56	61	1.69 (1.12-2.57)	1.57 (1.02-2.40)
Pelargonium sp. cf. inqinans (L.) L'Herit	Geraniaceae	12	Never	292	505		
			Ever	44	62	1.23 (0.78-1.93)	1.34 (0.84-2.12)
Acacia karroo	Mimosaceae	10	Never	292	517		
			Ever	44	50	1.42 (0.91-2.20)	1.33 (0.84-2.09)
Lantana rugosa Thunb.	Verbenaceae	10	Never	307	510		
			Ever	29	57	0.77 (0.46-1.27)	0.72 (0.43-1.21)
Mariscus congestus (Vahl.) C.B.Cl.	Cyperaceae	8	Never	312	521		
			Ever	24	46	0.90 (0.52-1.57)	0.94 (0.54-1.66)
Acokanthera oppositifolia (Lam.) Codd	Apocynaceae	8	Never	300	535		
			Ever	36	32	1.72 (1.02-2.90)	1.90 (1.12-3.23)
Hypoestes aristata (Vahl) Soland. Ex Roem. & Schult. Var. alba Balkwill	Acanthaceae	6	Never	310	535		
			Ever	26	32	1.34 (0.76-2.37)	1.52 (0.85-2.70)

Withania somnifera (L.) Dun	Solanaceae	5	Never	311	550		
			Ever	25	17	2.28 (1.19-4.38)	2.22 (1.14-4.33)
Brunsvigia sp.	Amaryllidaceae	3	Never	318	554		
			Ever	18	13	1.93 (0.92-4.05)	1.51 (0.70-3.29)
Turraea floribunda Hochst	Meliaceae	3	Never	324	556		
			Ever	12	11	1.79 (0.77-4.18)	1.34 (0.54-3.30)
Acacia caffra (Thumb.) Willd	Mimosaceae	3	Never	324	550		
			Ever	12	17	1.10 (0.51-2.40)	0.93 (0.41-2.11)
Solanum nigrum complex	Solanaceae	3	Never	324	550		
			Ever	12	17	1.10 (0.51-2.38)	0.74 (0.32-1.69)
Solanum mauritianum Scop.	Solanaceae	2.5	Never	327	553		
			Ever	9	14	1.10 (0.46-2.63)	1.01 (0.41-2.54)
Ficus craterostoma Warb. Ex Mild Br. & Burr.	Moraceae	1.6	Never	328	560		
			Ever	8	7	1.59 (0.56-4.51)	1.66 (0.58-4.71)
Maesa lanceolate Forsk. var. rufescens	Myrsinaceae	1.5	Never	329	560		
			Ever	7	7	1.38 (0.47-4.09)	1.48 (0.49-4.43)
Centella asiatica L.	Umbelliferae	1	Never	328	562		
			Ever	8	5	2.74 (0.87-8.65)	2.72 (0.83-8.89)
Pteridium aquilinum subsp. aquilinum	Dennstaedtiaceae	1	Never	333	563		
			Ever	3	4	0.84 (0.18-3.89)	0.87 (0.18-4.21)
Deinbollia oblongifolia (E. Mey. ex Arn.) Radlk.	Sapidaceae	1	Never	334	563		
			Ever	2	4	0.81 (0.14-4.57)	0.83 (0.14-4.74)
Datura stramonium L.	Solanaceae	1	Never	332	564		
			Ever	4	3	1.79 (0.37-8.28)	1.72 (0.36-8.13)
Catha edulis (Vahl.) Forsk ex Endl.	Celastraceae	1	Never	328	562		
			Ever	8	5	2.27 (0.71-7.19)	2.36 (0.73-7.66)
Dalbergia obovata E. Mey.	Fabaceae	1	Never	333	559		
			Ever	3	8	0.57 (0.15-2.22)	0.70 (0.18-2.72)
Convolvulus farinosus L.	Convolvulaceae	0.8	Never	334	562		
			Ever	2	5	0.55 (0.10-2.92)	0.44 (0.08-2.42)

Maytenus acuminata (L.f) Loes	Celastraceae	0.1	Never	336	566		
			Ever	0	1	-	-

[a]OR_1, adjusted for age, hospital, residence and years of education
[b]OR_2, further adjusted for total grams tobacco per day and total grams ethanol per week

7.3 Correlation Between Intake of Plants.

Information has been presented on a large numbers of plants and in an attempt to determine whether the intake of plants may be correlated, the kappa statistic was determined between plants that were observed to present a significant increase in OC risk. Hence the rationale was to determine whether the intake of one plant was associated with the intake of another plant beyond chance. Whereas the correlation coefficient is used for numerical data, the kappa statistic (also called the kappa coefficient), is used as a means of classifying agreement in categorical data. A kappa coefficient of 1 means a statistically perfect modeling whereas a 0 means every model value was different from the actual value. A kappa statistic of 0.7 or higher is generally regarded as good statistic correlation however the lower the value the lower the correlation. Plants presenting with an increased and significant association between intake and OC risk were further evaluated for an association. The data for males and females is presented in Tables 7.5, 7.6, 7.7 and 7.8 below.

Table 7.5 Association matrix using the kappa statistic between pairs of dietary plants used by males.

Dietary Plants	*R. lanceotus*	*C. esculenta*	*R. raphinastrum*	*R. nasturtio*	*S. dregei*	*C. rehmanii*
R. lanceotus						
C. esculenta	0.11					
R. raphinastrum	0.19	0.04				
R. nasturtio	0.07	0.09	0.08			
S. dregei	0.11	0.19	0.04	0.23		
C. rehmanii	0.11	0.23	0.03	0.20	0.35	

Table 7.6 Association matrix using the kappa statistic between pairs of dietary plants used by females.

Dietary Plants	*R. lanceotus*	*C. esculenta*	*S. oleraceous*	*R. raphinastrum*	*R. nasturtio*	*S. dregei*	*C. rehmanii*
R. lanceotus							
C. esculenta	0.17						
S. oleraceous	0.07	-0.05					
R. raphinastrum	0.11	0.01	0.47				
R. nasturtio	0.10	0.31	-0.02	0.03			
S. dregei	0.09	0.18	0.04	0.02	0.20		
C. rehmanii	0.14	0.20	0.01	0.03	0.15	0.21	

Table 7.7 Association matrix using the kappa statistic between pairs of medicinal plant used by males.

Medicinal Plants	*A. oppositifolia*	*T. reparium*	*E. autumnalis*	*Brunsvigia sp.*
A. oppositifolia				
T. reparium	0.21			
E. autumnalis	0.20	0.23		
Brunsvigia sp.	0.21	0.15	0.12	

Table 7.8 Association matrix using the kappa statistic between pairs of medicinal plant used by females.

Medicinal Plants	*A. oppositifolia*	*T. reparium*	*W. somnifera*	*A. afra*	*Brunsvigia sp.*
A. oppositifolia					
T. reparium	0.28				
W. somnifera	0.29	0.24			
A. afra	0.06	0.09	0.03		
G. purpensa	-0.004	0.02	0.11	0.002	

As can be seen from the above data none of the possible pairs of high risk plants were correlated.

7.4 DISCUSSION

7.4.1 Effect of Dietary Wild Plants

In section 6.2 it was observed that the consumption of *imifino* was associated with an almost 2-fold increased risk of developing OC. This aspect of the study further added weight to the previous findings by establishing which of the *imifino* plants were positively associated with OC development. In the study conducted by Sammon (1992), it was also observed that the plant *Solanum nigrum* had an increased association with OC. Those consuming this plant had a 3.6-fold increased risk of developing OC (OR=3.62, 95% CI 1.40 – 9.33). As an adjunct to this study, a further 30 cases and controls were interviewed and an analysis of the combined data still showed an increased risk of OC following intake of *S. nigrum* (OR=2.55, 95% CI 1.17-5.54). The possibility of a spurious association due to geographic distribution of the plant coinciding with the maximum incidence of the disease or by its better growth on soils deficient in molybdenum could not be ruled out (Schutte, 1966) although the chemical compound, solanide, an alkaloid contained in this plant has

been shown to produce fetal malformations in hamsters. A study conducted by Purchase *et al.* (1975) has also shown that rats fed a diet consisting of maize, beans, salt, *S. nigrum* and *Sonchus oleraceus* had, among other disorders, severe epithelial dysplasia of the oesophagus. A correlation was also observed between the intake of plants and OC in the island of Curacao, the Netherlands (Morton, 1968) where the indigenous dish, cadushi soup, made from the powdered flesh of the wild cactus, *Cereus repandus*, was consumed by 44 of the 49 oesophageal cancer patients. The role of plant-based chemicals in OC aetiology has also been enhanced by the findings that mate´, a tea-like infusion of the herb *Ilex paraguariensis* (Aquifoliaceae) is also positively associated with OC development in southern Brazil and in Uruguay (Victora *et al.*, 1990; Sewram *et al.*, 2003). Mate´ consumption was significantly associated with an increased risk of developing esophageal cancer and showed a clear dose response, with a relative risk of 2.84 [95% confidence interval (CI), 1.41–5.73] for those drinking more than 1 liter/day of mate´ as compared with nondrinkers.

It cannot be overlooked that the sustenance of many indigenous societies over many generations has been as a result of their reliance on a broad food base to supply their nutritional requirements. Through a process of trial and error experimentation in their environment, these indigenous communities acquired immense amounts of knowledge on (the use of) fauna and flora around them (Fox and Norwood Young 1982).Though the food base included animals and other food sources, edible wild plants formed the major food source. In many traditional African cultures a main meal comprises of a starch staple which is accompanied by supplement (the relish) of vegetables, meat or a combination of both which provides the other nutritional elements as well as flavour to the meals. The relish is therefore an important component of the diet. From these accounts it is evident that the broad base of wild food plants was a reliable food source for these communities which were more so during times of drought and famine when wild food plants provided a major nutritional supply (Reynolds 1989). Despite the use of these wild plants over centuries, the lack of acute toxicity does not equate to the lack of chronic toxicity. Due to the latency period of cancer, it would have been extremely difficult to associate long term use of certain wild plants to OC development just from anecdotal evidence.

7.4.2 Effect of Medicinal Plants

This study revealed that five medicinal plants were positively associated with OC development and it is ironic that the treatment of one disease is the unintended

cause of another. The selected high risk plants and their medicinal usages are captured in Table 7.9.

Table 7.9. Selected high risk plants and their medicinal uses

Selected high risk plants	Medicinal uses	Reference
Acokanthera oppositifolia	excessive and irregular menstruation, abdominal pains, toothache and septicaemia	Van Wyk & Gericke, 2000
Withania somnifera	Tonic, diarrhoea, abdominal discomfort, rheumatism	Van Wyk & Gericke, 2000
Eucomis autumnalis	Low backache, colic, fevers, healing of fractures, stomach ache	Watt & Breyer-Brandwijk, 1962
Brunsvigia sp.	purgative and emetic	Hutchings *et al.*, 1996
Artemisia afra	Colds and influenza, loss of apetite, colic, headache, blocked nasal passages	Hutchings, 1989

The lack of adequate health care systems in rural areas forces local people to treat themselves, either by using medicinal plants or by buying high-cost medicine in the rural markets. In the rural areas, as a whole, people begin by treating themselves before going to a traditional practitioner or a modern doctor. Medicinal plants are used at an early stage of the disease at low cost and conveniently replace the indiscriminate consumption of drugs without prescription. Recent research has shown that alternative medicine is flourishing in African society neither because users are dissatisfied with conventional medicine nor because they seek self-control over their health care decisions. The driving force of the majority of users appears to be the holistic belief that the health of body, mind and spirit are related and that this should be taken into account by whoever cares for their health. It is important to note

that even in contemporary rural Africa, there is no doubt about the efficacy of certain herbal medicines. However one must also bear in mind that the phytochemical constituents of plants may vary in concentration due to topography, season, as well as climatic and ecological conditions and hence the risk associated with their use may vary as a result of varying exposure levels. Furthermore the frequency of exposure may also present with a risk differential. Such has been the case with the plant *Pteridium aquilinum* (L.) Kuhn. This plant, commonly known as the bracken fern and used medicinally in South Africa for stomach ache, diarrhoea and deworming , did not present with any positive association in males and females although in other regions of the world where it forms part of the diet, there has been a positive association with cancer. Human epidemiological studies have been undertaken in populations exposed to bracken in an attempt to quantify the cancer risk in these population groups. In the past, bracken has been used as a famine food in Russia and on the Canary Islands (Jacobsen, 1983). Today, however, the young crosiers are a culinary delicacy in different regions of Japan (Shahin *et al.*, 1999), Venezuela (Alonso-Amelot *et al.*, 2001) and Brazil (Santos and Brasileiro-Filho, 1987). Epidemiological studies, however, show that exposure to this plant is associated with an increased risk of developing oesophageal and gastric cancers in Japan, Brazil and the Gwynedd region of North Wales. In Japan, the risk of developing oesophageal cancer (OC) following the consumption of bracken crosiers was estimated to be 2.1-fold and 3.7-fold in males and females, respectively (Kamon and Hirayama, 1975). Additionally, the study also reported that bracken consumption increased the risk of developing OC by as much as 3-times for daily consumers and 1.5-times for occasional consumers compared with a control group (Kamon *et al.*, 1975). An association between bracken consumption and cancer of the oesophagus and stomach has also been noted in Brazil (Marliere *et al.*, 1995). In view of the high incidence of gastric and esophageal cancers, a case-control study was carried out (Marliere *et el*, 2000) and results showed a correlation between bracken consumption and upper gastrointestinal tract neoplasia. Similarly, a higher prevalence of gastric cancer was observed among people who spent their childhood in farms containing bracken in the Gwynedd region of North Wales. Results of a case-control study reported a higher prevalence of gastric cancer in later stages of life among the people who spent their childhood in the farms containing bracken in this region (Galpin *et al.*, 1990) compared with others in the same area.

The notion of natural being safe is untrue. Plant chemical compounds, toxic to humans and livestock, are produced as part of the plant's defence against being

eaten by pests and herbivores or to gain an advantage over competing plants (Dowling & Mckenzie, 1993). Plant poisons are highly active substances that may cause acute effects when ingested in high concentrations and chronic effects when accumulated (Kofi-Tsekpo, 1997).

Poisoning or toxic principles as relates to vegetables generally fall into various phytochemical groups, which include alkaloids, glycosides, oxalates, phytotoxins (toxalbumins), resins, essential oils, amino acids, furanocoumarins, polyacetylenes, protein, peptides, coumarins, flavonoids and glycosides (Wat & Breyer-Brandwijk, 1962). Others are minerals and photosensitizing compounds. The toxic pyrrolizidine alkaloids are also a large group of related compounds that occur in plants, mainly in species of *Crotalaria*, *Senecio*, *Heliotropium*, *Trichodesma*, *Symphytum* and *Echium* and are poisonous. This has been discussed further in Chapter 8

Foods that are commonly eaten are presumed safe unless a significant risk has been identified. However, it must be emphasised that the absence of evidence of toxicity is not the same as evidence of the absence of toxicity under the proposed conditions of use. Epidemiological evidence and clinical case reports may be available in a few cases but even then usually would not be adequate to form the basis of a quantitative risk assessment. Often the available information is limited to acute toxicity (since this is more readily associated with recent exposure) and it is usually extremely difficult to obtain adequate chronic toxicity data from historical sources. However, for some botanical products, good epidemiological and clinical human studies addressing both efficacy and safety may be available (Ernst, 2002).

Today, many herbal remedies are being used prophylactically to maintain or enhance good health or prevent certain conditions from occurring. Since many of these herbal medications are popular and promoted as both safe and efficacious, it is not always possible for the long-term user to understand why this practice could be harmful. Symptoms can vary from trivial to severe and are particularly disconcerting when they effect the heart, blood pressure, liver, gastrointestinal tract and nervous or endocrine systems. Noteworthy are effects associated with ginseng, golden seal, milk thistle, cassia, saw-palmetto, valerian, and a variety of stimulants (D'Arcy, 1993; Ernst, 1998; O'Hara *et al.*, 1998) including those that contain caffeine, like guarana (*Paullinia cupana*) or maté (*Ilex paraguariensis*). The latter beverage has also been implicated in inducing oral cancers (Victora *et al.*, 1990). Also anthranoid laxatives

such as aloe, cascara, rhubarb, and senna, commonly considered as safe, may be a risk factor for colorectal cancer if used on a long-term basis (Siegers *et al.*, 1992).

In February 2002, the International Agency for Research on Cancer (IARC) following the meeting of an expert group concluded that herbal remedies containing plant species of the genus Aristolochia (e.g. European birthwort) were carcinogenic to humans. In 1992, an outbreak of rapidly progressing kidney failure afflicted more than 100 people in Belgium, mostly women, who were undergoing a body-weight loss regimen that involved consuming a mixture of Oriental herbs (Nortier *et al.*, 2000). In addition, consumption of the herbal mixture caused tumours of the renal pelvis, the ureter and the urinary bladder. Additional cases have subsequently been reported from at least five other countries in Europe and Asia (Arlt *et al.*, 2002). These herbal mixtures, possibly by accident, contained plants of the genus *Aristolochia*, which are traditionally considered medicinal plants in China but which contain mixtures of aristolochic acids which were classified as probably carcinogenic to humans.

Herbs containing anthraquinone derivatives that have been widely used as laxatives were also discussed. Epidemiological studies on the relation between the use of these preparations and human cancer incidence are weak and show no association, although in animal experiments 1-hydroxy-anthraquinone induces tumours in rats. Therefore, this compound is classified as possibly carcinogenic to humans. The herb Madder root (*Rubia tinctorum*), which contains the anthraquinone lucidin, is considered not classifiable as to its carcinogenicity to humans, on the basis of slight increases of the number of tumours in a single study in rats (IARC 2002b).

The pyrrolizidine alkaloid riddelliine is found in *Senecio riddellii* and other *Senecio* species, including *S. longilobus*, which is used as a herbal tea in certain regions of the world. Although no epidemiological data relating the use of herbal preparations containing riddelliine to cancer incidence in humans are available, riddelliine is clearly carcinogenic in rodents. It is therefore classified as possibly carcinogenic to humans.

The plants investigated in this chapter have been further evaluated for mechanistic evidence of carcinogenicity in the next chapter.

PART 2

CHAPTER 8: EVALUATION OF THE MUTAGENICITY OF DIETARY AND MEDICINAL WILD PLANTS INVESTIGATED IN THIS STUDY

8.1. Introduction

Southern Africa is exceptionally rich in plant diversity with some 30 000 species of flowering plants, accounting for almost 10% of the world's higher plants, many of which, through cultural diversity, are used daily either as food or medicine (Van Wyk and Gericke, 2000). As a source of food, the leaves of various plants are cooked traditionally with other pot herbs to form a relish that is served with maize meal. In addition to food, over 3000 species are used in the traditional systems of medicine with over 350 species being the most commonly used and traded medicinal plants. In South Africa, many of these plants continue to be used widely with minimal caution and little knowledge concerning their carcinogenic potential despite reports from international studies of plants possessing carcinogenic properties. Numerous naturally occurring carcinogens such as cycasin, pyrrolizidine alkaloids and ptaquiloside have been identified.

Laquer *et al.* (1963) found that rats fed crude cycad meal developed hepatocellular carcinomas, kidney tumours of both the epithelial and mesenchymal types, and intestinal tumours. Laquer (1964, 1965) subsequently found that the carcinogen in cycads was cycasin, which was first isolated from the seeds of *Cycas revoluta* Thunb (Nishida *et al.*, 1955). The cycad seeds are used as a source of starch and as a constituent of the bean paste "miso" in some parts of Amami and Okinawa, the southwestern islands of Japan (Whiting, 1963).

Pyrrolizidine alkaloids are widely distributed naturally occurring carcinogens of plant origin and were among the first group of natural products which have been found to induce tumours of the liver. Harris and Chen (1970) studied the carcinogenic activity of prolonged administration of a diet containing dried *Senecio longilobus* in rats, a plant that contains different analogues of the pyrrolizidine alkaloids. The carcinogenicity of another alkaloid, hydroxysenkirkine, found in *Crotalaria laburnifolia* L., which is used as a medicinal herb in Tanzania, was also studied by Schoental and Cavanagh (1972). They found that after a single intraperitoneal injection, one rat developed an astrocytoma of the cerebrum. When administered to rats in their diet, dried and powdered buds of the coltsfoot, *Tussilago farfara*, a member of the Senecioneae family used as a cough medicine in China and Japan, induced

hemangioendothelial sarcomas of the liver, hepatocellular adenomas, and carcinomas (Hirono *et al.*, 1976). *Symphytum officinale* is a herb of the family Boraginaceae, which includes the *Heliotropium* species, that contains high levels of pyrrolizidine alkaloids. This plant is called comfrey and is cultivated for use in Japan as a green vegetable or tonic. Rats fed diets containing 8 to 33% comfrey leaves or 1 to 8% roots for up to 600 days developed liver cell adenomas and hemangioendothelial sarcomas of the liver (Hirono *et al.*, 1978).

Kapadia *et al.* (1978) bioassayed 12 medicinal herbs to correlate a high incidence of oesophageal carcinoma in natives of South Carolina, Florida and South India. Outbred NIH Black rats were given 72 weekly subcutaneous injections of the total aqueous extracts. The tannin-rich extracts of the plants *Areca catechu* and *Rhus copallina* produced local tumors in 100 and 33% of the rats, respectively, while extracts of the plants *Diospyros virginiana*, *Sassafras albidum* and *Chenopodium ambrosiodes* were tumorigenic in over 50% of the rats treated.

Pteridium aquilinum, commonly known as the bracken fern has also been shown to cause neoplasia of the urinary bladder (Pamukcu *et al.*, 1967; Harbutt and Leaver, 1969) and in some circumstances, of the upper gut (Dobereiner *et al.*, 1967; Price and Pamukcu, 1968). Ptaquiloside (PT), a norsesquiterpenoid glucoside has been identified as the major carcinogen (Hoeven *et al.*, 1983; Hirono *et al.*, 1984; Hirono *et al.*, 1987). The young fronds and rhizomes of this plant are cooked and eaten locally by the Zulu and Tswana ethnic groups (Roberts, 1990) and also used medicinally as a vermifuge and for menstrual irregularities (Watt and Breyer-Brandwijk, 1962). The common terrestrial fern of the Himalayas, known as *Onychium contiguum* (Family Cryptogrammaceae) was also shown to cause tumours of the ileum, urinary bladder and mammary glands of guinea pigs following long term exposure (Dawra *et al.*, 2001). The roots of *Rubia tinctorum*, known as madder root, which contain a variety of hydroxyanthraquinones such as alizarin, purpurin, lucidin and rubiadin, was also shown to have carcinogenic potential in ACI rats, where non-neoplastic lesions related to the treatment were evident in the liver and kidneys (Westendorf *et al.*, 1998). Moreover, dose-dependent increases in benign and malignant tumour formation were observed in the liver and kidneys of treated animals.

Species belonging to the botanical family, Euphorbiaceae often contain skin-irritant and tumour-promoting toxins, diterpene esters (DTE) of the tigliane, ingenane and/or daphnane type (Evans and Soper, 1978). Direct or indirect exposure of people to

such plants or plant parts by occupation, diet or lifestyle or by treatment with phytomedicines may mean a potential risk of cancer. Previously it was shown that certain tumour initiators, together with direct exposure to a popular tea prepared from leaves of *Croton flavens* L. containing DTE-type tumour promoters, are responsible for a dietary risk of oesophageal cancer on the Caribbean Island of Curacao (Hecker *et al.*, 1983).

Much of the evidence for carcinogenicity discussed above has been revealed through animal studies and as a result the number of plants shown to contain natural carcinogens is few because detection of these carcinogens was accomplished entirely by animal experiments, which generally take a long time. Several substances in plants express cytotoxic and genotoxic activities and show correlation with the incidence of tumours (Ames, 1983). The *Salmonella* mutagenicity assay is being used extensively in genetic toxicology testing (Maron and Ames, 1983; Zeiger *et al.*, 1988) since the identification of substances capable of inducing mutations has become an important procedure in safety assessment and is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. The test relies on the observation that the most common cause of cancer is somatic mutations brought about by DNA damage. Chemicals that damage bacterial DNA, and induce mutations, are also likely to cause mutations of mammalian cells. It is estimated that over 80% of all carcinogens are also mutagens (McCann *et al.*, 1975) and with this mind, the Ames assay was used as a screening test (Levin *et al.*, 1982). Furthermore, the test has a high predictive value for rodent carcinogenicity when a mutagenic response is obtained (McCann *et al.*, 1975; Zeiger *et al.*, 1990).

The procedure employs bacterial tester strains that identify the reversions of missense and small frameshift mutations in the *his* operon. Certain strains of the *Salmonella typhimurium* were selected because they are unable to produce the amino acid histidine, which is essential for growth. The test substance and the bacterial tester strain are mixed together in a soft agar solution, which contains only small amounts of histidine. The histidine permits the inoculated test organism to undergo a limited number of divisions, but is insufficient to permit normal growth. If, however, the strain undergoes a reverse mutation (spontaneous or induced by the test substance), the bacteria no longer require histidine to grow and can produce a visible revertant colony. The greater the number of revertant colonies, the more mutagenic the chemical. The tests are performed both with and without S-9 (liver

homogenate) activation. The S-9 activation system is designed to simulate mammalian liver enzyme systems and is used to detect substances which undergo metabolic activation from non-mutagenic forms yielding active mutagenic metabolites.

Hence in view of the widespread habit in the use of dietary and medicinal wild plants in the Eastern Cape Province of South Africa, it is of considerable interest to elucidate their contribution to the overall load of environmental risk factors for cancer experienced by people exposed to these plants or plant parts. In this study, mutagenicity assays were performed on the aqueous plant extracts to determine whether the plants used contain mutagens that can alter the nucleic acid sequence of DNA.

8.2. Experimental Methods

8.2.1. Collection of Plant Material

Twenty dietary and 27 medicinal plants identified by 2000 patients attending three referral hospitals in the Eastern Cape Province of South Africa were collected from Flagstaff (Hala Location), Tsolo (Unga Location) and along the banks of the Umzimvubu River in Port St. Johns (Eastern Cape), and the Silverglen medicinal plant nursery (KwaZulu-Natal) during November 2002. Elderly men and women familiar with local botany and potential collection locations assisted during this component of the study. The identities of the plants were authenticated by comparison with reference specimens at the Kei Herbarium (University of Transkei) and Natal Herbarium (National Botanical Institute, KwaZulu-Natal). Plant voucher specimens were also deposited in these herbariums for future reference. A list of the plants collected and the parts used in the *Salmonella* mutagenicity assay is provided in Tables 8.1a and 8.1b.

Table 8.1a. Dietary wild plants tested in the *Salmonella* reverse mutation assay

Botanical name	Sampling location	Part of plant tested
Amaranthus asper	1	leaves
Amaranthus hybridus	1	leaves
Amaranthus thunbergii	1	leaves
Bidens pilosa	1	leaves
Centella asiatica	1	leaves
Chenopodium album	1	leaves
Coccinia rehmanii	1	root
Colocasia esculenta	1	leaves
Oxalis corniculata	1	leaves
Physalis viscosa	1	leaves
Raphanus nasturtio aquatica	1	leaves
Raphanus raphinastrum	1	leaves
Rumex lanceolatus	1	leaves
Sida dregei	1	leaves
Sida rhombifolia	2	leaves
Solanum nigrum	1	leaves
Solanum nodiflorum	1	leaves
Sonchus oleraceus	1	leaves
Urtica urens	1	leaves
Zantedeschia aethiopica	1	leaves

1 = Port St. Johns, 2 = Flagstaff (Hala Location)

Table 8.1b. Medicinal wild plants tested in the *Salmonella* reverse mutation assay

Botanical name	Sampling location	Part of plant tested
Acacia caffra	1	leaves
Acacia karroo	2	leaves
Acokanthera oppositifolia	2	leaves
Aloe ferox	1	leaves
Artemisia affra	3	leaves
Brunsvigia sp.	1	bulb
Catha edulis	4	leaves
Convolvulus farinosus	1	leaves
Dalbergia obovata	1	stem
Datura stramonium	1	leaves
Deinbollia oblongifolia	2	leaves and roots
Eucomis autumnalis	2	bulb
Ficus craterostoma	1	root
Gunnera perpensa	2	rhizomes
Helichrysum cymosum	1	leaves and twigs
Hypoestes aristata	2	leaves
Lantana rugosa	1	leaves
Lippia javanica	1	leaves
Maesa lanceolate	1	leaves
Mariscus congestus	1	leaves
Maytenus acuminate	1	leaves
Opuntia vulgaris	1	leaves
Pelargonium sp.	2	roots
Pteridium aquilinum	1	leaves
Solanum mauritianum	1	leaves
Turraea floribunda	2	leaves
Withania somnifera	1	leaves

1 = Port St. Johns, 2 = Silverglen Med. Plant Nursery,
3 = Flagstaff (Hala Location), 4 = Tsolo (Unga Location)

8.2.2. Preparation of Plant Extracts

The plants were washed in fresh running water to eliminate dust, dirt and possible parasites and then washed again with deionised water. The different parts of the plants used by the patients (bulb, stem, leaves, roots), were air dried and then ground into a fine powder using an electric mill. The milled plant material (30 grams) was then extracted with distilled water (500 ml) by heating for 1 hour at 75-85°C. This method of preparation is similar to the preparation method amongst the population. The mixture was cooled to room temperature (~3 hours) and filtered (Whatman No. 4 filter paper). The filtrate was subsequently frozen at -20°C for 12 hours and lyophilised. The lyophilised extracts were stored in a dessicator at -20°C until tested in the Ames assay. Depending on the solubility of the extracts in distilled water, varying concentrations of each extract were prepared ranging from 0.00125 g/ml – 0.08 g/ml. For each assay, a set of five concentrations were prepared to determine any trend in mutagenicity of the extracts. The highest concentration (depending on solubility) was prepared by weighing the appropriate quantity in a polypropylene tube (50 ml) and diluting in distilled water (20 ml). The extract was sonicated for 30 minutes and filtered through a glass fibre filter (GF52, Schleicher & Schuell) followed by filtration sequentially through a 0.45 μm (Cameo 30A) and then 0,22 μm (Cameo 25AS, Osmonics) syringe filters. Subsequent dilutions were prepared using sterile distilled water.

8.2.3. Chemicals, Media and Strains of Bacteria

Cumoylhydroperoxide (CHP) and hydrogen peroxide (H_2O_2) were purchased from Merck (Schuchardt, Germany), and Saarchem (SA) respectively. The mutagens, 2-acetamidofluorene (2-AAF) and aflatoxin B_1 (AFB_1) were purchased from Sigma Chemical Co. (SA) (purity >99%). Stock solutions of the mutagens were freshly prepared on the day of the experiment in dimethylsulphoxide (DMSO) purchased from BDH Laboratory Supplies (Poole, UK). Agar and nutrient broth No. 2 were purchased from the Difco Laboratories (Detroit, USA) and Oxoid (Hampshire, UK) respectively. All other reagents used were of analytical grade and purchased from Merck (Darmstadt, Germany). The *Salmonella typhimurium* bacterial strains, TA97a, TA98, TA100 and TA 102 were purchased from Xenometrix Inc. (CA, USA) and were received on dry ice.

8.2.4. Re-isolation of Tester Strains

The strains TA 97a, TA98, TA100 and TA102 were re-isolated by streaking the bacteria from the master copy on minimal glucose agar plates (1.5% Bacto-agar, 4% 25X Vogel-Bonner medium, 2% glucose) that was enriched with biotin (0.08%, m/v), histidine (4%, m/v) and ampicillin (5 μg/ml). With regards to the strain TA102, in addition to biotin, histidine and ampicillin, the plates were also supplemented with tetracycline at a final culture concentration of 2 μg/ml. After incubation at 37°C for 24 hours, single colonies were isolated using a sterile steel wire and grown overnight in Oxoid nutrient broth No. 2 (5 ml). The genotypes of the tester strains were confirmed by testing for the following: histidine requirement, rfa marker, uvrB deletion (except TA 102), R-factor and tetracycline resistance (TA102) according to the method outlined by Mortelmans and Zeiger (2000). Aliquots of each of the strains (1 ml) was added to Oxoid nutrient broth No. 2 (50 ml) and grown overnight in a waterbath maintained at 37°C with shaking. DMSO (0.9%) was added to the bacterial culture and aliquots (1 ml) were then transferred into cryovials and stored as working cultures at -80°C for use in the assay of the plant extracts.

8.2.5. Genetic Analysis of the Tester Strains

The tester strains were analysed for their genetic integrity and spontaneous mutation rate as follows:

8.2.5.1. Histidine Dependence

This characteristic was tested on minimal glucose agar plates by applying bacterial culture to two areas on the plate, one containing biotin histidine/biotin (0.5mM) and the other containing sterile distilled water as control. The plates were incubated overnight at 37°C. Since the *Salmonella* strains are histidine dependent, there should be no growth on the "control" side of the plate (Figure 8.1).

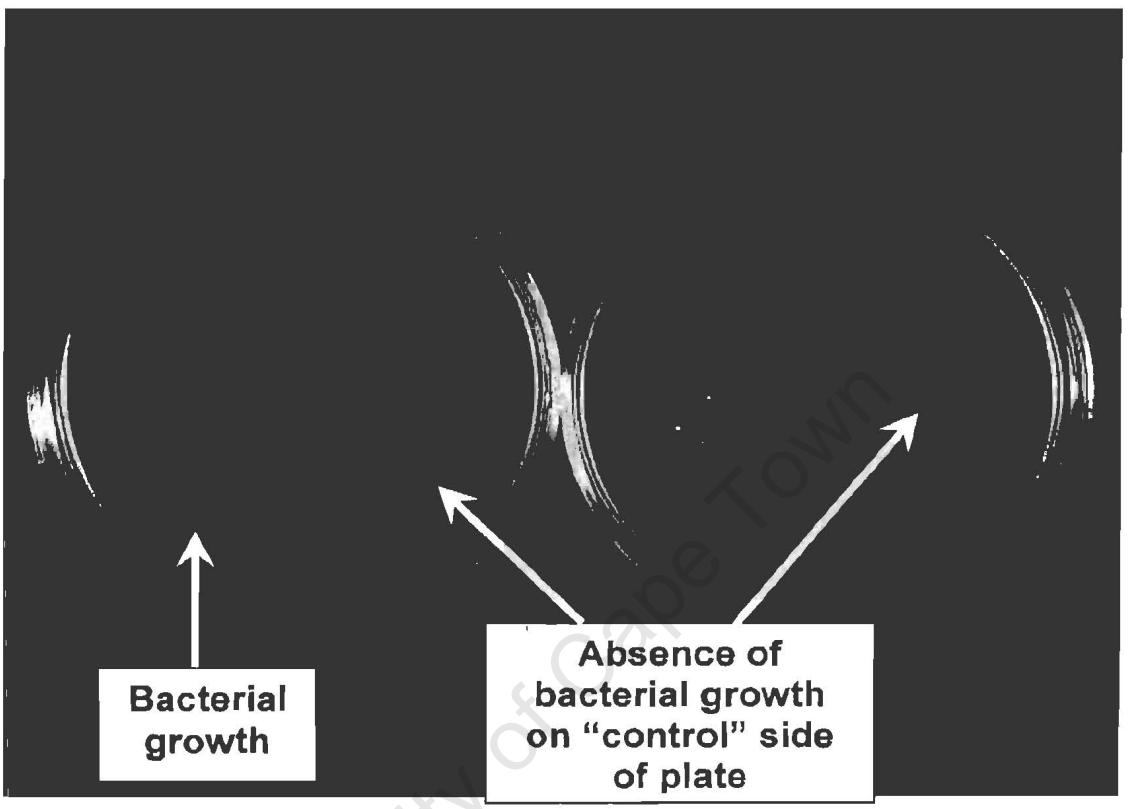


Figure 8.1. Absence of bacterial growth on the "control" side of the plate demonstrates that the *his* genetic deletion was intact in tester strain TA97a.

8.2.5.2. RFA Marker

The strains should have a *rfa* mutation that makes the outer membrane more permeable to large molecules. The *rfa* character was tested for crystal violet sensitivity on nutrient agar plates. The plates for the strains were each layered with top agar (2.5 ml) seeded with biotin (0.08% m/v), histidine (4% m/v) and the bacterial strain (0.1 ml). A sterile filter with crystal violet solution (0.1%, w/v) was placed in the center of each plate. The plates were incubated overnight at 37°C. A clear zone of inhibition around the filter disc should be seen after incubation (Figure 8.2).

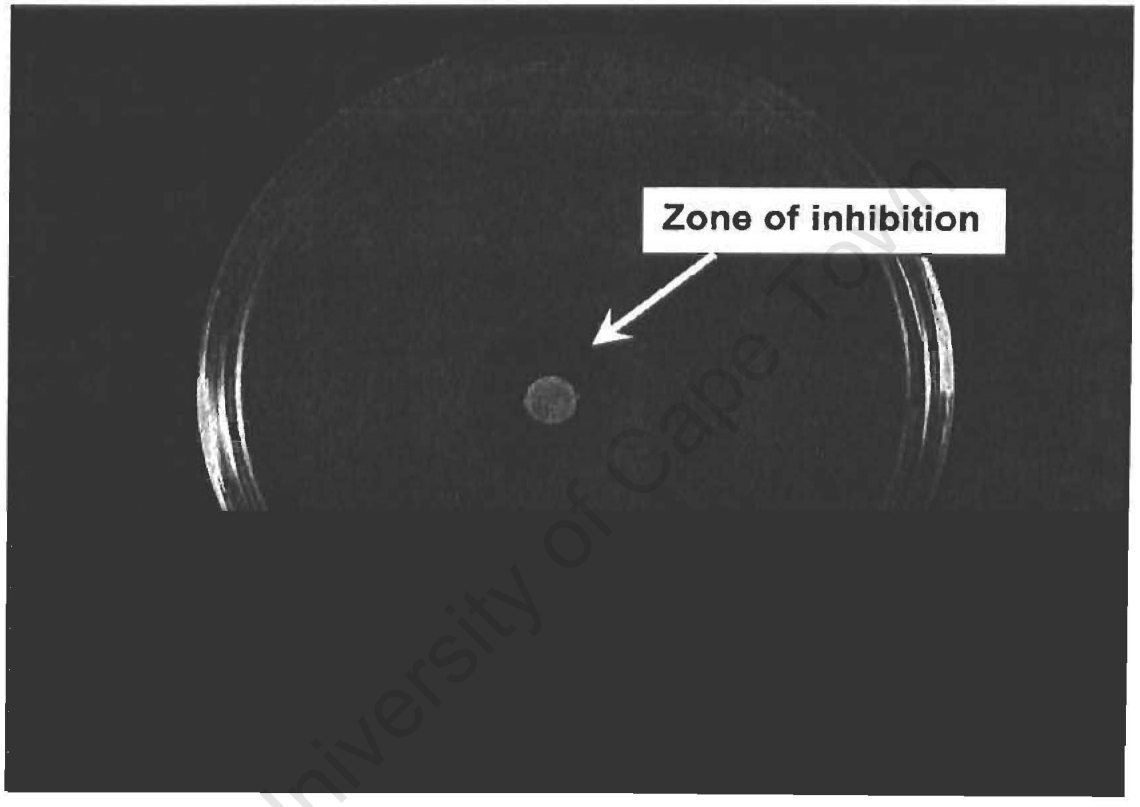


Figure 8.2. A clear zone of inhibition around the filter disc containing crystal violet solution reveals the *rfa* character of the strain TA97a.

8.2.5.3. uvrB Deletion

The uvrB mutation is confirmed by demonstrating UV sensitivity in strains that contain this mutation. This mutation deletes the *uvr*B gene to eliminate excision repair of DNA damage. The tester strains were streaked across a nutrient agar plate, in parallel stripes. A piece of cardboard was placed over half the plate and the plate irradiated with a 15W germicidal lamp for 8 sec at a distance of approximately 33 cm to the plate. The irradiated plates were incubated overnight 37°C. No growth should be evident on the irradiated part of the plates (Figure 8.3). The strains TA 97a, TA 98 and TA100 are R-factor strains while TA102 which contain wild-type excision repair enzymes are not affected.

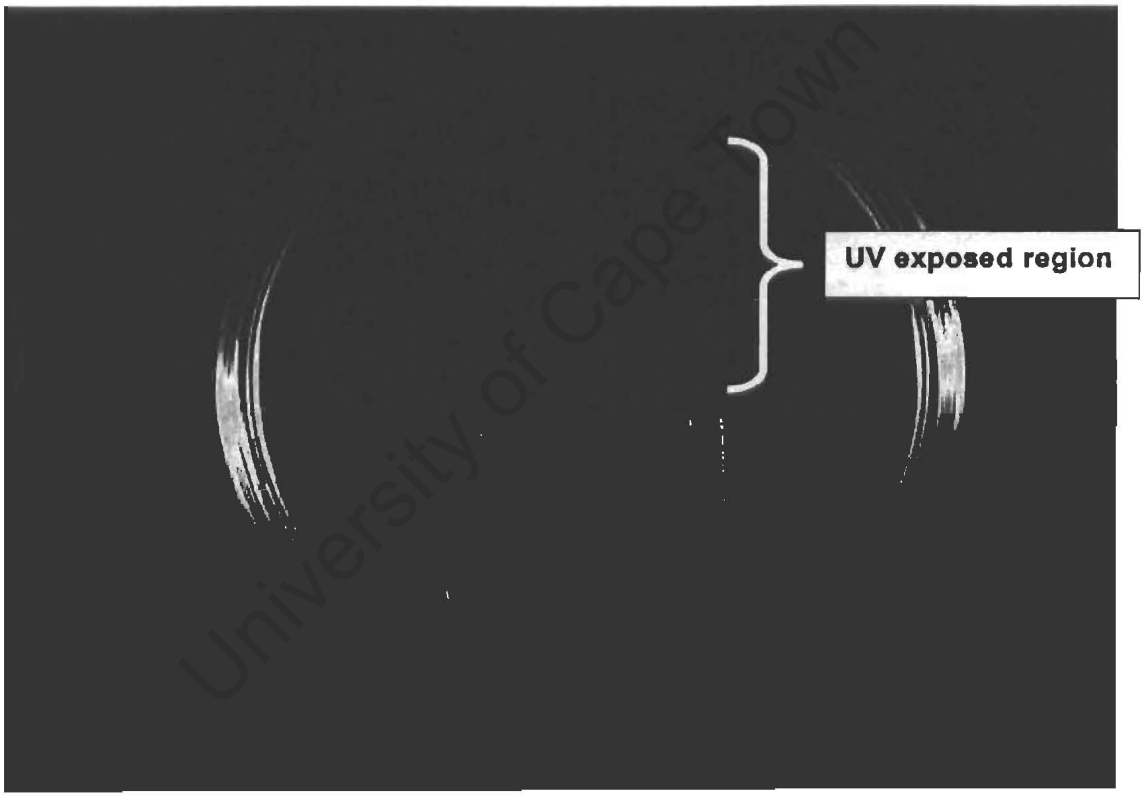


Figure 8.3. No growth in the UV exposed region confirms the *uvrB* gene deletion in the strain TA97a.

8.2.5.4. R-factor

All the strains used in this study were tested for the presence of the ampicillin resistance factor because the plasmid (pKM101), which increases error-prone repair of DNA damage is somewhat unstable and can be lost from the bacteria. The R-factor was tested by placing a disc impregnated with ampicillin on the nutrient plate after adding top agar (2.5 ml) containing histidine and biotin and the bacterial strain (0.1 ml). After incubation of the plates overnight at 37°C, there should be no inhibition of growth around the disc since all the strains contain a plasmid that makes them resistant to ampicillin (Figure 8.4).

Figure 8.4. Absence of zone of inhibition on strain TA97a indicates the presence of ampicillin resistance factor (R-factor) and hence the presence of plasmid pKM101.

8.2.5.5. Presence of Plasmid pAQ1 (Tetracycline Resistance)

The pAQ1 strain (TA102) was tested for tetracycline resistanceon minimal glucose agar plates. The strain was streaked across the plate. Tetracycline (2 μg/ml) was added to the plate and incubated overnight at 37°C. Growth should be observed.

8.2.5.6. Spontaneous Mutation Rate

Each of the tester strains revert spontaneously at a frequency that is characteristic of the strain. The standard plate incorporation assay procedure was used. The spontaneous mutation rate was determined after seeding the bacterial strain (0.1) in top agar (2.5 ml) both in the absence and presence of S-9 mixture. The plates were incubated for 48 hours at 37°C and the number of revertant colonies counted using a colony counter. Historical literature values and values provided by the supplier of the strains are listed in Table 8.2.

Table 8.2. Spontaneous revertant control values

Strain	Number of revertants		
	Literature values[1]		Supplier values[2]
	Without S9	With S9	Without S9
TA97a	75-200	100-200	196-256
TA98	20-50	20-50	-
TA100	75-200	75-200	-
TA102	100-300	200-400	500-588

[1]Values quoted from Mortelmans and Zieger (2000)
[2]Values quoted by the supplier, Xenometrix Inc., as per batch supplied.

8.2.6. Preparation of S9

The method was based on the procedure of Garner *et al.*, (1972). Induction of the rat liver enzymes was performed according to Maron and Ames (1983). Aroclor 1254, a polychlorinated biphenyl (PCB) mixture was used as enzyme inducer at a dosage of 500 mg/kg body weight. Animals were sacrificed after 1 week and the freshly excised livers were placed in sterile pre-weighed beakers containing cold KCl (0.15M, 1 ml/g wet liver). After weighing, the livers were washed with cold KCl (0.15M) and transferred to a beaker containing 3 volumes of KCl (0.15M) (3 ml/g wet liver). The liver samples were finely cut using a sterile scissors and homogenised for 5 minutes

using a Polytron homogeniser. The homogenate was centrifuged for 10 minutes at 9000 x g. The supernatant (S9 fraction) was aliquoted (2 ml) in sterile cryogenic vials, frozen in a bed of crushed dry ice, and stored immediately at -80°C. The above procedures were carried out under sterile conditions, while working on ice (4°C). The sterility of the preparation was determined by plating 0.1 ml on minimal agar containing histidine and biotin. The experimental protocol was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council, Tygerberg, South Africa.

8.2.7. Determination of Cytochrome P450 and Protein Concentration

The amount of cytP450 present in the rat liver microsomes was determined spectrophotometrically from difference spectra and expressed as nmol/mg protein. The microsomes were diluted 20 times in a volume of 8 ml Tris buffer (0.1M, pH 7.77). The sample was saturated with carbon monoxide for 1 minute and divided into two cuvettes. The baseline absorbance was read between 400 and 500 nm. Sodium dithionite was added to the sample cuvette and the difference in spectrum monitored between 400nm and 500 nm. The cyt P450 concentration was determined from the 450nm absorption peak using the formula and nmol extinction coefficient (91) given by Omura and Sato (1964) as follows:

$$\textit{Concentration of cytP450} = \left(A_{450-490} - \textit{correction of baseline}/100\right) \times \textit{sensitivity} \times \left(1000/91\right) \times \left(1/\textit{mg/ml protein}\right)$$

In this experiment, the cyt P450 concentration was found to be 0.7 nmol/mg protein (expected range 0.6 – 0.8 nmol/mg protein). The protein concentration was determined to be 27.08 mg/ml using the Bradford assay (Bradford, 1976) and was constant for the entire batch.

8.2.8. Preparation of S9 Mixture

The constituents of the standard S9 mixture (1 ml) were as follows: $MgCl_2$ (8mM), KCl (33mM), glucose-6-phosphate (5mM), NADP (4mM), sodium phosphate buffer (100mM, pH 7.40) and S9 fraction (0.04 ml, 1 mg protein). The S9 mix was prepared fresh before each assay and kept on ice until used.

8.2.9. Mutagenicity Assay (Standard Plate Incorporation Assay)

The mutagenic effects of the plant extracts were assayed according to the Ames test using *S. typhimurium* strains TA97a, TA98, TA100 and TA102 (Maron and Ames, 1983). Diluted plant extracts (0.1 ml) were added to the overnight-cultured strains (0.1 ml), with and without S9 mix (0.5 ml). Molten top agar (2 ml) containing 0.05mM biotin-histidine mixture was then added. The mixture was then gently mixed and poured onto a minimal glucose agar plate. When the top agar solidified, the plates were incubated in an inverted position at 37°C for 48 hours. The His^+ revertant colonies were then counted. Each sample was assayed using quadruplicate plates per run, and the data presented were the means of four experiments. An extract was considered mutagenic if it produced a reproducible, dose-related increase in the number of revertant colonies in one or more of the strains. The extract was classified as a weak mutagen if the number of revertant colonies was not double the background number of colonies at the highest concentration. A lack of a dose-related increase rendered the extract non-mutagenic.

8.2.10. Positive Control

In each experiment, positive controls were routinely included using diagnostic mutagens 2-AAF and CHP to confirm the reversion properties and specificity of each strain and the efficacy of the S9 mixture. 2-AAF was used as a positive control for the strains TA97a, TA98 and TA100 in the presence of S9 at concentrations of 200 μg/ml, 15 μg/ml and 90 μg/ml in DMSO respectively. The mutagen CHP was used as a positive control for the strain TA102 at a concentration of 1100 μg/ml.

8.2.11. Statistical Analyses

Basic statistics comprising the mean and standard deviation of quadruplicate experiments for each strain at each concentration were computed using Microsoft Excel XP software (Microsoft Corporation). Kruskal's gamma coefficient (γ) (Conover, 1999) was calculated to determine whether a trend existed in the number of revertant colonies at increasing levels of extract concentration. Kruskal's gamma coefficient estimates the difference between the probability of an monotonic increasing trend minus the probability of a monotonic decreasing trend. This means that if all the points at the next higher concentration are larger than those at the previous, then gamma = 1. Likewise, gamma = -1 if all the numbers were monotonically decreasing. It is a parametric measure which is specifically adjusted for the ties (in our case,

equal revertant colonies). A normal approximation was used to test for significance. A two-sided 1% significance level was used to compensate for multiple testing. Statistica v6.0 (Statsoft) was used for the trend analysis.

8.3. Results and Discussion

The plant extracts were evaluated for mutagenic potential against four bacterial strains of *Salmonella typhimurium*, *viz.*, TA97a, TA98, TA100 and TA102. These strains have different mutations in various genes in the histidine operon and each of these mutations is designed to be responsive to mutagens that act via different mechanisms. Twenty plants used as dietary supplements and 27 plants used as traditional medicines were evaluated using a range of concentrations with and without metabolic activation. The extracts were considered mutagenic if they produced a reproducible, dose-related increase in the number of revertant colonies in one or more of the strains. Where no dose-related increase was noted, the extract was considered non-mutagenic.

Of the 20 dietary plants assayed, 8 (40%) displayed weak mutagenicity towards the tester strain TA97a in the absence of metabolic activation (Table 8.3a). For the plants, *Rumex lanceolatus*, *Amaranthus hybridus, A. thunbergii, Oxalis corniculata, Raphanus raphinastrum, Sida dregei, S. rhombifolia* and *Solanum nodiflorum*, there was an increased monotonic dose-related response with increasing extract concentrations and when compared to the negative control. The Kruskal's gamma coefficient (γ) indicated that the trend was significant at the 1% level. Of these 8 plants, *R. lanceolatus* showed the highest dose-response. The strain TA97a contains an added cytosine resulting in a run of six cytosines at the mutated site in the histidine D gene. This strain is thus engineered to detect frameshift mutagens which are compounds that intercalate themselves between base pairs in the double helix of the DNA or between ring-stacked bases in a polynucleotide chain thus distorting the structure. This can be detrimental because the resulting amino acid sequence can be significantly altered or the start and stop signals can be disrupted.

The dietary plant extracts were also tested on TA97a with metabolic activation (Table 8.3b). For this, the rat liver S-9 microsomal fraction enriched in cytochrome P450 mixed function monooxygenases (MFO) and a fortified NADPH regenerating system, as outlined in the experimental section, was used. The number of plants that showed a mutagenic response was now 11 (55%) and two plants *viz.*, *Sonchus oleraceus* and *Zantedeschia aethiopica*, which did not show any response in the absence of S9

now displayed a toxic effect which manifested as a reduction in the revertant counts due to a sparse background growth. This decreased growth of revertants was significant at the 1% level (γ=-0.5910 and -0.4359 for *S. oleraceus* and *Z. aethiopica* respectively) (Figures 8.5a and 8.5b). The extracts of the plants *Centella asiatica* (γ=0.4640), *Colocasia esculenta* (γ=0.4510), *Physalis viscosa* (γ=0.5250) and *Solanum nigrum* (γ=0.6500) displayed weak mutagenicity upon metabolic activation. All of these responses were monotonic with significant trend at the 1% level. *R. lanceolatus* continued to show a significant dose-reponse relationship although the number of revertant colonies was moderately reduced following metabolic modification (Figure 8.6A).

Some carcinogenic chemicals are biologically inactive unless they are metabolised to active forms. In humans and animals, the cyrochrome-based P450 metabolic oxidation system, which is present mainly in the liver, is capable of metabolising a large number of these chemicals to DNA-reactive, electrophilic forms. Since bacteria do not have this metabolic capability and hence are unable to metabolize chemicals via cytochromes P450, an exogenous mammalian metabolic activation system consisting of rat liver homogenate (S-9 microsomal fraction) was introduced into the assay in the presence of NADP and cofactors for NADPH-supported oxidation (Maron and Ames, 1983). Hence from the above results is can be concluded that some plants contain promutagens which only become activated upon metabolic activation while others contain direct acting mutagens.

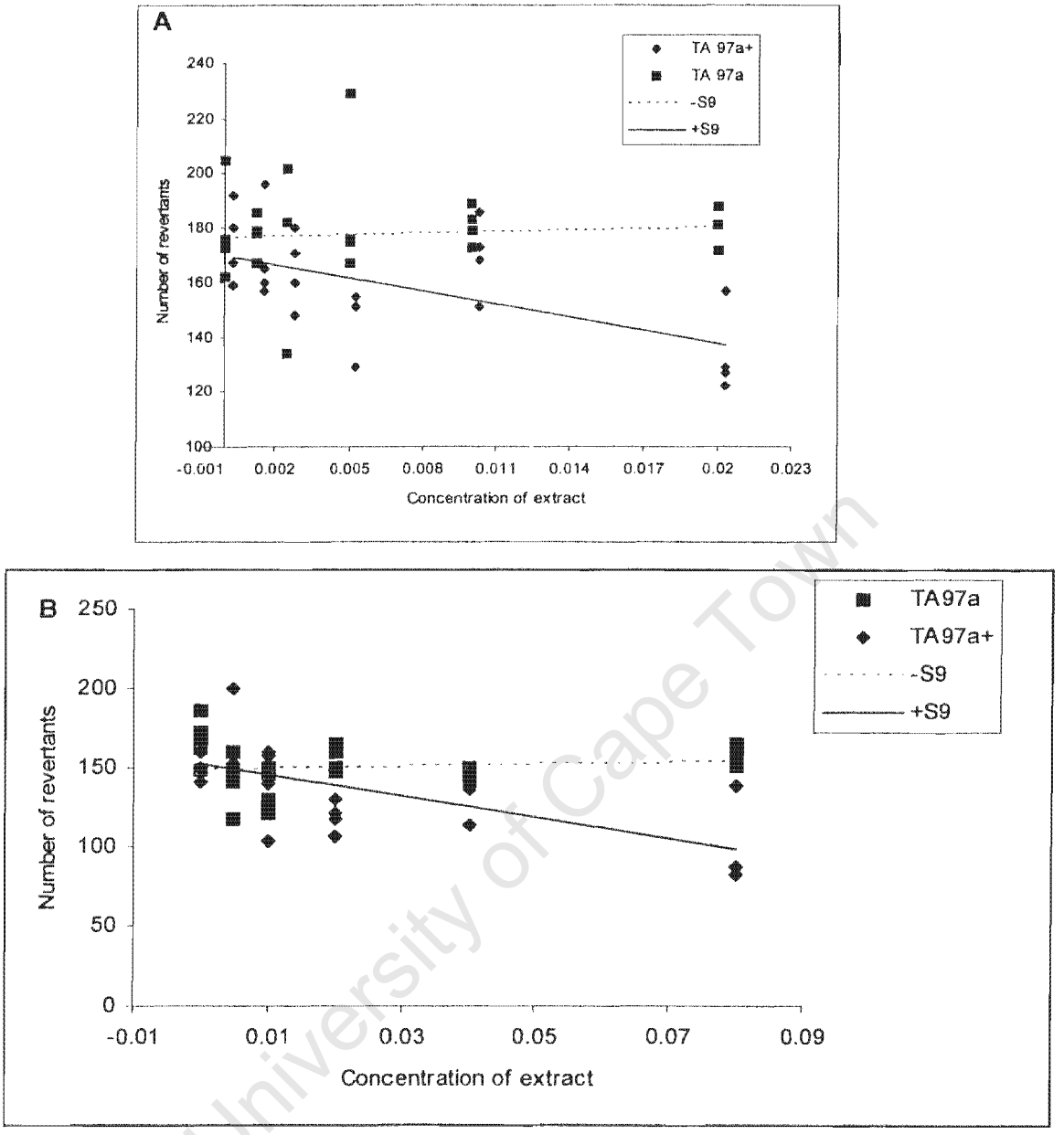


Figure 8.5. Effect of metabolic activation on the response of (A) *Z. aethiopica* extract and (B) *S. oleraceus* extract towards *S. typhimurium* strain TA97a. Data points represent the actual values of quadruplicate experiments at each concentration.

Table 8.3a: Mutagenicity of water extracts from dietary wild plants towards *S. typhimurium* TA97a in the absence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]							
	-S9							
	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)						γ^d
		0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Amaranthus asper	181±27	±	±	199±14	167±7	191±30	158±8	183±12	-0.1120
Amaranthus hybridus	147±17	-	-	142±10	157±6	157±17	177±13	172±13	0.5640*
Amaranthus thunbergii	186±22	-	-	200±17	183±17	219±20	207±24	238±23	0.4940*
Bidens pilosa	193±24	-	-	200±18	199±18	197±21	219±18	217±20	0.3610
Centella asiatica	186±16	-	-	187±16	175±26	190±15	191±16	210±21	0.3000
Chenopodium album	127±10	-	144±11	117±23	145±22	146±21	133±10	-	0.1000
Coccinia rehmanii	188±11	-	-	183±7	162±11	168±17	188±12	197±9	0.1740
Colocasia esculenta	180±5	-	-	179±15	176±27	169±12	180±22	183±24	-0.0130
Oxalis corniculata	170±8	-	-	161±13	172±11	184±35	181±18	207±20	0.4370*
Physalis viscosa	181±22	-	-	187±16	146±26	176±25	189±13	186±14	0.1210
Raphanus nasturtio aquatica	182±18	-	-	185±25	186±9	189±15	171±17	195±9	0.1020
Raphanus raphinastrum	171±16	-	-	173±10	177±16	186±4	188±16	205±35	0.4530*
Rumex lanceolatus	161±27	-	-	179±15	183±32	216±8	241±13	307±51	0.8739*
Sida dregei	138±10	-	138±17	150±8	163±26	173±15	170±11	-	0.6190*
Sida rhombifolia	167±6	-	-	177±10	185±8	178±24	197±13	213±20	0.6640*
Solanum nigrum	186±14	-	-	179±7	164±14	153±12	167±8	260±10	-0.0090
Solanum nodiflorum	185±16	-	-	207±14	223±19	213±13	242±14	253±38	0.6530*
Sonchus oleraceus	167±16	-	-	142±18	137±14	156±8	144±4	159±6	0.0090
Urtica urens	146±14	162±23	152±18	164±23	150±11	175±21	-	-	0.2240
Zantedeschia aethiopica	179±18	178±8	163±34	187±28	181±7	178±8	-	-	0.0932

[a] Data are means ± S.D. of four plates.
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1 ml of extract was added to each plate
[d] = Kruskal's gamma coefficient
* = significant at 1% level

Table 8.3b: Mutagenicity of water extracts from dietary wild plants towards *S. typhimurium* TA97a in the presence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]								
	+S9								γ^e
	Control (-)[b]	Control (+)[c]	Concentration of plant extract per plate[d] (g/ml)						
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Amaranthus asper	166±6	443±17	±	±	164±22	156±16	174±14	161±8	163±25	-0.0980
Amaranthus hybridus	104±13	492±121	-	-	117±11	122±25	139±18	130±11	125±18	0.3420
Amaranthus thunbergii	142±12	530±33	-	-	158±32	138±18	161±4	184±7	168±9	0.4530*
Bidens pilosa	145±7	528±27	-	-	154±22	173±30	163±29	164±11	199±15	0.4710*
Centella asiatica	150±27	583±100	-	-	150±12	133±16	147±14	173±9	201±23	0.4640*
Chenopodium album	134±22	466±139	-	156±13	112±9	96±12	120±5	124±23	-	-0.1950
Coccinia rehmanii	173±12	561±130	-	-	168±16	165±14	170±3	168±21	190±23	0.1130
Colocasia esculenta	145±12	573±87	-	-	136±12	138±20	146±17	155±12	173±13	0.4510*
Oxalis corniculata	150±7	556±114	-	-	170±13	176±11	177±16	167±8	177±11	0.3560
Physalis viscosa	175±20	508±64	-	-	173±13	157±22	175±16	210±8	218±21	0.5250*
Raphanus nasturtio aquatica	189±17	717±51	-	-	175±12	168±10	161±16	188±13	206±14	0.2630
Raphanus raphinastrum	150±14	582±96	-	-	156±10	171±4	162±14	192±13	177±15	0.5970*
Rumex lanceolatus	154±13	600±98	-	-	155±17	148±15	154±4	193±41	295±51	0.4576*
Sida dregei	133±9	353±73	-	138±17	143±15	153±13	153±21	164±25	-	0.4590*
Sida rhombifolia	150±10	512±133	-	-	152±22	150±22	154±24	185±19	189±14	0.4770*
Solanum nigrum	170±9	403±18	-	-	184±15	174±13	191±6	191±7	301±26	0.6500*
Solanum nodiflorum	111±23	536±79	-	-	140±21	154±15	180±17	236±23	247±13	0.8740*
Sonchus oleraceus	155±11	405±31	-	-	166±23	141±26	119±10	134±14	99±27#	-0.5910*
Urtica urens	127±28	497±73	113±13	130±9	122±13	111±13	120±6	-	-	-0.1060
Zantedeschia aethiopica	175±15	646±95	170±18	165±14	141±14	170±14	134±16	-	-	-0.4359*

[a] Data are means ± S.D. of four plates.
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] Control (+) = number of revertants in the presence of 2-Acetamidofluorene (20 μg)
[d] 0.1 ml of extract was added to each plate
[e] = Kruskal's gamma coefficient, * = significant at 1% level, # = toxic effect

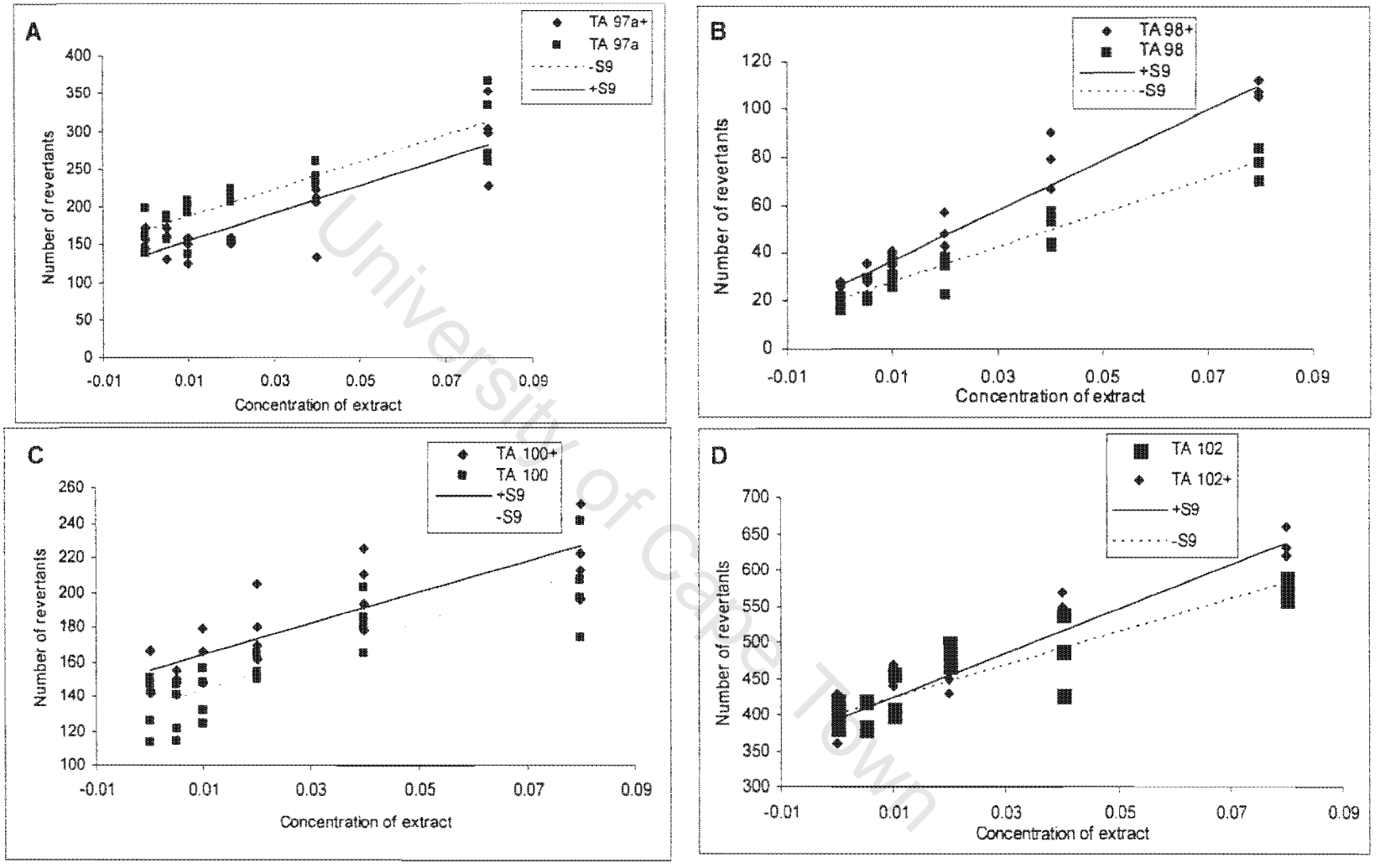


Figure 8.6. The effect of *Rumex lanceolatus* extract on (A) Strain TA97a, (B) Strain TA98, (C) Strain TA100 and (D) Strain TA 102 both in the presence and absence of metabolic activation. Data points represent the actual values of quadruplicate experiments at each concentration. All the trends are significant at the 1% level.

The tester strain TA98 was also used to detect frameshift mutations. In the absence of S-9 mix, only *R. lanceolatus* displayed mutagenicity (Table 8.4a). At the highest concentration of plant extract, the revertant colonies were approximately 4-times that of the negative control. A significant trend at the 1% level was observed over a series of 5 concentrations (γ=0.8979) (Figure 8.4a). In the presence of S-9 mix, however, 9 plants exhibited a significant trend in mutagenicity although the level of response varied (Table 3.4b). The extracts of *Amaranthus hybridus* (γ=0.5060), *A. thunbergii* (γ=0.5540), *Bidens pilosa* (γ=0.6910), *Sida rhombifolia* (γ=0.4550), *Solanum nigrum* (γ=0.6370) and *S. nodiflorum* (γ=0.6360) were considered to display a weak mutagenic response while extracts of *Centella asiatica* (γ=0.6300), *Physalis viscosa* (γ=0.7800) and *Rumex lanceolatus* (γ=0.9409) displayed a strong response since the number of revertants was greater than 2-times the spontaneous revertant count.

Table 8.4a: Mutagenicity of water extracts from dietary wild plants towards *S. typhimurium* TA98 in the absence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]								
	-S9								
	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)							γ^d
		0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Amaranthus asper	25±8	-	-	24±10	25±6	24±4	28±4	27±7	0.1490
Amaranthus hybridus	23±3	-	-	22±2	22±7	20±5	17±5	24±1	-0.0530
Amaranthus thunbergii	27±6	-	-	28±5	29±8	24±5	25±5	34±12	-0.0040
Bidens pilosa	24±3	-	-	25±5	29±3	34±15	28±4	27±4	0.2430
Centella asiatica	30±6	-	-	22±4	32±9	26±7	25±7	30±4	0.0260
Chenopodium album	28±4	-	21±2	26±4	26±8	24±4	25±3	-	-0.0260
Coccinia rehmanii	17±1	-	-	17±2	17±5	21±3	21±8	21±3	0.3780
Colocasia esculenta	20±3	-	-	22±2	19±3	15±1	20±5	21±4	-0.1470
Oxalis corniculata	19±4	-	-	22±2	18±4	19±5	24±3	25±3	0.3910
Physalis viscosa	24±6	-	-	22±6	22±5	25±8	26±3	22±5	0.0490
Raphanus nasturtio aquatica	22±5	-	-	23±6	23±5	22±5	21±4	33±6	0.2420
Raphanus raphinastrum	19±2	-	-	29±6	18±1	20±3	25±5	22±3	0.1300
Rumex lanceolatus	20±3	-	-	23±5	30±5	33±7	50±7	78±6	0.8979*
Sida dregei	25±6	-	23±2	27±9	20±2	29±3	31±4	-	0.2910
Sida rhombifolia	24±7	-	-	16±5	28±14	28±12	24±3	34±19	0.2780
Solanum nigrum	24±5	-	-	24±4	23±4	28±5	23±4	37±9	0.3300
Solanum nodiflorum	14±5	-	-	29±3	24±7	28±5	26±5	23±2	0.1550
Sonchus oleraceus	26±7	-	-	22±7	23±4	25±5	22±3	28±6	0.1070
Urtica urens	22±3	24±3	24±6	23±6	20±8	24±2	-	-	0.0140
Zantedeschia aethiopica	26±2	27±3	36±6	29±7	25±2	25±6	-	-	-0.2735

[a] Data are means ± S.D. of four plates.
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1 ml of extract was added to each plate
[d] = Kruskal's gamma coefficient
* = significant at 1% level

Table 8.4b: Mutagenicity of water extracts from dietary wild plants towards *S. typhimurium* TA98 in the presence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]									
	+S9									γ^e
	Control (-)[b]	Control (+)[c]	Concentration of plant extract per plate[d] (g/ml)							
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Amaranthus asper	32±2	155±5	-	-	36±2	28±3	31±3	31±5	30±4	-0.1840
Amaranthus hybridus	41±7	208±10	-	-	45±5	46±9	43±8	54±7	59±8	0.5060*
Amaranthus thunbergii	26±2	238±19	-	-	30±3	34±10	37±9	47±6	39±5	0.5540*
Bidens pilosa	36±7	186±23	-	-	37±2	39±4	46±2	47±4	51±10	0.6910*
Centella asiatica	28±8	195±43	-	-	31±5	28±2	38±10	48±5	66±6	0.6300*
Chenopodium album	30±5	191±13	-	28±3	35±4	31±2	67±22	30±7	-	0.2460
Coccinia rehmanii	29±2	149±16	-	-	32±6	31±6	31±9	36±10	38±7	0.3130
Colocasia esculenta	32±5	226±31	-	-	25±5	35±5	28±5	31±4	33±9	0.1080
Oxalis corniculata	29±4	190±9	-	-	31±2	25±3	24±4	30±4	31±3	0.0000
Physalis viscosa	25±5	225±82	-	-	34±3	38±6	38±4	39±2	53±4	0.7800*
Raphanus nasturtio aquatica	30±5	196±20	-	-	30±2	32±10	41±6	35±9	38±11	0.2690
Raphanus raphinastrum	26±5	222±20	-	-	25±2	29±7	25±4	32±7	33±7	0.3240
Rumex lanceolatus	26±3	181±31	-	-	29±7	38±3	47±8	73±14	107±3	0.9409*
Sida dregei	29±7	153±19	-	37±3	36±10	36±6	34±12	34±9	-	0.1000
Sida rhombifolia	26±3	171±19	-	-	27±3	26±4	27±2	33±8	45±16	0.4550*
Solanum nigrum	32±8	169±4	-	-	32±4	37±7	40±4	44±10	59±6	0.6370*
Solanum nodiflorum	32±6	216±26	-	-	36±9	33±3	35±5	45±5	58±7	0.6360*
Sonchus oleraceus	35±7	216±36	-	-	34±3	36±4	29±1	35±9	38±11	-0.0390
Urtica urens	28±4	179±22	32±9	31±7	24±5	26±3	28±7	-	-	-0.0870
Zantedeschia aethiopica	37±8	228±18	36±8	38±2	43±11	35±5	35±6	-	-	-0.0553

[a] Data are means ± S.D. of four plates.
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] Control (+) = number of revertants in the presence of 2-Acetamidofluorene (1.5 μg)
[d] 0.1 ml of extract was added to each plate
[e] = Kruskal's gamma coefficient
* = significant at 1% level

When tested on the tester strain TA100 in the absence of metabolic activation, only 8 plants exhibited a weak mutagenic response (Table 8.5a). This strain is designed to detect mutagens that cause base-pair substitutions (Levin and Ames, 1986). The plants were *Amaranthus hybridus* (γ=0.5020), *A. thunbergii* (γ=0.7820), *Bidens pilosa* (γ=0.7410), *Raphanus nasturtio aquatica* (γ=0.6370), *Rumex lanceolatus* (γ=0.7815), *Sida rhombifolia* (γ=0.4410), *Solanum nigrum* (γ=0.5700) and *Sonchus oleraceus* (γ=0.4360). Upon metabolic activation, 7 plants displayed mutagenic activity (Table 8.5b). Three of the plants that had shown mutagenic behaviour in the absence of S-9 mix, revealed no activity under metabolic activation leading one to believe that the mutagens in the extract may have become deactivated rendering them unable to cause base-pair substitutions. These plants were *Amaranthus hybridus*, *Bidens pilosa* and *Sida rhombifolia*. In contrast, two plants, *viz. Centella asiatica* (γ=0.7310) and *Physalis viscosa* (γ=0.6360) were found to exhibit mutagenic behaviour.

Table 8.5a: Mutagenicity of water extracts from dietary wild plants towards *S. typhimurium* TA100 in the absence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]								
	-S9								
	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)							γ^d
		0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Amaranthus asper	120±16	-	-	142±17	131±9	131±14	142±12	144±5	0.3600
Amaranthus hybridus	109±15	-	-	94±8	106±13	104±13	134±11	145±22	0.5020*
Amaranthus thunbergii	109±8	-	-	117±7	123±9	136±16	143±19	177±9	0.7820*
Bidens pilosa	124±11	-	-	119±12	127±8	137±9	151±12	178±14	0.7410*
Centella asiatica	161±43	-	-	162±23	139±23	127±5	127±4	172±18	-0.1190
Chenopodium album	137±18	-	134±10	136±11	131±10	135±16	160±13	-	0.2910
Coccinia rehmanii	-	-	-	-	-	-	-	-	-
Colocasia esculenta	124±22	-	-	127±12	134±14	129±15	134±10	165±13	0.3700
Oxalis corniculata	106±13	-	-	113±11	116±11	116±11	119±14	122±20	0.2410
Physalis viscosa	140±15	-	-	143±9	150±7	144±22	147±10	167±21	0.3310
Raphanus nasturtio aquatica	108±6	-	-	125±13	117±15	134±21	138±14	158±17	0.6370*
Raphanus raphinastrum	101±11	-	-	97±13	108±9	109±14	105±7	120±18	0.3140
Rumex lanceolatus	134±18	-	-	131±16	140±15	158±7	183±16	205±28	0.7815*
Sida dregei	119±17	-	135±6	133±10	127±17	114±21	120±14	-	-0.1490
Sida rhombifolia	104±9	-	-	118±6	112±20	122±26	116±6	145±18	0.4410*
Solanum nigrum	114±17	-	-	120±7	115±12	118±6	140±11	192±10	0.5700*
Solanum nodiflorum	175±14	-	-	139±8	149±16	141±2	163±13	171±14	0.1490
Sonchus oleraceus	117±13	-	-	114±9	130±12	125±14	125±35	143±8	0.4360*
Urtica urens	117±16	124±8	122±13	146±9	120±27	129±9	-	-	0.1950
Zantedeschia aethiopica	148±9	140±16	133±19	137±7	135±10	152±22	-	-	0.0345

[a] Data are means ± S.D. of four plates.
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1 ml of extract was added to each plate
[d] = Kruskal's gamma coefficient
* = significant at 1% level

Table 8.5b: Mutagenicity of water extracts from dietary wild plants towards *S. typhimurium* TA100 in the presence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]									
	+S9									
	Control (-)[b]	Control (+)[c]	Concentration of plant extract per plate[d] (g/ml)							γ^e
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Amaranthus asper	126±14	363±34	-	-	128±16	141±13	139±6	134±5	139±11	0.2570
Amaranthus hybridus	134±13	380±37	-	-	121±3	124±18	139±12	123±5	142±19	0.1420
Amaranthus thunbergii	117±12	402±45	-	-	123±14	116±6	131±11	145±14	161±16	0.6530*
Bidens pilosa	140±15	452±35	-	-	175±17	123±8	158±7	174±12	140±18	0.0990
Centella asiatica	130±9	388±31	-	-	120±19	134±13	148±14	164±13	202±18	0.7310*
Chenopodium album	128±3	411±70	-	129±12	113±13	119±6	137±30	138±17	-	0.1140
Coccinia rehmanii	-	-	-	-	-	-	-	-	-	-
Colocasia esculenta	127±20	457±54	-	-	132±16	134±9	145±12	129±7	156±6	0.3390
Oxalis corniculata	111±10	387±26	-	-	109±15	113±3	106±17	114±14	123±12	0.1240
Physalis viscosa	129±9	390±32	-	-	113±12	126±13	143±23	158±14	173±11	0.6360*
Raphanus nasturtio aquatica	122±10	363±15	-	-	126±10	132±11	140±17	127±3	177±8	0.4890*
Raphanus raphinastrum	112±12	492±49	-	-	112±7	104±5	113±18	116±12	127±12	0.2570
Rumex lanceolatus	155±14	508±104	-	-	149±6	165±13	179±19	202±20	221±23	0.7384*
Sida dregei	125±10	358±39	-	150±18	136±12	126±18	141±22	139±6	-	0.1780
Sida rhombifolia	121±17	300±36	-	-	135±55	115±25	119±15	128±13	149±6	0.3870
Solanum nigrum	115±5	370±29	-	-	123±6	132±4	121±8	138±6	224±14	0.6950*
Solanum nodiflorum	169±13	484±32	-	-	161±22	157±23	166±2	183±6	181±2	0.3670
Sonchus oleraceus	126±21	425±24	-	-	123±15	116±16	146±15	135±7	154±10	0.4550*
Urtica urens	116±8	313±42	116±20	98±8	114±20	120±15	98±4	-	-	-0.2030
Zantedeschia aethiopica	141±17	411±75	149±11	153±10	152±16	17±136	148±10	-	-	-0.0088

[a] Data are means ± S.D. of four plates.
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] Control (+) = number of revertants in the presence of 2-Acetamidofluorene (9 μg)
[d] 0.1 ml of extract was added to each plate, [e] = Kruskal's gamma coefficient,
* = significant at 1% level

The strain TA102 was used for detecting mutations due to oxidative damage. In the absence of S-9 mix, the plants *Amaranthus asper* (γ=0.4380), *A. hybridus* (γ=0.6320), *A. thunbergii* (γ=0.7980), *Bidens pilosa* (γ=0.6220), *Centella asiatica* (γ=0.5300), *Colocasia esculenta* (γ=0.4400), *Rumex lanceolatus* (γ=0.7908), *Sida rhombifolia* (γ=0.6780), *Solanum nigrum* (γ=0.7930) and *Solanum nodiflorum* (γ=0.7600) all displayed mutagenic activity (Table 8.6a). In the presence of S-9 mix, a further three plants, *viz. Chenopodium album* (γ=0.7450), *Coccinia rehmanii* (γ=0.6050) and *Physalis viscosa* (γ=0.5130) showed mutagenic activity (Table 8.6b) while the extract of *S. nodiflorum* did not reveal mutagenic activity upon metabolic activation.

Table 8.6a: Mutagenicity of water extracts from dietary wild plants towards *S. typhimurium* TA102 in the absence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]									
	-S9									γ^e
	Control (-)[b]	Control (+)[c]	Concentration of plant extract per plate[d] (g/ml)							
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Amaranthus asper	375±25	1588±103	-	-	364±42	370±36	400±27	411±13	453±69	0.4830*
Amaranthus hybridus	275±17	1110±73	-	-	323±37	328±6	393±28	326±33	454±26	0.6320*
Amaranthus thunbergii	324±5	1250±182	-	-	366±14	378±38	430±29	405±13	557±33	0.7980*
Bidens pilosa	442±37	1743±165	-	-	423±21	481±25	465±76	555±38	548±49	0.6220*
Centella asiatica	441±19	1423±204	-	-	476±14	443±46	468±45	486±32	568±51	0.5300*
Chenopodium album	411±15	1325±209	-	392±25	376±34	417±31	429±24	428±71	-	0.2170
Coccinia rehmanii	368±29	1675±185	-	-	406±46	373±10	401±27	385±20	471±55	0.3950
Colocasia esculenta	470±31	1495±116	-	-	449±11	433±13	480±14	495±31	546±59	0.4400*
Oxalis corniculata	388±42	1303±236	-	-	434±40	387±38	444±29	431±23	455±35	0.3790
Physalis viscosa	368±78	1160±127	-	-	450±47	403±21	415±34	435±58	485±62	0.3740
Raphanus nasturtio aquatica	458±5	1417±104	-	-	425±25	420±26	431±17	463±30	523±72	0.2590
Raphanus raphinastrum	370±16	950±56	-	-	359±24	345±37	358±43	411±26	455±77	0.4070
Rumex lanceolatus	402±16	1323±79	-	-	392±19	417±27	480±14	488±45	577±15	0.7908*
Sida dregei	378±29	1325±185	-	338±34	358±39	373±45	368±34	368±46	-	0.0390
Sida rhombifolia	317±15	1573±92	-	-	327±20	336±23	337±15	365±27	433±39	0.6780*
Solanum nigrum	343±24	1275±87	-	-	318±17	360±29	386±50	483±13	540±14	0.7930*
Solanum nodiflorum	354±24	1533±177	-	-	388±63	420±24	443±52	483±36	520±55	0.7600*
Sonchus oleraceus	440±12	1588±250	-	-	430±39	398±26	423±10	450±39	490±34	0.2980
Urtica urens	383±56	1393±26	370±52	398±34	380±27	398±45	443±63	-	-	0.2030
Zantedeschia aethiopica	445±47	1403±134	420±59	423±26	408±31	443±41	430±86	-	-	-0.0702

[a] Data are means ± S.D. of four plates.
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] Control (+) = number of revertants in the presence of cumene hydroperoxide (110 μg)
[d] 0.1 ml of extract was added to each plate
[e] = Kruskal's gamma coefficient
* = significant at 1% level

Table 8.6b: Mutagenicity of water extracts from dietary wild plants towards *S. typhimurium* TA102 in the presence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]								
	+S9								
	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)							γ^d
		0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Amaranthus asper	348±33	-	-	353±26	415±44	400±62	463±45	415±13	0.4810*
Amaranthus hybridus	323±19	-	-	320±10	332±27	355±37	367±38	483±10	0.6560*
Amaranthus thunbergii	346±9	-	-	340±37	412±10	443±31	506±5	573±17	0.8900*
Bidens pilosa	464±26	-	-	450±27	478±40	573±31	538±62	565±33	0.6210*
Centella asiatica	434±31	-	-	455±10	470±41	486±6	509±24	579±69	0.8120*
Chenopodium album	354±31	-	395±34	413±27	418±33	459±31	512±66	-	0.7450*
Coccinia rehmanii	380±36	-	-	398±21	363±29	408±43	485±48	503±28	0.6050*
Colocasia esculenta	427±49	-	-	419±18	500±34	513±62	518±20	553±36	0.6480*
Oxalis corniculata	438±33	-	-	415±48	453±25	427±19	473±22	455±66	0.1560
Physalis viscosa	433±22	-	-	425±19	448±21	430±22	515±59	530±64	0.5130*
Raphanus nasturtio aquatica	493±54	-	-	420±27	438±33	503±30	485±37	525±61	0.3010
Raphanus raphinastrum	378±19	-	-	355±37	345±38	368±33	385±29	448±50	0.3830
Rumex lanceolatus	386±33	-	-	442±27	440±29	450±16	545±38	623±33	0.8814*
Sida dregei	390±44	-	348±45	345±37	390±62	383±41	390±41	-	0.1480
Sida rhombifolia	244±21	-	-	263±33	237±23	323±67	328±67	448±52	0.6260*
Solanum nigrum	400±8	-	-	413±13	422±27	483±53	525±13	528±15	0.7910*
Solanum nodiflorum	422±28	-	-	445±28	445±44	425±13	535±78	468±32	0.4080
Sonchus oleraceus	448±36	-	-	420±16	418±37	453±46	450±47	455±26	0.1820
Urtica urens	430±54	415±6	443±29	413±41	410±22	428±46	-	-	0.0140
Zantedeschia aethiopica	400±47	455±70	423±59	403±77	430±28	393±19	-	-	-0.0940

[a] Data are means ± S.D. of four plates.
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1 ml of extract was added to each plate
[d] = Kruskal's gamma coefficient
* = significant at 1% level

With regards to the 27 plants used as traditional medicines, 5 plants showed weak mutagenic potential with a significant positive trend with increasing extract concentrations when assayed on the strain TA97a without metabolic activation (Table 8.7a). These plants were *Aloe ferox* (γ=0.5680), *Datura stramonium* (γ=0.7930), *Hypoestes aristata* (γ=0.4320), *Maesa laceolate* (γ=0.5760) and *Turraea floribunda* (γ=0.4120). The extract of the plant *Convolvulus farinosus* (γ=-0.6790), in contrast, showed extreme toxicity towards the strain and a significant dose-related decrease in the number of revertant colonies was observed. At the highest extract concentration (0.08 g/ml), the number of revertant colonies decreased by 2.4 times that of the spontaneous revertants. The toxicities were even more evident when S-9 mix was added to the test system (Figure 8.7).

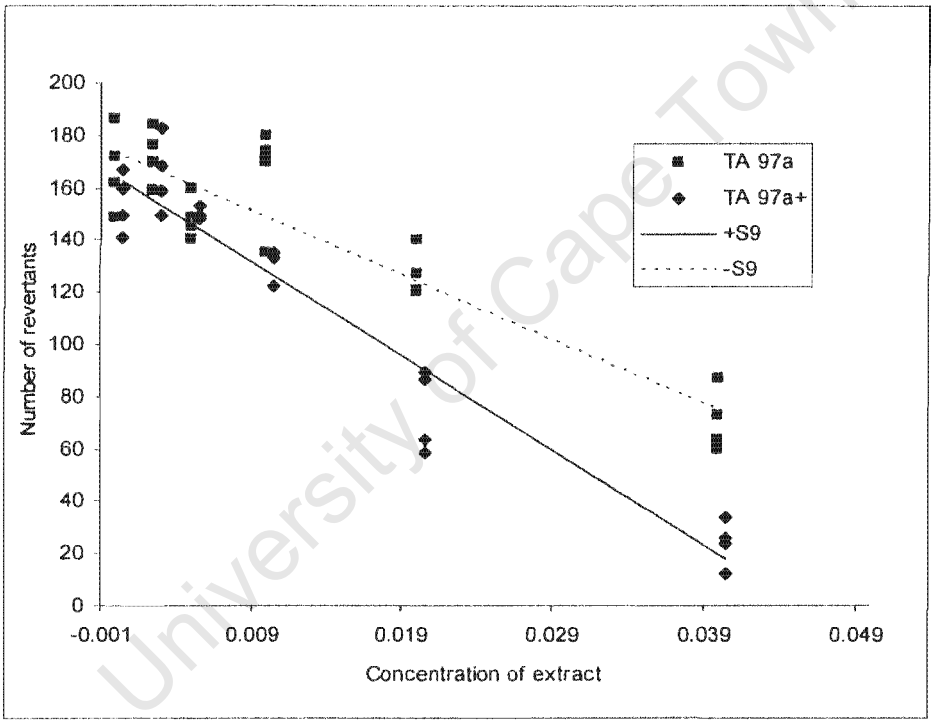


Figure 8.7. The effect of *Convolvulus farinosus* extract on Strain TA97a in the absence and presence of metabolic activation. Data points represent the actual values of quadruplicate experiments at each concentration. The decreased trend is indicative of cytotoxicity and is significant at the 1% level.

A similar observation was also made for the extract of *Ficus craterostoma* where upon metabolic activation, a significant trend in toxicity was observed (γ=-0.6020) (Figure 8.8).

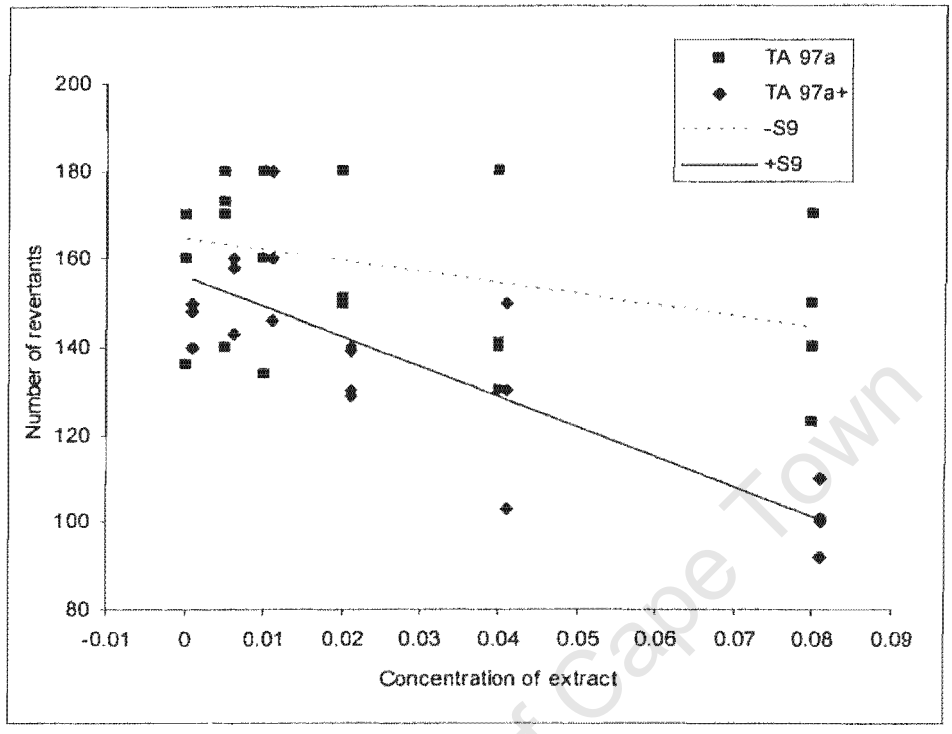


Figure 8.8. The effect of *Ficus craterostoma* extract on Strain TA97a in the absence and presence of metabolic activation. Data points represent the actual values of quadruplicate experiments at each concentration. The decreased trend is indicative of cytotoxicity but is only significant at the 1% level in the presence of metabolic activation.

These toxicities were evident through the thinning of the background lawn which was accompanied by a decrease in the number of revertant colonies. In certain instances pinpoint non-revertant colonies were observed. A decrease in the number of revertant colonies to levels below the spontaneous reversion level may on occasion be seen with thinning. A complete absence of background lawn indicates a high level of toxicity with the inability of the bacteria to grow and form a lawn although this was not the case in this experiment. Occasionally numerous small, non-revertant colonies were present on the plate. These colonies are referred to as "pinpoint colonies" and consist of histidine-dependent bacteria that survived high chemical toxicity. These

colonies are readily visible to the naked eye and can be mistaken for revertant colonies. However microscopic inspection of the plates revealed that there was a total absence of background lawn. The pinpoint colonies arose due to the fact that the high level of toxicity resulted in more histidine being available to the surviving *His*$^-$ bacteria on a per cell basis. Therefore these bacteria can undergo additional cell divisions until the depletion of the histidine. The plants are listed in Table 8.7b and can be distinguished from others by the negative Kruskal's gamma coefficient. A decrease in the number of revertants is also illustrated for *Pelargonium sp.* (Figure 8.9A) and *Pteridium aquilinum* (Figure 8.10A). Of the 27 medicinal plants tested, only two converted from non-mutagenic (-S9) to mutagenic in the presence of S-9 mix. These two plants were *Deinbolia oblongifolia* (γ=0.6140) and *Opuntia vulgaris* (γ=0.5170) which showed a significant trend. The extract of *D. oblongifolia* was classified as a strong mutagen due to its 2-fold increase in the number of revertant colonies compared to the negative control. The extracts of *Maesa lanceolate* and *Turraea floribunda* which showed weak mutagenic behaviour in the absence of S-9 were classified as non-mutagenic in the presence of S-9 mix.

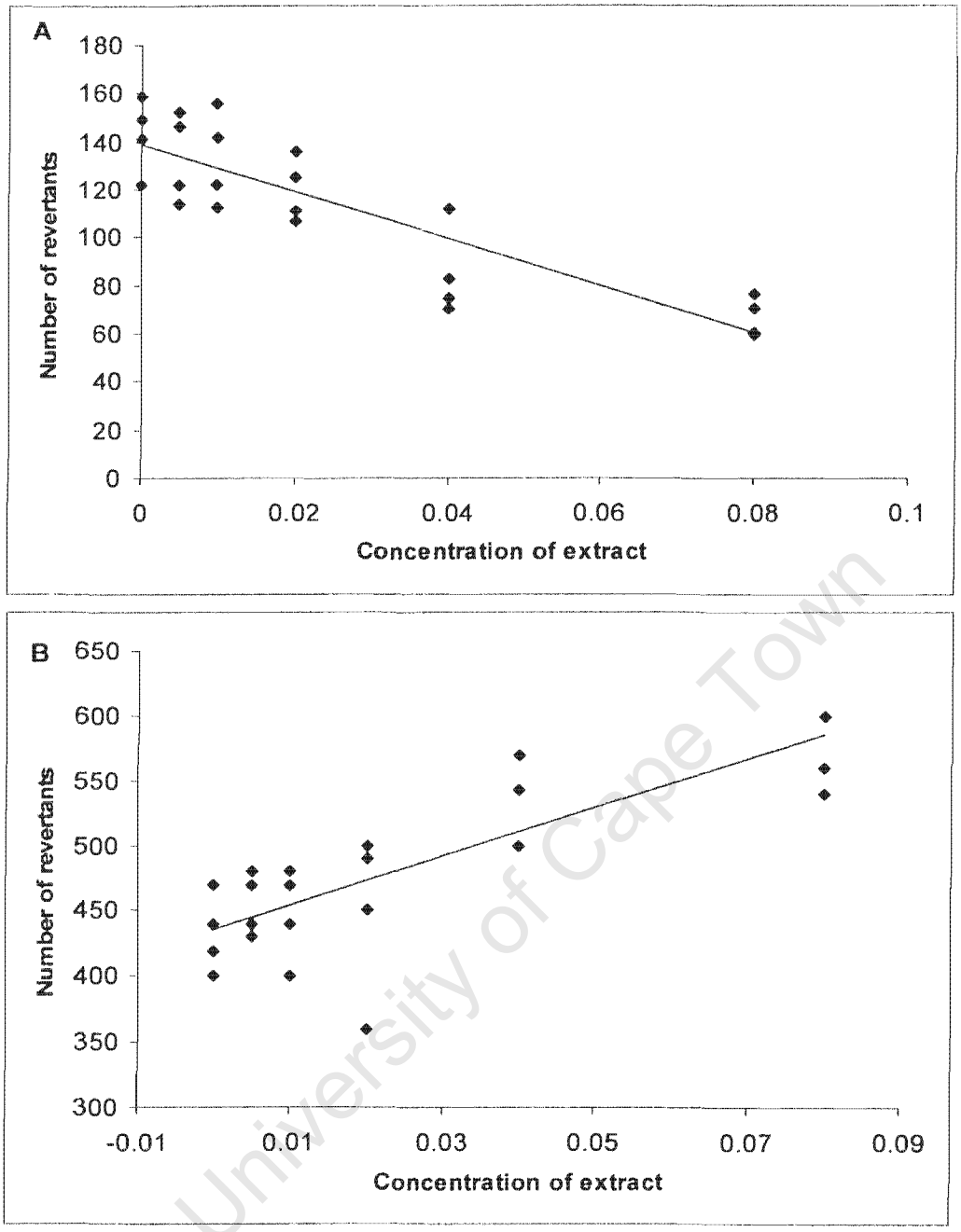


Figure 8.9. The effect of *Pelargonium extract* on (A) Strain TA97a in the presence of metabolic activation and (B) Strain TA102 in the absence of metabolic activation. Data points represent the actual values of quadruplicate experiments at each concentration. The trend is significant at the 1% level in both test systems.

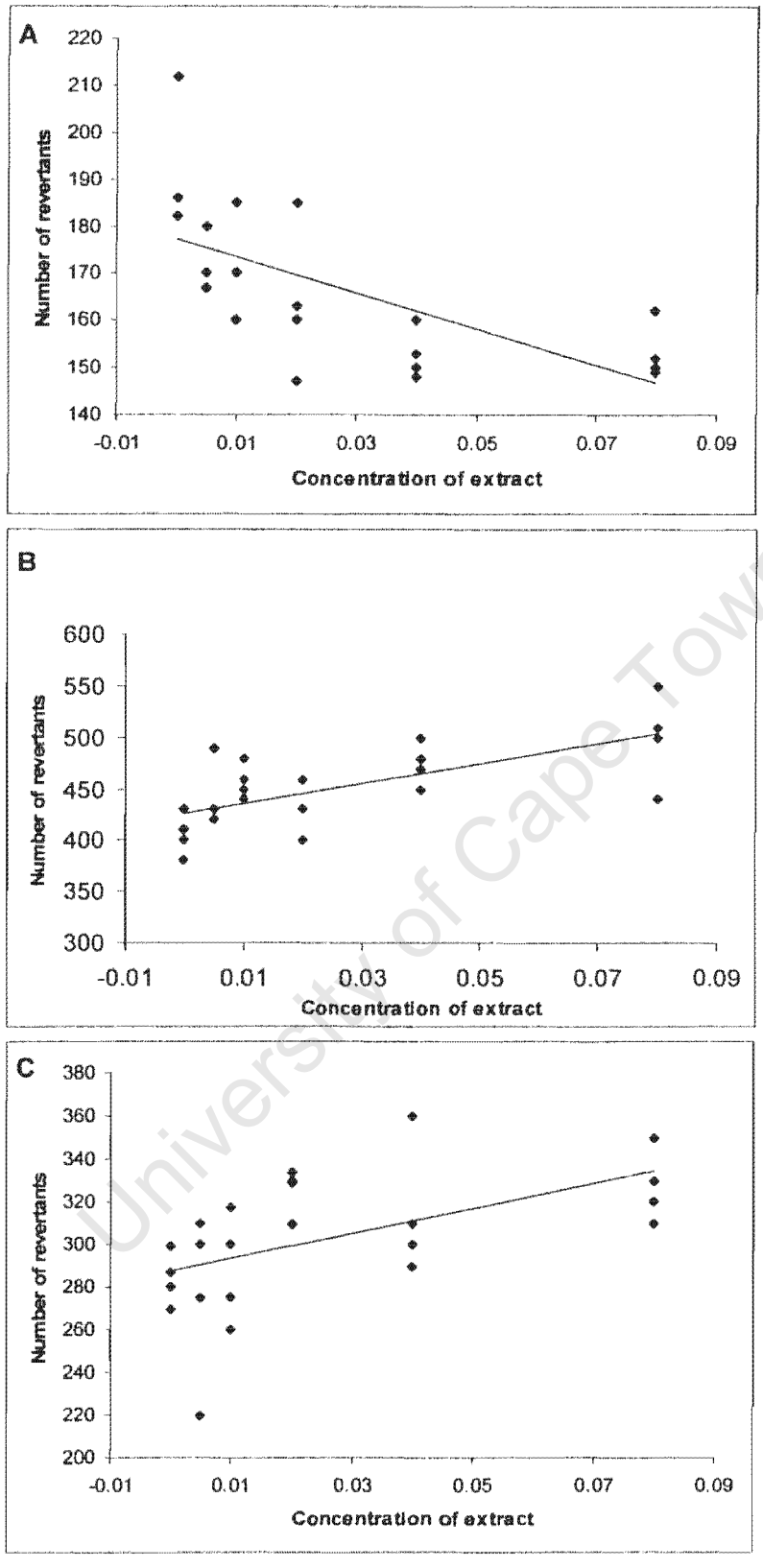


Figure 8.10. The effect of *Pteridium aquilinum* extract on (A) Strain TA97a in the presence of metabolic activation, (B) Strain TA100 in the presence of metabolic activation and (C) Strain TA102 in the absence of metabolic activation. Data points represent the actual values of quadruplicate experiments at each concentration.

Table 8.7a: Mutagenicity of water extracts from medicinal wild plants towards *S. typhimurium* TA97a in the abscence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]							
	-S9							
	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)						γ^d
		0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Acacia caffra	124±17	-	-	135±19	109±12	124±28	128±14	145±7	0.2200
Acacia karroo	158±10	-	-	186±12	157±12	183±5	181±16	190±12	0.4060
Acokanthera oppositifolia	170±13	-	-	177±14	158±4	156±7	161±4	167±16	-0.2696
Aloe Ferox	157±17	-	-	179±28	171±14	181±20	191±21	207±19	0.5680*
Artemisia affra	157±20	-	-	178±17	168±5	164±31	214±52	159±28	0.1480
Brunsvigia sp.	135±13	-	-	135±24	153±5	150±12	148±5	135±19	0.0700
Catha edulis	192±14	-	-	190±9	187±23	163±9	191±16	159±18	-0.3810
Convolvulus farinosus	167±16	-	-	172±11	149±9	165±20	127±9	71±12#	-0.6790*
Dalbergia obovata	196±27	-	-	203±29	176±18	195±11	214±21	232±5	0.3360
Datura stramonium	168±15	-	-	143±13	205±17	223±12	265±31	280±47	0.7930*
Deinbollia oblongifolia	185±12	-	-	171±9	213±31	227±33	184±16	196±10	0.1980
Eucomis autumnalis	160±20	-	-	163±19	152±23	169±23	155±7	152±16	-0.1480
Ficus craterostoma	159±16	-	-	166±18	164±22	165±17	148±22	146±20	-0.2070
Gunnera perpensa	152±18	-	-	160±36	147±9	157±18	160±4	161±15	0.3010
Helichrysum cymosum	212±12	-	-	160±8	170±10	187±23	188±14	192±7	0.0890
Hypoestes aristata	161±14	-	-	164±15	177±16	165±21	181±15	194±9	0.4320*
Lantana rugosa	182±17	-	-	138±14	172±14	173±22	168±13	183±19	0.1910
Lippia javanica	169±20	-	-	167±19	167±14	176±8	170±8	170±11	0.0000
Maesa lanceolate	135±13	-	-	135±11	151±8	170±12	148±12	173±21	0.5760*
Mariscus congestus	180±15	-	-	183±13	169±12	161±13	167±11	171±15	-0.2880
Maytenus acuminata	147±29	-	-	158±13	169±7	163±31	143±7	161±7	0.0220
Opuntia vulgaris	192±21	-	-	179±24	167±13	207±34	179±16	223±26	0.2100
Pelargonium sp.	109±32	-	-	150±25	166±19	177±21	166±4	129±27	0.2100
Pteridium aquilinum	204±25	-	-	181±14	169±10	178±5	163±20	188±15	-0.1802
Solanum mauritianum	186±14	-	-	160±8	148±10	152±15	137±7	175±6	-0.2840
Turraea floribunda	157±17	-	-	153±5	151±14	185±25	174±26	182±22	0.4120*
Withania somnifera	148±19	-	156±8	159±21	148±22	157±5	140±29	-	-0.0750

[a] Data are means ± S.D. of four plates., [b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1 ml of extract was added to each plate, [d] = Kruskal's gamma coefficient, * = significant at 1% level, # = toxic effect

Table 8.7b: Mutagenicity of water extracts from medicinal wild plants towards *S. typhimurium* TA97a in the presence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]									
	+S9									
	Control (-)[b]	Control (+)[c]	Concentration of plant extract per plate[d] (g/ml)							γ^e
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Acacia caffra	128±11	458±64	-	-	106±11	91±21	105±17	101±7	61±21#	-0.5350*
Acacia karroo	181±17	570±69	-	-	180±19	182±23	191±5	538±67	408±61	0.5630*
Acokanthera oppositifolia	161±17	480±37	-	-	132±9	131±8	140±11	158±10	133±4	-0.1224
Aloe Ferox	151±18	418±80	-	-	156±11	163±19	183±14	171±17	183±17	0.5510*
Artemisia affra	154±23	410±27	-	-	145±24	180±31	158±19	156±43	78±25#	-0.3300
Brunsvigia sp.	134±5	450±42	-	-	144±12	147±14	140±18	145±13	133±24	0.0550
Catha edulis	177±19	593±38	-	-	154±6	137±13	132±13	128±7	139±14#	-0.5400*
Convolvulus farinosus	155±11	405±31	-	-	165±14	150±2	128±7	74±16	24±9#	-0.8550*
Dalbergia obovata	218±30	490±64	-	-	186±5	158±10	178±17	223±10	237±15	0.2570
Datura stramonium	138±4	423±87	-	-	122±26	147±16	163±10	176±10	163±31	0.5830*
Deinbollia oblongifolia	218±55	485±32	-	-	211±23	220±25	220±22	259±42	427±54	0.6140*
Eucomis autumnalis	145±6	530±37	-	-	157±29	138±22	157±11	172±22	129±24	0.0680
Ficus craterostoma	147±5	420±95	-	-	155±8	162±14	135±6	128±19	101±7#	-0.6020*
Gunnera perpensa	153±6	410±10	-	-	128±21	122±8	124±9	121±20	122±6	-0.3390
Helichrysum cymosum	210±11	630±25	-	-	202±40	213±23	159±20	160±11	181±30	-0.3890
Hypoestes aristata	148±7	637±95	-	-	143±11	151±18	165±13	160±6	168±10	0.5250*
Lantana rugosa	171±8	415±26	-	-	154±24	150±23	138±22	92±22	84±21#	-0.7280*
Lippia javanica	169±22	613±97	-	-	139±21	88±13	66±24	63±14	65±16#	-0.7630*
Maesa lanceolate	134±5	450±42	-	-	125±4	115±10	106±13	132±5	138±14	0.0400
Mariscus congestus	174±12	725±29	-	-	166±5	158±5	149±25	131±10	121±8#	-0.7690*
Maytenus acuminata	163±18	485±38	-	-	138±6	136±27	128±9	120±4	130±8	-0.4400*
Opuntia vulgaris	141±4	398±61	-	-	153±17	132±3	149±19	180±8	181±12	0.5170*
Pelargonium sp.	143±16	492±23	-	-	134±18	133±19	120±13	85±19	66±8#	-0.7370*
Pteridium aquilinum	192±14	462±17	-	-	172±6	171±10	164±16	153±5	153±6	-0.7056*
Solanum mauritianum	170±9	403±18	-	-	153±8	149±9	122±7	120±8	100±11#	-0.8820*
Turraea floribunda	136±17	418±80	-	-	162±16	164±11	150±12	144±17	161±17	0.1120
Withania somnifera	140±27	455±79	-	140±8	151±10	143±15	159±12	121±18	-	-0.1740

[a] Data are means ± S.D. of four plates, [b] Control (-) = number of spontaneous revertants without plant extracts,
[c] Control (+) = number of revertants in the presence of 2-Acetamidofluorene (20 μg), [d] 0.1 ml of extract was added to each plate
[e] = Kruskal's gamma coefficient, * = significant at 1% level, # = toxic effect

Six plant extracts showed mutagenicity towards the tester strain TA 98 (Table 8.8a). Of these 6 plants, 4 were classified as strongly mutagenic due to their high number of revertant colonies per plate in comparison to the spontaneous revertants. These plants were *Acacia caffra* (γ=0.6720), *Artemisia affra* (γ=0.7610), *Datura stramonium* (γ=0.9490) and *Maesa lanceolate* (γ=0.6670). These plant extracts continued to show mutagenic potential in the presence of S-9 with the extract of *D. stramonium* providing revertant colonies of up to 9-times that of the spontaneous revertants (Figure 8.11) and 1.5-times that of the positive control indicating that this plant has great potential in causing frameshift mutations (Table 8.8b).

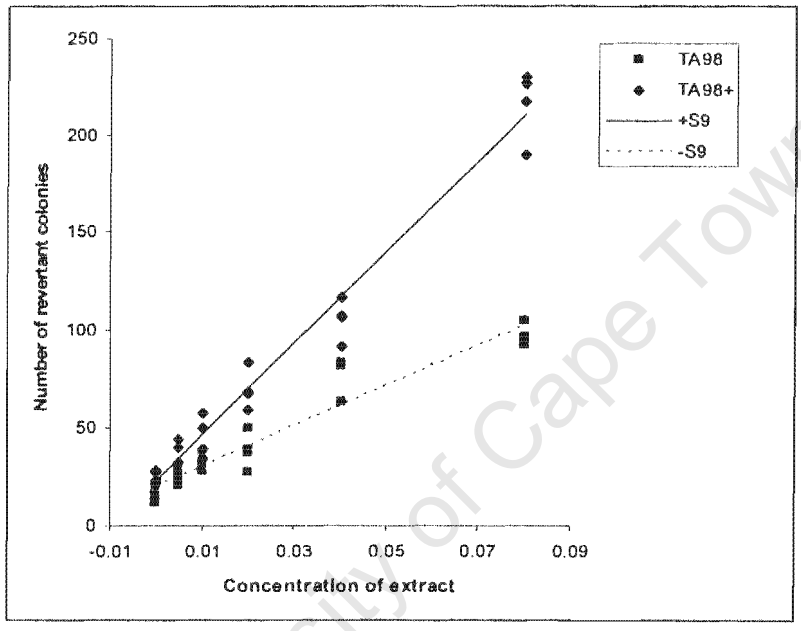


Figure 8.11. The effect of *Datura stramonium* extract on Strain TA98 in the absence and presence of metabolic activation. Data points represent the actual values of quadruplicate experiments at each concentration. The increased trend is siginificant at the 1% level and the number of revertant colonies shows this plant to be highly mutagenic to the bacterial strain.

The extracts of *Lantana rugosa* and *Mariscus congestus* showed moderate cytotoxic effects which manifested as a reduction in the revertant counts due to a sparse background growth. The extract of *Solanum mauritianum* which was non-mutagenic in the absence of S-9 showed a significant trend in cytotoxicity in presence of S-9 mix. *Solanum* extacts are known to contain high levels of solanine alkaloids (Vogel and Gutzwiller, 1993). In this study cytotoxicity could be attributed to the glycoalkaloids which are non-mutagenic (Friedman and Henika, 1992).

Table 8.8a: Mutagenicity of water extracts from medicinal wild plants towards *S. typhimurium* TA98 in the abscence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]								
	-S9								
	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)							γ^d
		0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Acacia caffra	21±2	-	-	20±3	25±2	23±1	32±2	43±9	0.6720*
Acacia karroo	27±3	-	-	29±3	30±7	27±3	39±6	47±5	0.5930*
Acokanthera oppositifolia	24±8	-	-	23±2	25±1	23±3	26±3	27±3	0.1150
Aloe Ferox	27±2	-	-	27±4	28±9	28±5	28±4	35±4	0.3360
Artemisia affra	25±9	-	-	21±3	26±6	35±2	44±4	77±6	0.7610*
Brunsvigia sp.	19±3	-	-	22±9	20±3	22±6	24±6	25±3	0.2800
Catha edulis	25±4	-	-	19±3	22±6	23±3	20±2	29±4	0.1640
Convolvulus farinosus	26±7	-	-	28±10	23±5	21±1	21±2	22±7	-0.1980
Dalbergia obovata	26±5	-	-	33±3	26±3	30±3	28±7	29±3	0.0000
Datura stramonium	18±4	-	-	24±3	31±4	38±9	73±11	97±6	0.9490*
Deinbollia oblongifolia	28±5	-	-	42±32	33±3	29±2	34±7	37±12	0.2430
Eucomis autumnalis	30±6	-	-	24±3	22±4	31±2	24±8	23±1	-0.1890
Ficus craterostoma	23±5	-	-	19±5	20±4	18±3	19±5	17±1	-0.3020
Gunnera perpensa	23±2	-	-	28±6	26±5	21±1	24±2	27±6	0.0230
Helichrysum cymosum	33±9	-	-	28±7	30±6	33±7	30±5	23±5	-0.2720
Hypoestes aristata	18±5	-	-	20±3	19±7	21±2	18±3	23±5	0.1390
Lantana rugosa	26±3	-	-	23±4	24±5	24±3	26±8	20±5	-0.1980
Lippia javanica	23±4	-	-	24±5	28±2	26±4	27±4	28±6	0.3030
Maesa lanceolate	19±3	-	-	19±5	19±3	21±4	32±4	40±6	0.6670*
Mariscus congestus	25±3	-	-	21±8	28±3	25±6	24±4	22±7	-0.0260
Maytenus acuminata	21±2	-	-	24±2	18±2	20±1	20±1	26±7	-0.0390
Opuntia vulgaris	24±7	-	-	20±3	21±2	21±4	27±4	31±2	0.4720*
Pelargonium sp.	21±4	-	-	21±2	23±6	22±7	23±3	24±1	0.2480
Pteridium aquilinum	23±11	-	-	26±6	23±6	25±6	23±4	23±3	-0.1207
Solanum mauritianum	24±5	-	-	23±4	21±3	26±4	24±3	22±3	0.0700
Turraea floribunda	27±2	-	-	26±4	25±8	26±2	23±5	30±3	0.0450
Withania somnifera	20±4	-	20±2	22±4	24±7	25±3	24±4	-	0.3390

[a] Data are means ± S.D. of four plates, [b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1 ml of extract was added to each plate, [d] = Kruskal's gamma coefficient, * = significant at 1% level

Table 8.8b: Mutagenicity of water extracts from medicinal wild plants towards *S. typhimurium* TA98 in the presence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]									
	+S9									
	Control (-)[b]	Control (+)[c]	Concentration of plant extract per plate[d] (g/ml)							γ^e
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Acacia caffra	25±5	158±28	-	-	19±3	25±8	30±6	32±9	70±12	0.5450*
Acacia karroo	46±9	173±26	-	-	39±4	34±10	43±11	55±8	71±9	0.4600*
Acokanthera oppositifolia	37±8	193±20	-	-	37±2	36±4	41±7	31±2	39±3	0.0442
Aloe Ferox	40±2	169±8	-	-	33±4	42±2	34±2	32±5	33±7	0.3130
Artemisia affra	33±4	146±5	-	-	29±4	31±6	36±6	35±7	57±4	0.4850*
Brunsvigia sp.	31±2	167±20	-	-	27±8	27±5	36±6	37±10	33±11	0.2050
Catha edulis	31±6	167±15	-	-	27±7	24±4	25±3	26±3	26±3	-0.1470
Convolvulus farinosus	35±7	208±38	-	-	38±4	33±10	32±7	31±5	33±7	0.2210
Dalbergia obovata	40±7	160±18	-	-	44±11	48±5	41±7	35±10	31±5	-0.3900
Datura stramonium	25±3	146±10	-	-	39±5	45±10	69±10	105±10	216±18	0.9500*
Deinbollia oblongifolia	60±20	175±13	-	-	70±5	67±4	70±15	84±4	101±8	0.5730*
Eucomis autumnalis	31±6	152±6	-	-	32±6	30±3	38±3	36±5	46±8	0.5190*
Ficus craterostoma	23±8	176±19	-	-	30±10	30±4	27±5	27±4	25±6	-0.0170
Gunnera perpensa	31±8	195±6	-	-	29±9	28±7	28±3	23±3	24±4	-0.2860
Helichrysum cymosum	44±5	170±8	-	-	43±11	38±6	45±12	36±3	35±6	-0.3190
Hypoestes aristata	32±5	201±32	-	-	27±5	30±5	32±6	31±6	32±2	0.0920
Lantana rugosa	35±4	184±32	-	-	38±8	32±5	31±5	33±10	24±6	-0.4440*
Lippia javanica	45±5	194±31	-	-	48±6	34±8	27±5	30±4	32±8	-0.5190*
Maesa lanceolate	31±2	167±20	-	-	28±5	27±1	29±6	35±4	57±7	0.4790*
Mariscus congestus	34±4	189±34	-	-	29±3	26±3	28±4	26±2	26±3	-0.4360*
Maytenus acuminata	33±6	172±11	-	-	22±5	27±6	20±2	23±4	21±4	-0.3680
Opuntia vulgaris	30±6	184±28	-	-	33±7	31±5	33±8	24±6	30±9	-0.1620
Pelargonium sp.	35±4	173±14	-	-	24±2	21±3	20±3	23±5	26±5	-0.2750
Pteridium aquilinum	37±3	200±40	-	-	40±8	34±4	29±5	31±3	34±7	-0.3391
Solanum mauritianum	32±8	169±4	-	-	33±3	38±3	38±9	36±8	51±8	-0.4870*
Turraea floribunda	40±2	169±8	-	-	36±5	37±5	44±1	39±10	41±4	0.2040
Withania somnifera	37±6	176±26	-	39±5	39±10	30±4	38±7	39±4	-	-0.0180

[a] Data are means ± S.D. of four plates, [b] Control (-) = number of spontaneous revertants without plant extracts

[c] Control (+) = number of revertants in the presence of 2-Acetamidofluorene (1.5 μg), [d] 0.1 ml of extract was added to each plate

[e] = Kruskal's gamma coefficient, * = significant at 1% level

Seven medicinal plants showed a weak and one showed a strong mutagenic response towards the strain TA100 in the absence of S9 (Table 8.9a). Despite the variation in the number of revertant colonies, there was a significant monotonic dose-response with these plant extracts. In the presence of S-9, 4 plants that were classified as non-mutagenic in the absence of S-9, displayed weak (*M. lanceolate*, *O. vulgaris* and *Pteridium aquilinum*) and strong (*D. oblongifolia*) mutagenic responses (Table 8.9b). Figure 3.10B illustrates the response of the bacteria towards the extract of *P. aquilinum*. A similar observation was reported by Pamukcu *et al.*, (1980) where *P. aquilinum* demonstrated mutagenicity for *S. typhimurium* strain TA100 but not for TA98.

Table 8.9a: Mutagenicity of water extracts from medicinal wild plants towards *S. typhimurium* TA100 in the abscence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]							
	-S9							
	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)						γ^d
		0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Acacia caffra	107±12	-	-	121±11	120±12	120±11	125±13	141±24	0.3770*
Acacia karroo	174±31	-	-	190±14	258±21	360±36	358±57	348±49	0.7230*
Acokanthera oppositifolia	120±11	-	-	135±8	133±6	126±11	145±12	140±12	0.2658
Aloe Ferox	125±13	-	-	122±12	119±11	117±22	112±9	144±25	-0.0220
Artemisia affra	118±10	-	-	137±16	125±26	139±16	156±5	178±19	0.6680*
Brunsvigia sp.	119±7	-	-	111±7	118±12	124±12	142±15	132±16	0.4380*
Catha edulis	113±15	-	-	117±20	124±23	116±16	112±15	133±15	0.1600
Convolvulus farinosus	117±13	-	-	110±11	151±6	130±8	148±5	137±9	0.3830
Dalbergia obovata	147±6	-	-	162±6	133±13	124±8	150±16	140±16	-0.1740
Datura stramonium	134±5	-	-	161±1	150±20	155±19	166±7	181±17	0.5790*
Deinbollia oblongifolia	140±9	-	-	116±20	122±14	134±15	130±13	178±15	0.2910
Eucomis autumnalis	124±26	-	-	108±14	137±2	115±22	133±19	134±14	0.2200
Ficus craterostoma	124±5	-	-	84±15	121±8	113±17	122±15	113±9	0.0630
Gunnera perpensa	110±8	-	-	122±12	111±6	134±5	112±10	116±9	0.1030
Helichrysum cymosum	138±14	-	-	117±11	135±11	114±10	132±5	137±15	-0.0100
Hypoestes aristata	113±9	-	-	121±12	132±16	131±26	141±10	161±13	0.6150*
Lantana rugosa	119±19	-	-	97±12	116±19	144±13	126±11	132±9	0.3300
Lippia javanica	167±16	-	-	169±21	204±17	208±9	190±10	197±29	0.2790
Maesa lanceolate	94±56	-	-	127±14	124±5	124±13	134±13	128±13	0.2800
Mariscus congestus	125±12	-	-	117±15	118±10	102±11	104±10	119±12	-0.2180
Maytenus acuminata	105±11	-	-	115±6	106±6	112±15	118±10	122±6	0.3690
Opuntia vulgaris	140±18	-	-	135±8	125±8	132±19	136±8	150±17	0.1400
Pelargonium sp.	121±17	-	-	123±14	126±17	121±23	138±8	154±21	0.3950
Pteridium aquilinum	269±10	-	-	263±39	232±16	259±28	253±15	294±42	0.0796
Solanum mauritianum	114±17	-	-	103±7	129±8	119±9	127±12	154±8	0.5640*
Turraea floribunda	125±13	-	-	113±16	113±5	145±13	140±14	158±22	0.5540*
Withania somnifera	128±9	-	120±8	118±13	129±15	116±9	163±24	-	0.1960

[a] Data are means ± S.D. of four plates, [b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1 ml of extract was added to each plate, [d] = Kruskal's gamma coefficient, * = significant at 1% level

Table 8.9b: Mutagenicity of water extracts from medicinal wild plants towards *S. typhimurium* TA100 in the presence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]									
	+S9									
	Control (-)[b]	Control (+)[c]	Concentration of plant extract per plate[d] (g/ml)							γ^e
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Acacia caffra	100±7	342±26	-	-	116±5	128±14	120±26	120±12	142±22	0.4310*
Acacia karroo	176±20	433±34	-	-	296±52	250±59	305±34	262±19	295±19	0.3360
Acokanthera oppositifolia	118±7	355±24	-	-	127±5	126±16	140±5	132±3	127±8	0.1864
Aloe Ferox	126±19	385±31	-	-	118±15	120±8	116±10	131±14	154±8	0.4060
Artemisia affra	109±9	313±26	-	-	135±30	133±29	134±18	124±11	179±11	0.4340*
Brunsvigia sp.	114±5	357±33	-	-	131±8	119±24	128±10	123±5	141±14	0.4270
Catha edulis	129±11	434±6	-	-	115±2	111±10	115±7	118±4	129±24	-0.0340
Convolvulus farinosus	126±21	425±24	-	-	116±6	123±8	120±21	129±8	138±19	0.3020
Dalbergia obovata	140±18	350±24	-	-	148±6	142±6	144±21	146±17	152±11	0.1160
Datura stramonium	165±8	418±61	-	-	134±11	142±7	159±13	215±20	225±19	0.5620*
Deinbollia oblongifolia	158±13	365±13	-	-	162±32	159±7	189±28	206±26	293±44	0.6740*
Eucomis autumnalis	125±11	340±45	-	-	129±13	122±7	135±11	133±15	137±11	0.3100
Ficus craterostoma	113±5	283±30	-	-	127±13	112±22	100±15	114±7	113±17	-0.0990
Gunnera perpensa	122±10	373±43	-	-	113±11	134±27	103±5	116±11	115±11	-0.1380
Helichrysum cymosum	128±4	380±54	-	-	140±7	127±10	123±22	138±8	139±15	0.1550
Hypoestes aristata	115±13	425±38	-	-	107±6	121±12	128±16	135±12	151±8	0.6130*
Lantana rugosa	108±29	325±25	-	-	125±10	129±12	114±6	125±19	144±16	0.2770
Lippia javanica	140±14	488±29	-	-	258±67	302±22	295±19	191±17	168±10	-0.0310
Maesa lanceolate	114±5	357±33	-	-	124±8	108±19	143±10	135±10	127±11	0.4310*
Mariscus congestus	131±8	359±9	-	-	136±19	106±12	125±3	120±4	116±6	-0.3190
Maytenus acuminata	115±11	383±61	-	-	104±7	116±14	117±9	112±10	114±12	0.0960
Opuntia vulgaris	129±9	346±32	-	-	114±7	125±4	120±14	139±9	142±13	0.4320*
Pelargonium sp.	152±9	375±25	-	-	128±9	130±9	138±4	127±15	158±31	-0.0040
Pteridium aquilinum	284±12	430±25	-	-	276±40	288±25	326±11	315±31	328±17	0.5459*
Solanum mauritianum	115±5	420±98	-	-	103±14	118±2	132±10	122±11	172±35	0.5480*
Turraea floribunda	126±19	385±31	-	-	122±25	143±13	119±18	138±12	149±20	0.2720
Withania somnifera	124±11	298±19	-	133±13	129±21	126±18	129±17	136±11	-	0.1260

[a] Data are means ± S.D. of four plates, [b] Control (-) = number of spontaneous revertants without plant extracts

[c] Control (+) = number of revertants in the presence of 2-Acetamidofluorene (9 μg), [d] 0.1 ml of extract was added to each plate

[e] = Kruskal's gamma coefficient, * = significant at 1% level

When evaluated using the tester strain TA102, 8 plants a showed significant dose-response relationship with increasing concentration of the plant extracts although the level of response led them to be classified as weak mutagens (Table 8.10a). These plants were *D. stramonium* (γ=0.4630), *H. aristata* (γ=0.6020), *O. vulgaris* (γ=0.5590), *Pelargonium sp.* (γ=0.6880), *P. aquilinum* (γ=0.6195), *S. mauritianum* (γ=0..6810), *T. floribunda* (γ=0.5970) and *W. somnifera* (γ=0.4450). Figure 8.9B illustrated the response brought about by the extract of *Pelargonium sp.*, indicating that while the extract showed cytotoxicity towards the strain TA97a, it was mutagenic towards TA102 indicating that the mutations were the result of oxidative DNA damage. Figure 8.10C illustrates the response induced by the extract of *P. aquilinum* in the absence of metabolic activation. In the presence of the S-9 fraction, the plant *D. stramonium* continued to show mutagenic potential in a significant dose-related manner with the number of revertant colonies much higher than when assayed in the absence of the S-9 fraction (Table 8.10b). The plants *A. karroo* (γ=0.4980) and *A. affra* (γ=5390), which showed no mutagenicity in the absence of S-9 mix subsequently displayed moderate mutagenic activity, whilst the extracts of the plants *A. caffra* (γ=-0.6120) and *M. acuminata* (γ=-0.4630) showed moderate cytotoxicities as a result of a reduction in the revertant counts below that of the spontaneous revertants.

It is important to bear in mind when evaluating mutagenicity data with strain TA102, that nucleophilic sites in peptides, proteins and DNA are most susceptible target sites for electrophilic chemicals, which are found in plants. While S-9 mix is meant to provide a protective environment due to its detoxification mechanism by reaction of the phytochemical with glutathione, this resultant decrease of glutathione concentration not only exposes other nucleophilic targets to alkylation, but also increases the susceptibility to other toxic effects, such as internal oxidative stress. Alkylation of nuceophilic sites in DNA is the primary reaction triggering mutagenesis and subsequent carcinogenesis.

Table 8.10a: Mutagenicity of water extracts from medicinal wild plants towards *S. typhimurium* TA102 in the abscence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]									
	-S9									
	Control (-)[b]	Control (+)[c]	Concentration of plant extract per plate[d] (g/ml)							γ^e
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Acacia caffra	330±22	1325±132	-	-	343±22	358±40	325±29	350±42	370±22	0.2560
Acacia karroo	440±62	1475±132	-	-	470±26	473±30	490±74	500±71	540±62	0.2830
Acokanthera oppositifolia	430±36	1613±85	-	-	470±25	415±21	435±21	468±10	423±26	-0.0796
Aloe Ferox	348±43	1075±65	-	-	335±51	398±38	405±40	383±56	405±29	0.3670
Artemisia affra	330±50	1150±108	-	-	368±32	373±34	373±49	355±60	405±26	0.3160
Brunsvigia sp.	303±33	1575±194	-	-	315±17	353±26	340±45	403±57	260±32	0.0710
Catha edulis	450±42	1650±100	-	-	421±15	420±43	420±56	448±60	448±73	0.0390
Convolvulus farinosus	440±12	1588±250	-	-	380±24	395±24	378±17	423±41	423±39	0.0230
Dalbergia obovata	450±34	1638±75	-	-	405±40	413±31	420±18	459±16	480±36	0.3450
Datura stramonium	282±14	1525±119	-	-	281±13	301±28	290±56	328±48	335±33	0.4630*
Deinbollia oblongifolia	425±52	1738±170	-	-	435±60	420±45	438±39	413±67	500±29	0.2800
Eucomis autumnalis	303±33	1500±135	-	-	330±24	393±8	330±24	353±57	363±43	0.3200
Ficus craterostoma	310±32	1250±122	-	-	290±33	325±21	308±33	313±26	315±34	0.1150
Gunnera perpensa	356±20	1725±87	-	-	310±26	375±13	380±42	345±13	390±18	0.3100
Helichrysum cymosum	425±45	1413±63	-	-	418±42	470±25	468±28	454±32	455±13	0.2660
Hypoestes aristata	388±25	1315±110	-	-	380±21	429±70	440±26	470±17	456±15	0.6020*
Lantana rugosa	423±66	1463±85	-	-	373±46	375±25	363±24	408±66	383±34	-0.0880
Lippia javanica	358±45	1433±76	-	-	365±54	445±31	430±42	428±28	423±44	0.3480
Maesa lanceolate	303±33	1575±194	-	-	371±40	335±29	353±45	363±38	403±70	0.3870
Mariscus congestus	435±36	1667±104	-	-	543±17	422±11	548±20	525±19	526±21	0.2540
Maytenus acuminata	323±26	1213±125	-	-	328±36	365±29	340±49	333±33	318±21	-0.0560
Opuntia vulgaris	355±47	1438±63	-	-	338±22	373±38	370±36	388±37	455±24	0.5590*
Pelargonium sp.	433±30	1538±131	-	-	455±24	448±36	450±64	546±33	575±30	0.6880*
Pteridium aquilinum	405±21	1575±126	-	-	443±32	458±17	430±24	475±21	500±45	0.6195*
Solanum mauritianum	343±24	1275±87	-	-	413±38	373±30	390±37	468±28	493±33	0.6810*
Turraea floribunda	348±43	1075±65	-	-	343±21	353±24	413±54	410±73	485±42	0.5970*
Withania somnifera	400±57	1600±122	-	408±46	393±66	477±15	475±33	485±66	-	0.4450*

[a] Data are means ± S.D. of four plates, [b] Control (-) = number of spontaneous revertants without plant extracts

[c] Control (+) = number of revertants in the presence of cumene hydroperoxide (110 μg), [d] 0.1 ml of extract was added to each plate

[e] = Kruskal's gamma coefficient, * = significant at 1% level

Table 8.10b: Mutagenicity of water extracts from medicinal wild plants towards *S. typhimurium* TA102 in the presence of S9 mixture

Botanical name	His$^+$ revertants per plate[a]								
	+S9								γ^d
	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)							
		0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
Acacia caffra	370±24	-	-	380±35	338±41	305±21	283±15	298±17	-0.6120
Acacia karroo	465±41	-	-	480±55	445±52	448±57	598±26	640±52	0.4980*
Acokanthera oppositifolia	475±24	-	-	410±6	460±6	438±30	418±23	418±32	-0.3036
Aloe Ferox	430±42	-	-	348±30	348±15	355±44	405±29	420±28	0.1890
Artemisia affra	335±19	-	-	375±53	375±39	375±31	420±10	417±21	0.5390*
Brunsvigia sp.	315±26	-	-	345±44	370±37	423±28	398±63	308±22	0.1700
Catha edulis	473±17	-	-	439±8	485±63	483±39	510±42	446±42	0.0820
Convolvulus farinosus	448±36	-	-	403±39	368±17	373±22	455±37	423±39	0.0090
Dalbergia obovata	442±42	-	-	468±25	413±41	450±50	463±29	388±26	-0.2210
Datura stramonium	282±19	-	-	257±14	303±22	306±11	345±10	473±22	0.7970*
Deinbollia oblongifolia	405±31	-	-	483±36	468±53	530±22	528±17	590±22	0.7700*
Eucomis autumnalis	391±60	-	-	335±39	340±42	315±40	353±25	415±37	0.1340
Ficus craterostoma	328±31	-	-	340±48	278±43	328±13	263±10	288±30	-0.3690
Gunnera perpensa	378±31	-	-	318±10	338±29	353±36	368±38	401±28	0.2800
Helichrysum cymosum	473±33	-	-	470±27	493±15	445±33	387±32	437±21	-0.4420*
Hypoestes aristata	400±7	-	-	419±14	445±30	430±12	474±14	550±35	0.8060*
Lantana rugosa	400±27	-	-	380±48	413±43	418±47	403±41	395±66	0.0800
Lippia javanica	425±44	-	-	485±33	508±37	448±63	405±37	432±37	-0.2140
Maesa lanceolate	315±26	-	-	333±30	358±26	343±34	338±38	413±34	0.4710*
Mariscus congestus	437±24	-	-	419±19	412±7	463±26	505±10	535±19	0.6940*
Maytenus acuminata	400±41	-	-	415±47	353±25	345±37	330±61	338±38	-0.4630*
Opuntia vulgaris	375±45	-	-	353±22	355±39	328±21	330±41	398±25	-0.0360
Pelargonium sp.	413±56	-	-	440±22	415±39	430±26	460±20	453±63	0.3050
Pteridium aquilinum	463±68	-	-	450±57	458±46	452±56	493±77	398±42	-0.1342
Solanum mauritianum	390±22	-	-	515±21	440±34	448±36	443±25	508±39	0.2840
Turraea floribunda	430±42	-	-	390±55	418±36	420±34	438±81	438±83	0.2050
Withania somnifera	408±74	-	413±65	398±21	390±35	460±34	535±33	-	0.4110*

[a] Data are means ± S.D. of four plates, [b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1 ml of extract was added to each plate, [d] = Kruskal's gamma coefficient, * = significant at 1% level

It is quite evident that due to the chemical complexity of the plant extracts assayed, a variety of response profiles were obtained in the Ames assay. It is not surprising that such a large number of extracts exhibited mutagenicity in one or more of the strains tested either with or without metabolic activation. Studies carried out on medicinal plants used in Brazil revealed the plants *Myrciaria tenella* (Myrtaceae), *Smilax campestris* (Smilaceae) *Tripodanthus acutifolius* (Loranthaceae) and *Cassia corymbosa* (Leguminosae) also possessed mutagenic activity. The observed mutagenicity was attributed to the presence of selected flavonoids, tannins and anthraquinones in the extracts (Ferreira and Vargas, 1999). The roots of *Rubia tinctorum*, known as madder root, which contain a variety of hydroxyanthraquinones such as alizarin, purpurin, lucidin and rubiadin, was also reported to be mutagenic in the *Salmonella*/micosome assay (Yasui and Takeda, 1983). Hence the varied responses in this study can be attributed to the different point mutations that the chemical compounds induced in the genome of the organism.

With regards to the dietary wild plants a large proportion of the extracts were mutagenic towards strains TA97a (75%) and TA102 (65%) whilst with respect to the medicinal plants, most of the extracts exhibited mutagenicity towards TA100 (44%) and TA102 (48%). When comparing the metabolically activated dietary plant extracts against those assayed without metabolic modification, it is apparent that the number of extracts testing positive were increased. With regards to the medicinal plant extracts, however, the responses varied from decreased mutagenicity to cytotoxicity. In the presence of cytochrome P450, a number of reactions can occur with the chemical components of the extract. These include oxidation, reduction, hydrolysis, dealkylation, deamination, dehalogenation, ring formation, and ring breakage hence rendering the coumpound/s mutagenic or toxic.

The strain TA102 has an A–T base-pair at the critical site for reversion hence it detects a variety of oxidative mutagens, active forms of oxygen, and has an intact excision-repair pathway (Levin *et al.*, 1982). Oxidation of endogenous macromolecules can generate electrophiles capable of forming mutagenic adducts in DNA which have been implicated in cancer. The genotoxicity of oxidizing agents, both exogenous and endogenous, arises from direct damage to DNA (Breen and Murphy, 1995) or from reactions with other biomolecules that lead to the formation of DNA-reactive electrophilic species (Lindahl, 1993). Oxidative damage can also be caused by superoxide radicals, hydrogen peroxide, or hydroxide radicals with the most important type of oxidative damage being the formation of 8-oxo-guanine which will pair with adenine and generate transversions. A large number of the

dietary plant extracts displayed mutagenicity resulting from oxidative DNA damage. Such damage has been known to arise from furanocoumarins and other compounds of similar structural design which are able to intercalate in the DNA molecule forming mono- and di-adducts, and they also induce the production of singlet oxygen superoxide radicals (Pathak and Joshy, 1984; Bianchi *et al.*, 1996). As evidenced from the literature, many plants also contain flavonoids which may be related to the *in vitro* lipid peroxidation and pro-oxidant responses. A common flavonoid which is responsible for such an activity is quercetin as reported by Laughton *et al.* (1989). Pro-oxidant activity in quercetin is derived from its ability to reduce iron and correlates with lipid peroxidation induced by the microsome-ascorbic acid-iron system.

With regards to the medicinal plants, a large number of the extracts displayed cytotoxicity towards strain TA97a in the presence of S-9 mix. Such a phenomenon has been seen with plants containing sequiterpene lactones (SQL) which have been implicated for cyotoxic effects (Ramos, *et al.*, 2001). Cytotoxicity is a common feature shared by most SQLs, partly dependent on the presence of an α-methylene γ-lactone moiety (Kupchan *et al.*, 1971). Michael addition of thiol groups from cysteine and GSH to the exocyclic methylene, probably leading to both enzyme inactivation and/or oxidative stress has been proposed as a mechanism for cell cytotoxicity in these compounds (Robles *et al.*, 1995). SQLs are able to form double adducts through the α-methylene group of the γ-lactone and the endocyclic double bond on the cyclopentenone ring. (Picman *et al.*, 1979). In this study, the cytotoxicity of the extracts was enhanced by S-9.

This study revealed that while the use of dietary and medicinal wild plants play a critical role in the cultural habits of the residents of the Eastern Cape Province of South Africa, use of these plants is accompanied by a high risk of mutagenesis. Such mutations are known to initiate tumour formation and when coupled by exposure to tumour promoters, will increase the likelihood of these residents developing tumours at a high rate. The plants used in traditional medicine are assumed to be safe due to long-term use by traditional healers. This has been assumed due to a lack of safety data. The detection of mutagenic effects reveals that these plants should be administered with caution and after rigorous toxicological investigations so as to reduce the risk of exposure to these mutagenic chemicals. Plants identified with mutagenic activity can alter the structure of DNA of somatic cells and can induce cancer. Additional studies are however needed to identify the phytochemical substances that might contribute to the effects observed.

The observations resulting from the mutagenicity assay in combination with the epidemiological evidence discussed in Chapter 7 made possible the identification of plants that pose a high risk in the development of OC. In addition to this, the trace elemental composition of these plants were also evaluated and the data presented in the next Chapter.

CHAPTER 9: TRACE METAL ANALYSES OF DIETARY AND MEDICINAL WILD PLANTS INVESTIGATED IN THIS STUDY

9.1 Introduction

Apart from mutagenicity, the determination of the metal content in medicinal, seasoning, and aromatic plants has been one of great interest and has received increased attention during recent years because of their association with human health (Majid *et al.*, 1995). Several investigators have performed numerous studies on the residual levels of toxic metals in medicinal herbs (Schilcher, 1982; Ali, 1983, 1987; Peters and Schilcher, 1986; Schilcher *et al.*, 1987). Medicinal plants occurring wild have been found to show more anomalous values than cultivated herbs and in some cases may exceed the permissible levels (Peters and Chilcher, 1986; Schilcher *et al.*, 1987). The reason being that plants grown wild are more difficult to control for all the potential ways of contamination since heavy metal contents in plants depend on climatic factors, plant species, air pollution, and other environmental factors (Sovljanski *et al.*, 1989).

In Bahrain, Al-Saleh and Chudasama (1994) reported that a large portion of the plants examined contained high concentrations of toxic metals and some of them exceeded the limits of toxicity indicating a potential health hazard. Abou-Arab and Abou-Donia (2000) determined the heavy metal content of several Egyptian spices and medicinal plant samples intended for export. The maximum levels of trace elements in the analysed samples were 14.2, 2.4, 33.8, 2.9, 0.1, 68.8, 343.0, 11.4 and 1046.3 μg/g for Pb, Cd, Cr, Ni, Sn, Zn, Mn, Cu, and Fe, respectively. Karavoltos *et al.* (2002) determined the cadmium content of a wide variety of foodstuffs from a Greek market and found that leafy vegetables contained levels of Cd as high as 28.3 ng/g.

A variety of metals have been identified as human carcinogens by epidemiological studies and as animal carcinogens by laboratory testing (Furst, 1978). Human carcinogens identified in this manner are compounds of arsenic, chromium, nickel, cadmium and beryllium (Doll, 1981). Animal carcinogens include the human carcinogens, with the exception of arsenic (Pershagen, 1981), as well as compounds of cobalt, lead, manganese, and iron dextran (Sunderman, 1981; Kushner, 1981; Piscator, 1981; Norseth, 1981; Kazantzis, 1981).

Suspicions on the association between trace metals and oesophageal cancer have been aroused from reports from South Africa and China where deficiencies in Mo and Se in soils in high-risk areas of the former Transkei region of South Africa (Burrell et al., 1966; Warwick and Harington, 1973; Kibblewhite *et al.*, 1984) and China (Yang, 1980) were reported to affect directly or indirectly the incidence of oesophageal cancer (OC). Whole blood selenium levels were significantly lower in the high OC risk areas than those of the control groups from low-risk areas of Transkei (Jaskiewicz *et al.*, 1988b). Deficiencies of Mn and Zn in soils have also been implicated as risk factors for OC (Yang, 1980; Kibblewhite *et al.*, 1984), along with other elements such as B, Cu, Ni, Mg, Fe, K, Na, and P. Pb and V levels however in soils from high-risk areas were not significantly different from low-risk areas, but V is antagonistic to Mo and this could exacerbate any deficiency of Mo (Kibblewhite *et al.*, 1984). Van Rensburg (1985) showed that deficiencies of Zn, Mo, Se and Mg markedly enhanced susceptibility to carcinogens and the ability of these elements to inhibit chemically induced oesophageal carcinogeneis has been demonstrated in animal models (Fong, *et al.*, 1984; Van Rensburg *et al.*, 1986). The above investigations have focused on the analysis of soil samples in relation to oesophageal cancer but none focused on the levels of these trace elements in plants.

9.2 Aim

The aim in this study was to determine the trace element concentrations in plants consumed for dietary and/or medicinal purposes by residents of the Eastern Cape Province.

9.3 Objectives

The objectives of this study were to:

5. To collect plant samples from the high OC regions,
6. To prepare the samples for analysis of selected trace elements by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).
7. To determine the levels of the following trace elements in plants; Mo, Se, Mn, Zn and Mg (due to their association with OC from previous studies) and Co, Cr, Cd, As and V (due to their known toxicities and carcinogenic potential as discussed in this chapter).

9.4 Experimental Methods

9.4.1 Sample Digestion

Approximately 25 milligrams of dry sample material was weighed accurately into Teflon beakers (7 ml). Ultra-clean concentrated nitric acid (2 ml) and hydrogen peroxide (0.8 ml) were added to each beaker and the samples were left to digest unheated and uncovered in a fume-cupboard for 48 hours. The samples were subsequently evaporated to complete dryness on a hotplate. The dried residue was re-dissolved in ultra-clean concentrated nitric acid (4 ml) and heated for 12 hours with the beaker lids firmly closed. Following this step, the samples were again evaporated to complete dryness and finally dissolved in ultra-clean 5% nitric acid. The final solutions analysed represented 1000 and 5000 times dilutions of the original solid samples (all dilutions were accurately determined using a high precision balance).

9.4.2 ICP-MS Analysis

All samples were analysed using a Perkin Elmer/Sciex Elan 6000 inductively coupled plasma mass spectrometer. Typical instrument operating conditions during the analyses were as follows:

nebuliser gas flow:	0.82 $L.min^{-1}$
auxiliary gas flow:	0.75 $L.min^{-1}$
main gas flow:	15.0 $L.min^{-1}$
ICP RF power:	1150 W
dwell time:	35-50 milliseconds per mass peak
number of sweeps:	20
number of replicates:	3
total analytical time:	67.92 s
sample flush time:	40 s
read-delay time:	20 s

wash-time between samples: 180 s (in 5% ultra-clean nitric acid).

The instrument was optimized daily to minimize the formation of oxides (CeO/Ce < 0.030) and doubly-charged ions (Ba^{2+}/Ba^{+} < 0.001). The average instrument sensitivity during the days of analysis was 23 687 $cps.ppb^{-1}$ for ^{103}Rh.

Instrument calibration was achieved by external standardisation against a set of four standards made up accurately from commercial synthetic multi- and single-element standard solutions. Instrument drift was corrected by internal standardization against ^{103}Rh, ^{115}In and ^{209}Bi.

9.4.3 Reproducibility of Analytical Procedure

In order to determine the reproducibility of the analytical procedure, selected raw materials and one lyophilized material was prepared in duplicate for analysis. The reproducibility was ascertained from the results obtained following analysis of each of these samples.

9.4.4 Statistical Analysis

Basic statistics comprising the mean and the relative standard deviation for the samples were computed using Microsoft Excel software (Microsoft Corporation). The mean and standard deviation of the reproducibility experiments were calculated from duplicate sample preparation and analyses.

9.5 Results

For the analysis of the heavy metals, a total procedural blank sample was prepared and analysed together (and under identical conditions) with each batch of samples. Analytical precision (based on with-in run statistics and repeat analyses) was typically less than 5% for most of the metals (Table 9.1).

The plants evaluated in this study have been divided into two categories, *viz.* those consumed as part of the traditional diet (Table 9.2) and those used predominantly as traditional medicines (Table 9.3) and the results of their heavy metal concentrations are reflected accordingly. For the dietary plants, the mean concentration of heavy metals indicate the following order: Mg>Mn>Zn>Cr>Mo>V>Co>Se>Cd>As, whilst the mean levels in plants used as traditional medicines are of the following order: Mg>Mn>Zn>Cr>V>Co>Mo>As>Se>Cd.

Table 9.1. Analysis of trace elements in selected wild plants showing the repeatability of the analytical method.

Trace Elements	*Acacia caffra* (Raw material) (mg/kg)			*Lantana rugosa* (Raw material) (mg/kg)			*Zantedeschia aethiopica* (Raw material) (mg/kg)			*Maytenus acuminate* (Lyophilised extract) (mg/kg)		
	Analysis 1	Analysis 2	Mean	Analysis 1	Analysis 2	Mean	Analysis 1	Analysis 2	Mean	Analysis 1	Analysis 2	Mean
Cr	2.10	2.25	2.18 (0.05)*	3.44	3.40	3.42 (0.01)	1.57	1.66	1.62 (0.04)	1.06	1.04	1.05 (0.01)
Mn	84.8	84.4	84.60 (0)	310	345	327.34 (0.07)	517	534	525.70 (0.02)	821	804	812.22 (0.02)
Mg	3118	3141	3130 (0.01)	6350	6934	6642 (0.06)	2505	2446	2476 (0.02)	9285	9003	9144 (0.02)
Co	0.69	0.73	0.71 (0.04)	0.43	0.47	0.45 (0.07)	0.29	0.27	0.28 (0.04)	0.22	0.23	0.23 (0.03)
Se	0.07	0.052	0.061 (0.21)	0.13	0.18	0.16 (0.23)	0.17	0.21	0.19 (0.15)	0.17	0.18	0.18 (0.04)
Zn	12.9	13.5	13.20 (0.03)	33.9	35.7	34.82 (0.04)	104	104	104.02 (0.01)	33.5	33.8	33.63 (0.006)
As	0.055	0.054	0.05 (0.01)	0.087	0.079	0.08 (0.07)	0.088	0.086	0.09 (0.02)	0.32	0.31	0.31 (0.03)
Cd	*n.d.*	*n.d.*	-	0.050	0.058	0.05 (0.11)	0.050	0.052	0.05 (0.02)	0.12	0.12	0.12 (0)
Mo	0.14	0.16	0.15 (0.09)	n.d.	n.d.	-	2.42	2.43	2.43 (0)	0.12	0.13	0.13 (0.06)
V	0.36	0.35	0.36 (0.02)	0.47	0.47	0.47 (0)	0.30	0.29	0.30 (0.02)	0.16	0.15	0.16 (0.05)

*numbers in parentheses indicate the relative standard deviation

Table 9.2. Distribution levels of selected trace elements in dietary wild plants used in the Eastern Cape Province of South Africa.

Botanical name	Sampling Location	Trace element concentration (mg/kg)									
		Cr	Mn	Mg	Co	Se	Zn	As	Cd	Mo	V
Amaranthus asper	1	1.23	232	8163	0.33	0.12	1060	0.065	0.61	0.76	0.23
Amaranthus hybridus	1	1.68	43.5	11233	0.3	n.d.	39.8	0.12	0.066	1.43	0.43
Amaranthus thunbergii	1	1.47	51.6	16858	0.58	0.075	75.7	0.06	0.14	3.06	0.47
Bidens pilosa	1	2.13	85.2	4966	0.24	0.028	49.4	0.079	0.093	0.44	0.65
Centella asiatica	1	2.34	1607	5572	2.86	0.17	106	0.24	0.34	0.26	1.34
Chenopodium album	1	1.16	107	9252	0.31	n.d.	97.3	0.072	0.06	3.48	0.31
Coccinia rehmanii	1	1.09	96.9	7194	0.21	0.084	34.9	0.09	0.16	0.17	0.41
Colocasia esculenta	1	0.99	299	3466	0.16	n.d.	17.1	0.056	0.051	0.19	0.067
Oxalis corniculata	1	1.18	202	2749	0.062	0.14	21.9	0.044	0.12	0.068	0.1
Physalis viscosa	1	2.45	44.4	5649	0.45	0.5	56.2	0.19	0.025	1.54	1.05
Raphanus nasturtio aquatica	1	2.45	119	4413	1.1	0.27	47	0.17	0.16	0.58	1.28
Raphanus raphinastrum	1	2.74	47.8	5927	0.47	1.07	35.7	0.14	0.09	2.33	1.32
Rumex lanceolatus	1	1.68	78.8	6599	0.36	0.38	18.5	0.12	0.043	0.26	0.38
Sida dregei	1	1.32	158	5405	0.19	0.3	33.2	0.037	0.12	0.82	0.15
Sida rhombifolia	2	3.18	71.7	6765	0.56	0.1	43.6	0.14	0.1	0.94	0.9
Solanum nigrum	1	1.64	68.8	2921	0.49	0.046	39.3	0.088	0.27	1.54	0.24
Solanum nodiflorum	1	1.99	67.3	5115	0.47	0.062	26.4	0.21	0.22	2.86	0.8
Sonchus oleraceus	1	3	77.8	3773	0.37	0.14	51.8	0.34	0.071	0.74	0.91
Urtica urens	1	2.59	149	9498	0.6	0.12	26.3	0.34	0.015	0.31	1.5
Zantedeschia aethiopica	1	1.57	517	2478	0.29	0.19	104	0.088	0.05	2.43	0.3
Average		**1.93**	**204.83**	**6307.00**	**0.53**	**0.23**	**48.64**	**0.14**	**0.12**	**1.23**	**0.66**

1 = Port St. Johns, 2 = Flagstaff (Hala Location)

Table 9.3. Distribution levels of heavy metals in medicinal plants used in the Eastern Cape Province of South Africa.

Botanical name	Sampling location	Trace metal concentration (mg/kg)									
		Cr	Mn	Mg	Co	Se	Zn	As	Cd	Mo	V
Acacia caffra	1	2.18	84.6	3130	0.71	0.061	13.2	0.05	n.d.	0.15	0.36
Acacia karroo	2	1.75	33.1	2497	0.15	0.064	17.1	0.1	0.013	0.022	0.37
Acokanthera oppositifolia	2	1.4	192	1454	0.13	0.047	51.5	0.13	0.067	0.15	0.17
Aloe Ferox Mill.	1	1.57	37.9	15751	0.15	0.025	12.9	0.029	n.d.	0.79	0.18
Artemisia affra	3	1.87	28.9	2325	0.23	0.12	25.1	0.078	0.057	0.11	0.5
Brunsvigia sp.	1	1.82	162	2411	0.64	0.074	13.5	0.13	0.3	0.054	0.69
Catha edulis	4	1.65	18	2373	0.073	0.11	52.2	1.4	0.17	0.069	0.19
Convolvulus farinosus	1	2.07	101	2289	0.37	0.13	26.8	0.21	0.013	0.06	0.97
Dalbergia obovata	1	1.21	89.9	2369	0.2	0.032	35.6	0.12	0.033	1.05	0.21
Datura stramonium	1	1.7	41.1	2830	0.3	0.045	35.9	0.13	0.12	1.39	0.58
Deinbollia oblongifolia	2	1.5	29.7	3758	0.072	0.019	31.1	2.74	0.086	0.17	0.12
Eucomis autumnalis	2	1.07	6.06	2011	0.013	0.14	18.7	0.17	0.011	0.091	n.d.
Ficus craterostoma	1	1.72	1.4	817	n.d.	0.027	4.92	0.024	0.015	n.d.	0.012
Gunnera perpensa	2	1.38	489	4635	0.61	0.41	6.89	0.086	0.04	0.04	0.24
Helichrysum cymosum	1	2.98	162	1989	0.57	0.046	29	0.14	0.22	0.16	1.56
Hypoestes aristata	2	1.9	111	3108	0.32	0.04	121	0.38	0.07	1.37	0.42
Lantana rugosa	1	3.4	345	6642	0.47	0.16	35.7	0.079	0.058	n.d.	0.47
Lippia javanica	1	1.63	190	3408	0.97	0.11	29.2	0.1	0.01	n.d.	0.73
Maesa lanceolate	1	1.45	63.9	2044	0.19	0.15	11.6	0.051	n.d.	0.26	0.22
Mariscus congestus	1	1.06	832	1015	0.29	0.1	5.22	0.059	0.06	0.14	0.079
Maytenus acuminate	1	1.48	614	3686	0.23	0.26	33.7	0.1	0.18	0.015	0.27
Opuntia vulgaris	1	1.08	377	11260	1.71	0.6	316	0.065	0.25	0.59	0.21
Pelargonium sp.	2	3.09	100	2950	0.26	0.087	42.6	1.14	0.021	0.27	0.85
Pteridium aquilinum	1	1.08	435	1995	1.03	n.d.	31.2	0.07	0.055	0.18	0.42
Solanum mauritianum	1	2.68	121	3669	1.19	0.46	34.3	0.073	0.044	n.d.	0.55
Turraea floribunda	2	1.64	15.5	3464	0.11	0.1	57.9	0.08	0.1	0.25	0.24
Withania somnifera	1	2.68	36.5	4850	0.21	0.15	30.1	0.23	0.035	0.42	1.15
Average		**1.80**	**178.19**	**3676.92**	**0.42**	**0.14**	**42.68**	**0.30**	**0.08**	**0.35**	**0.46**

1 = Port St. Johns, 2 = Silverglen Med. Plant Nursery, 3 = Flagstaff (Hala Location), 4 = Tsolo (Unga Location)

9.5 Discussion

It is not surprising that Mg, Mn and Zn are present at relatively higher levels (6307, 205, 49 mg/kg, and 3677, 178 and 43 mg/kg for dietary and medicinal plants, respectively). These elements are the natural essential components of coenzymes and they are important for growth, photosynthesis, and respiration. Despite previous reports of Mg, Mn and Zn deficiencies being implicated as a risk factor for OC in South Africa (Kibblewhite *et al.*, 1984), the results indicate that the plants used by residents of the Eastern Cape Province do contain these essential elements hence deficiency of these elements in the population would depend largely on the quantity and frequency of intake. Rheeder *et al.* (1994) also published evidence showing that essential elements were present in the soil further lending support to our findings. Zinc is arguably one of the most important trace metals in the body (with iron) and functions in multiple body processes. There is adequate evidence that zinc is required for important antioxidants (metallothionein (MT); Zn, Cu-superoxide dismutase) that protect against free radical damage of both chemicals and metals. Lack of Zn can increase oxidant damage to various cell components (Hennig *et al.*, 1999; DiSilvestro and Carlson, 1994) including DNA (Fong *et al.*, 1996). Some DNA-repair enzymes also require zinc to function (Fong *et al.*, 1996). In this study, the dietary plant, *Amaranthus asper*, was found to contain the highest level of Zn (1060 mg/kg). The recommended dietary allowance (RDA) of Zn is 11 mg/day for healthy males and 8 mg/day for healthy females while the upper limit (UL) is set between 34 – 40 mg/day (Trumbo *et al.*, 2001) indicating that this plant may provide a good source of Zn although the UL could easily be reached if this herb is eaten consistently. However, *Colocasia esculenta*, which only contains 17.1 mg/kg Zn would have to be consumed more frequently and at much larger quantities for the RDA to be reached.

The plant *Centella asiatica* L. contained the highest level of Mn amongst all the dietary wild plants (607 mg/kg). The adequate intake (AI) level of Mn is 2.2 -2.3 mg/day for healthy males and 1.6 – 1.8 mg/day for healthy females (Trumbo *et al.*, 2001) with an UL of 6 – 11 mg/day. One is likely to achieve the maximum level of the UL by consuming approximately 7 grams of this plant on a daily basis. This plant is eaten as a herb by 26% males and 40% females interviewed and was associated with a 14% increased risk of developing OC in males only (see Chapter 7). It must be noted however that manganese (II) is considered to be a weak animal carcinogen and is mutagenic to bacteria, phage, and yeast (Flessel, 1979). Manganese (II) also causes mutations in mammalian cells (Oberly et al., 1982). Since Mn (II) can substitute for Mg (II) as an activator in DNA polymerases, with concomitant increased infidelity of DNA replication (Zakour *et al.*, 1981), it is a reasonable assumption

that much of the mutagenic activity might be explained on the basis of errors in replication due to excess exposure to Mn.

The mean levels of selenium in the dietary and medicinal plants were 0.23 and 0.14 mg/kg respectively. With a RDA for Se of 55 μg/day (Trumbo *et al.*, 2001), an average of 250 grams of dietary wild plants is needed per individual on a daily basis for them to receive the RDA although other dietary sources of selenium may be available. The UL of 400 μg/day is unlikely as one would have to consume approximately 1800 grams of wild herbs to achieve this level. A deficiency in selenium has been postulated to play a role in the development of OC through increased susceptibility upon exposure to carcinogens. A population-based study in the former Transkei region has shown whole blood Se levels to be significantly lower (58 to 71 ng/ml) amongst the population in high OC incidence areas than in low incidence areas (114 to 177 ng/ml) (Jaskiewicz *et al.*, 1988b). Selenium protects membranes from peroxidation through the action of a Se-dependent enzyme glutathione peroxidase and enhances immune response with potentially increased resistance to cancer initiation and growth (Watson and Leonard, 1986). Four plants had no detectable levels of Se, i.e. *Amaranthus hybridus, Chenopodium album, Colocasia esculenta* and *Pteridium aquilinum.*

Molybdenum was present in the dietary plants at a mean level of 1.23 mg/kg while a lower mean level (0.35 mg/kg) was present in medicinal plants. The RDA for males and females is 43 – 45 μg/day (Trumbo *et al.*, 2001) indicating that at least 37 grams of herbs must be eaten on a daily basis. Deficiencies of this element have been reported to increase susceptibility to carcinogens (Van Rensburg, 1985). These low levels are unlikely to provide much usefulness especially since vanadium has also been detected in both the dietary and medicinal plants at mean levels of 0.66 and 0.46 mg/kg respectively. It has been reported that V is antagonistic to Mo and hence the uptake of Mo can be reduced.

The AI of chromium for males and females is 30 – 35 μg/day and 20 – 25 μg/day respectively (Trumbo *et al.*, 2001). The data indicate that the mean levels of Cr in the dietary and medicinal plants were observed to be 1.93 and 1.80 mg/kg respectively. Hence at least between 13 -18 grams of plant needs to be eaten per day. The levels of Cr in the dietary plants range from 0.99 – 3.18 mg/kg, hence depending on the plants consumed, these levels are likely to be exceeded, although the UL in humans has not been determined. Concerns for chromium exposure stems from its exhibition of mutagenic properties in the *Salmonella typhimurium* bacterial assay (Tindall *et al.*, 1979) and has been detected as a genotoxic agent in a number of other *in vitro* biosassays (Sirover, 1981). While the toxicity of Cr is

dependent on its oxidation state, it is unknown as to what oxidation states are present in these plants. Chrome-6 resembles organic carcinogens in its ability to cause genetic effects (Flessel, 1979) and has been shown to enter animal cells, where it is reduced to chromium III, which remains inside the cells since it is not able to cross cellular membranes (Jennette, 1979). Chromium III is then able to form DNA-protein cross-links, single strand breaks in DNA, and interstrand cross-links (Tsapakis *et al.*, 1981; Fornace *et al.*, 1981). DNA synthesis occurs with loss of fidelity in the presence of chromium III (Sirover and Loeb, 1981). Concerns from Cr toxicity is unlikely to stem from the medicinal plants as they contain similar quantities as the dietary plants and are used in far lesser quantities. Industrial sources of chrome-6 are negligle as the Transkei region is rural with minimal industries in the high OC regions.

The levels of cobalt are also somewhat similar in the dietary (mean = 0.53 mg/kg) and medicinal (0.42 mg/kg) plants. Cobalt occurs in vegetables via uptake from soil, and vegetables account for the major part of human dietary intake of cobalt while animal-derived foods, particularly liver, contain cobalt in the form of vitamin B12. Concerns stem from the fact that cobalt and cobalt compounds are possibly carcinogenic to humans (Group 2B) (IARC, 1980). Moderate levels of Co were found in the dietary and medicinal wild plants and did not appear to result in increased risk of developing OC.

Cadmium does not have any biochemical or physiological importance and is considered as a very toxic pollutant (Sovljanski *et al.*, 1989) and has the ability to displace and replace molecules such as Zn and Ca that have pivotal controlling functions in the body (Thiesen and Bach, 1991). This metal has been frequently under heavy scrutiny since the 1970s and is known for its high risk of toxicity (Page *et al.*, 1987), stability and bioaccumulation (Spiro and Stigliani, 1996, Cabrera *et al.*, 1998). Uptake through food and digestion is regarded as the most significant source of cadmium for the general population with elevated exposure via contaminated water and crops grown on polluted soil and through cigarette smoking (Hutton *et al.*, 1987; WHO, 1989a). The mean levels found in the plants were 0.12 mg/kg (dietary) and 0.08 mg/kg (medicinal). It is well known that Cd is widely distributed into the environment at relatively low concentrations while higher concentrations are found in hot spots related to human activities and in agricultural lands where high concentrations of phosphate fertilizers and manure are applied (Scoullos *et al.*, 2001). Atmospheric deposition from urban and agricultural areas may also play an important role in the enrichment of agricultural produce in cadmium. Since many of these plants have been collected in the wild, it is expected that the levels of Cd would be low and while it does not have any known useful

function in humans, its biological half-life is estimated as from 5 to 10 years in the liver and from 10 to 30 years in the kidney. The (IARC) classified cadmium and cadmium compounds as Class 1 human carcinogens (IARC, 1993b). Hence its bioaccumulation through continued low level exposure could reach levels of toxicity. Since the tolerable intake of Cd is 7 μg/kg of body weight (bw)/week (using 70 kg of bw) as established by the International Program on Chemical Safety of the World Health Organization (IPCS/WHO) (WHO, 2001), it is evident that whilst a plant such as *Bidens pilosa* which contains 0.093 mg/kg of Cd would not pose a risk even if eaten on a daily basis (600 g) by a 70 kg adult. However daily intake of at least 120 g of *Amaranthus asper* by a 70 kg adult would result in a weekly intake of 7.32 μg Cd.

Chronic arsenic exposure can lead to leukaemia, skin cancer, cancer of the lung, kidney, liver and bladder (Engel *et al.*, 1994; Graeme and Pollack, 1998; Basu *et al.*, 2001). Hence exposure to arsenic is considered a major public health concern, particularly because of its clear carcinogenic potential (IARC, 1980). The mean concentrations of arsenic in dietary and medicinal plants were found to be 0.14 and 0.30 mg/kg respectively. The previously established tolerable level of exposure of 15 mg/kg bw/week is now deemed to be unsafe (WHO, 1989b). Hence assuming that a 70 kg adult were to consume at least 400 grams of herbs on a daily basis, which is unlikely, the As exposure would be 0.012 mg/kg bw/week assuming a daily exposure of 0.30 mg/kg of plant material. Hence it is unlikely that As exposure at such low levels poses a risk for the development of OC.

Much attention has been drawn to the metal content of herbal remedies (Zimmers, 1985; Friedman and Levin, 1989). Metal contamination in the form of arsenic, cadmium, lead and mercury has been demonstrated in traditional Chinese (Abt *et al.*, 1995) and South American remedies (Hindmarsh and Gatt, 1980). The effect of processing of plant materials on the levels of the metal contents in the final product has been an area of concern, whether plants are applied topically or used in an oral dosage form. In this study, the effect of extraction on the heavy metal content was evaluated on the most frequently used plant, *Artemisia affra* (used by 57% of the patients interviewed) and the data presented in Table 9.4. The metals were transferred from the plant tissue into the water at different rates. Arsenic and cadmium were completely transferred into the extract while all other metals were between 59 – 78 % extracted. A report by Abou-Arab and Abou-Donia (2000) indicated that higher concentrations of heavy metals were transferred into hot water by boiling than simply by immersion in hot water (tea method).

Table 9.4. The effect of processing of *Artemisia affra* on the heavy metal content

Trace metals	Raw material (mg/kg)	Lyophilised material (mg/kg)	% extracted
Cr	1.87	1.11	59
Mn	28.90	18.10	63
Mg	2325.00	1773.00	76
Co	0.23	0.18	78
Se	0.12	n.d.	-
Zn	25.10	15.30	61
As	0.08	0.061	76
Cd	0.06	0.045	75
Mo	0.11	n.d.	-
V	0.50	0.34	68

This is the first report on heavy metal contents in dietary and medicinal plants used in the Eastern Cape Province and in particular, the former Transkei region. The levels of heavy metals vary and their exposure levels depend entirely on the frequency of intake of the dietary plants. With regards to the plants used as traditional medicines, the levels are quite low and it is not anticipated that a risk would be imposed. While it is well known that the soil is the primary source of heavy metals, additional contamination could arise from traffic (plants growing alongside the roads), animal droppings, and the dumping of corroding automobile structure frames and tires. This investigation reveals that exposure to heavy metals which are known carcinogens can occur through wild plant consumption, while beneficial elements such as Se, are severely lacking.

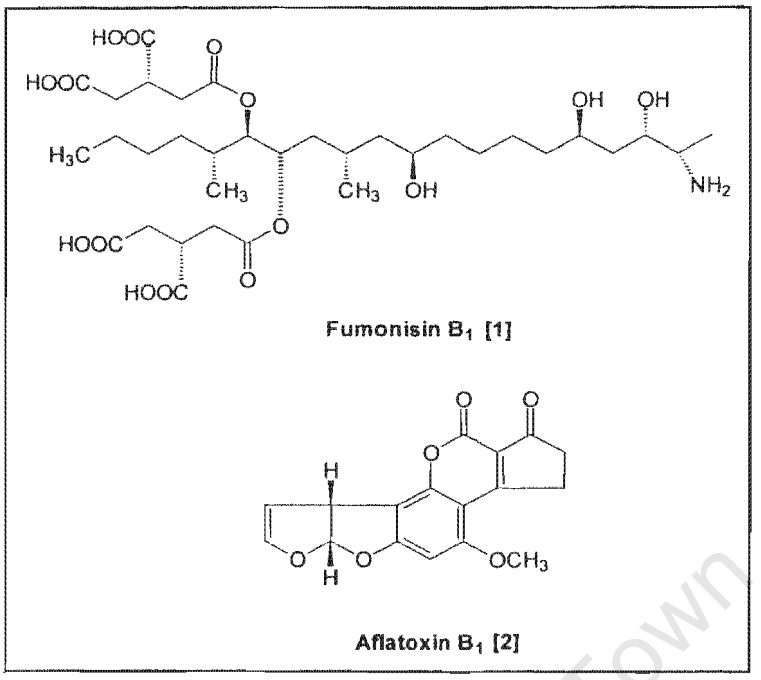
CHAPTER 10: MYCOTOXIN CONTAMINATION OF DIETARY AND MEDICINAL WILD PLANTS IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA

10.1 Introduction

Mycotoxins are secondary metabolites produced by a wide range of fungi known to contaminate a variety of food and agricultural commodities worldwide (Shephard *et al.* 1996) and thus have great significance in human health. There are five mycotoxins, or groups of mycotoxins, that frequently occur in food: deoxynivalenol/nivalenol; zearalenone; ochratoxin; fumonisins; and aflatoxins. The food-borne mycotoxins of most interest in South Africa are the fumonisins and aflatoxins and reports from literature indicate that these toxins can also be found in a variety of medicinal plants.

The fumonisins (diesters of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-polyhydroxy-eicosanes) are toxic and carcinogenic secondary metabolites, produced primarily by the fungus *Fusarium verticillioides* (Sacc.) Nirenberg (formerly known as *F. moniliforme* Sheldon) (Gelderblom *et al.* 1988). The fumonisins have been shown to affect several target tissues in animals resulting in disease syndromes such as equine leukoencephalomalacia and porcine pulmonary oedema as well as causing nephrotoxicity, hepatotoxicity and hepatocellular carcinoma in rats (Marasas 1995). The International Agency for Research on Cancer (IARC) has classified toxins produced by *F. moniliforme* as possible human carcinogens (Group 2B) (Vainio *et al.* 1993). Currently, twenty-eight structural fumonisin analogs are known (Rheeder *et al.*, 2002), but most research has focused on the most commonly and widely occurring natural form, fumonisin B_1 (FB_1) **[1]**. The IARC recently classified FB_1 itself as a group 2B carcinogen (IARC 2002b).

The consumption of fumonisin-contaminated maize has been statistically associated with the development of endemic levels of human OC in the Transkei region of the Eastern Cape Province of South Africa (Rheeder *et al.* 1992) and Hebei and Henan Provinces in China (Chu and Li 1994). These ecological associations have also been shown for primary liver cancer in Henan Province (Ueno *et al.* 1997). Although it has been well established that FB_1 is a natural contaminant primarily in maize, research undertaken in Portugal has indicated the natural occurrence of fumonisin B_1 in medicinal plants as well (Martins *et al.* 2001). In a study of 69 samples of four medicinal plants, *viz. Citrus sinensis*, *Tillia grandifollia*, *Stigmata maydis* and *Matricaria chamomilla*, 59% of these samples contained FB_1 ranging from 20 – 700 μg/kg.



Fumonisin B_1 [1]

Aflatoxin B_1 [2]

The aflatoxins are secondary metabolites produced by some strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*, in areas of the world with hot, humid climates and are known to contaminate rice, cottonseed, peanuts, peanut products, maize and maize products (Pohland and Ewood, 1987). This group of highly toxic metabolites have closely similar structures and form a unique group of highly oxygenated, naturally occuring heterocyclic compounds with specific forms designated as B_1 (6-Methoxydifurocoumarone) **[2]**, B_2, G_1, G_2, M_1 and M_2.

Because aflatoxins, especially aflatoxin B_1 (AFB_1), are potent carcinogens, there is interest in the effects of long-term human exposure to low levels of these important mycotoxins. In 1987, the IARC placed AFB_1 on the list of human carcinogens as a result of numerous epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and Liver Cell Cancer (LCC) (IARC, 1987). Additionally, the expression of aflatoxin-related diseases in humans may be influenced by factors such as age, sex, nutritional status, and/or concurrent exposure to other causative agents such as viral hepatitis (HBV) or parasite infestation.

Although the highest concentrations of aflatoxin are produced as a result of post-harvest spoilage of commodities stored under warm moist conditions, significant concentrations may also be produced in the field before harvest. This arises from endophytic associations

between these moulds and plants (Hill *et al.*, 1985). A recent survey demonstrated that relatively low levels of aflatoxin can occur in herbs and spices and these are not reduced by domestic cooking (Macdonald and Castle, 1996). An investigation into the aflatoxin contamination of plants used as medicines in Bihar, India showed that 14/15 of the samples analysed were positive for AFB_1 (Roy *et al.* 1988). The highest level of AFB_1 contamination was detected in the seeds of *Piper nigrum* (1.20 μg/g), followed by the level detected in the seeds of *Mucuna prurita* (1.16 μg/g), and the lowest level was detected in the bark of *Acacia catechu* (0.09 μg/g). Of the 158 isolates of *A. flavus* obtained from these plants, 49 were found to be toxigenic. In another study conducted in Andhra Pradesh, India, chillies (*Capsicum annum*) were shown to contain AFB_1 (Reddy *et al.* 2001). Of the 182 chilli samples tested, 59% of the samples were contaminated with AFB_1 and 18% contained the toxin at non-permissable levels (>30 μg/kg). *Aspergillus* spp. were also detected in six Asian medicinal plants (Abeywickrama and Bean, 1991) however only one, i.e. *Aerva lanata*, contained AFB_1 at a level of 0.5 μg/kg. Mycotoxin-producing fungi, *viz. A. flavus*, *Alternaria alternata*, *Penicillium chrysogenum* and *Fusarium verticilliodes* have also been found in the leaves of *Catha edulis* in Yemen (Mahmoud, 2000). AFB_1 contamination was also found in 6 crude herbal drug preparations in Nigeria (Efuntoye, 1999) where levels as high as 0.8 μg/g were detected.

These studies provide evidence that medicinal plants can be naturally contaminated with mycotoxins and it is ironic if the treatment of one disease is the unintended cause of another. Therefore in the light of current evidence, South African medicinal and dietary wild plants were investigated for mycotoxin contamination. Due to their carcinogenic potential, plants were evaluated for the presence of FB_1 and AFB_1.

10.2. Experimental Methods

10.2.1. Chemicals and Solvents

A pure standard of FB_1 was isolated at PROMEC Unit according to the method of Cawood *et al.* (1991). A stock solution of FB_1 was prepared at a concentration of 250 □g/ml in acetonitrile-H_2O mixture (1:1). This solution was used to prepare a working solution at a concentration of 50 μg/ml. AFB_1 was purchased from Sigma (Missouri, USA) and prepared at a stock solution concentration of 125 μg/ml. From this, a working solution of 1.25 μg/ml was used in the analytical determination. Formic acid (analytical grade), KH_2PO_4, NaCl and methanol were obtained from Merck (Darmstadt, Germany). Acetonitrile (HPLC grade) was obtained from Romil (Cambridge, England) while water for general laboratory use and HPLC

mobile phase was deionized on a Milli-Q water purification system (Millipore, Bedford, MA, USA).

10.2.2. Collection of Plants

Nineteen dietary and 30 medicinal plants plants identified by 2000 patients attending three referral hospitals in the Eastern Cape Province of South Africa were collected from Flagstaff (Hala Location), Tsolo (Unga Location) and along the banks of the Umzimvubu River in Port St. Johns (Eastern Cape), and the Silverglen Medicinal plant nursery (KwaZulu-Natal) during November 2002. Elderly men and women familiar with local botany and potential collection locations assisted during this component of the study. The identities of the plants were authenticated by comparison with reference specimens at the Kei Herbarium (University of Transkei) and Natal Herbarium (National Botanical Institute, KwaZulu-Natal). Plant voucher specimens were also deposited in these herbariums for future reference.

10.2.3. Preparation of Plant Extracts

The plants were washed in fresh running water to eliminate dust, dirt and possible parasites and then washed again with deionised water. The different parts of the plants used by the patients (bulb, stem, leaves), were air dried and then ground into a fine powder. Ground sample of each plant (2 g) and sodium chloride (0.4 g) was homogenized in a mixture of methanol-water (80:20, 30 ml) for 5 minutes. The extract was then filtered (Whatman No. 4 filter paper) and the filtrate collected in a clean vessel. Ten millilitre quantities of this extract were each taken for FB_1 and AFB_1 cleanup respectively.

10.2.4. Cleanup for FB_1 Determination

The filtered extract (10 mL) was diluted with a solution of phosphate buffered saline (PBS, pH 7.0) (40 ml) containing 0.5% Tween 20 solution. The extract was then filtered through a microfibre filter (Schleicher & Schuell, Germany) and the filtrate transferred into a polypropylene syringe barrel, which was attached to the FumoniTest™ immunoaffinity (IA) column (Vicam, USA). The extract was passed through the IA column at a rate of about 1-2 drops/second until air passed through column. Thereafter, PBS (15 ml) was passed through the column at a rate of 1-2 drops/second. The FB_1 was eluted from the IA column under gravity by passing HPLC grade methanol (3 ml) through column at a rate of 1 drop/second and the eluate collected into a glass vial. The eluate was dried down under a stream of nitrogen at 60°C and concentrated at the base of a small vial (4 ml capacity).

10.2.5. Cleanup for AFB_1 Determination

The filtered extract (10 mL) was diluted with a solution of 10% Tween 20 (20 ml). The extract was then filtered through a microfibre syringe filter into a polypropylene syringe barrel, as above, which was attached to the AflaTest®-P immunoaffinity (IA) column (Vicam, USA). The extract was passed through the IA column at a rate of about 1-2 drops/second until air passed through the column. Thereafter, water (15 ml) was passed through the column at a rate of 1-2 drops/second. The AFB_1 was eluted from the IA column under gravity by passing HPLC grade methanol (3 ml) through column at a rate of 1 drop/second and the eluent collected into a glass vial. The eluent was dried down under a stream of nitrogen at 60°C and concentrated at the base of a small vial (4 ml capacity).

10.2.6. HPLC Analysis

FB_1 analysis was carried out by reconstituting the residue into methanol (200 □l) and aliquots were derivatized with *o*-phthaldialdehyde (OPA) prior to reversed-phase HPLC separation and fluorescence detection according to the method of Sydenham *et al.* (1996). Standards were similarly derivatised and chromatographed (Figure 7.1). AFB_1 was determined after reconstituting the residue into methanol (200 □l) and injecting 5 □l aliquots onto the column according to the method of Thiel *et al.* (1986). In the latter method, the HPLC mobile phase was adjusted to contain 0.01 M KH_2PO_4-acetonitrile-methanol in the ratio 690:220:75 (v/v/v).

10.2.7. HPLC-MS Analysis

HPLC-MS analysis was performed using a SpectraSERIES P2000 HPLC pump equipped with an AS 1000 autosampler containing a 20 □L injection loop. The HPLC column was attached on-line to a Finnigan MAT LCQ ion trap mass spectrometer setup for positive ion electrospray ionization (ESI). The test samples were filtered through a 0.45 □m syringe filter prior to injections. Binary gradient reversed-phase HPLC was performed on a 150 x 4.6 mm I.D. Luna C_{18} column packed with 5 μm ODS-2. Solvents A and B consisted of water-acetonitrile-formic acid in the ratios 90:10:0.1 and 10:90:0.1, respectively and pumped at 0.7 mL/min. Full scan MS-MS between *m/z* 330 and *m/z* 730 was undertaken to monitor FB_1 in the plants. Collision energy of 34% was used to fragment the protonated molecular ions and the resulting product ions for FB_1 were monitored as diagnostic indicators for its presence. The HPLC eluate entered the mass spectrometer without splitting at a source voltage of 4.5

kV and a capillary voltage of 40 V, while the heated capillary temperature was maintained at 220 °C and the sheath to auxiliary gas ratio was set at 4:1.

10.2.8. Statistical Analysis

Basic statistics comprising the mean and relative standard deviation for the spiked samples were computed using Microsoft Excel software (Microsoft Corporation) following duplicate experiments.

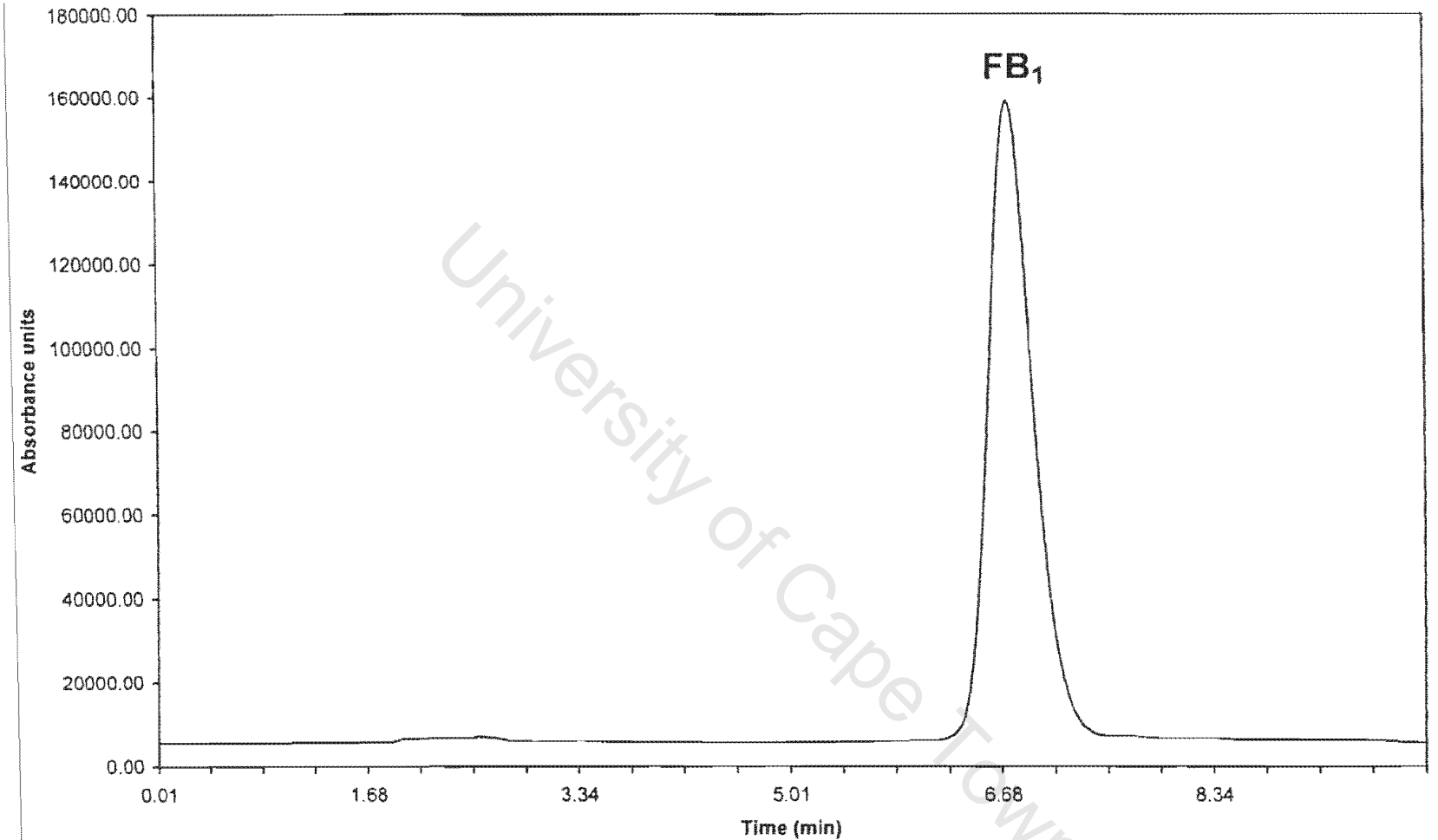


Figure 7.1. Reversed-phase HPLC Chromatogram of 50 μg/ml FB_1 standard solution. Injection volume = 10 μl

10.3. Results

The results of this study indicated that while none of the plants contained detectable levels of AFB_1, 8/47 (17%) of the plants analysed contained FB_1 ranging between 8.3μg/kg and 1553 μg/kg (Table 10.1). Four of the plants were identified as dietary supplements while the remaining four were frequently used as traditional medicine.

Table 10.1. Levels of FB_1 found in indigenous dietary and medicinal wild plants

Indigenous plants	Level of FB_1 detected (μg/kg)
Dietary	
Solanum nodiflorum	34
Raphanus raphinastrum	69
Zantedeschia aethiopica	105
Rumex lanceolatus	524
Medicinal	
Dalbergia obovata	8.6
Catha edulis	38
Datura stramonium	42
Brunsvigia sp.	1553

10.4. Discussion

The plant *Rumex lanceolatus*, which is cooked as a vegetable, contained 524 μg/kg FB_1 (Figure 10.2) and displayed an OC odds ratios of 1.86 (95% CI 1.31 – 2.63) and 1.68 (95% CI 1.18 – 2.41) for males and females respectively. This indicated that persons consuming this plant were almost twice more likely to develop OC than those who did not consume this plant. Similarly, the bulb of *Brunsvigia* sp., which is used medicinally as a purgative and emetic (Hutchings *et al.*, 1996), displayed an odds ratio of 1.80 (95% CI 1.12 – 2.88) for males and was found to contain 1553 μg/kg FB_1 (Figure 10.3). Bulb decoctions of this plant are also administered as enemas for renal and liver complaints (Watt and Breyer-Brandwijk, 1962) thus arousing serious concerns since FB_1 has been shown to be nephrotoxic (Suzuki *et al.*, 1995), hepatotoxic and to induce hepatocellular carcinoma in rats (Gelderblom *et al.*, 1991).

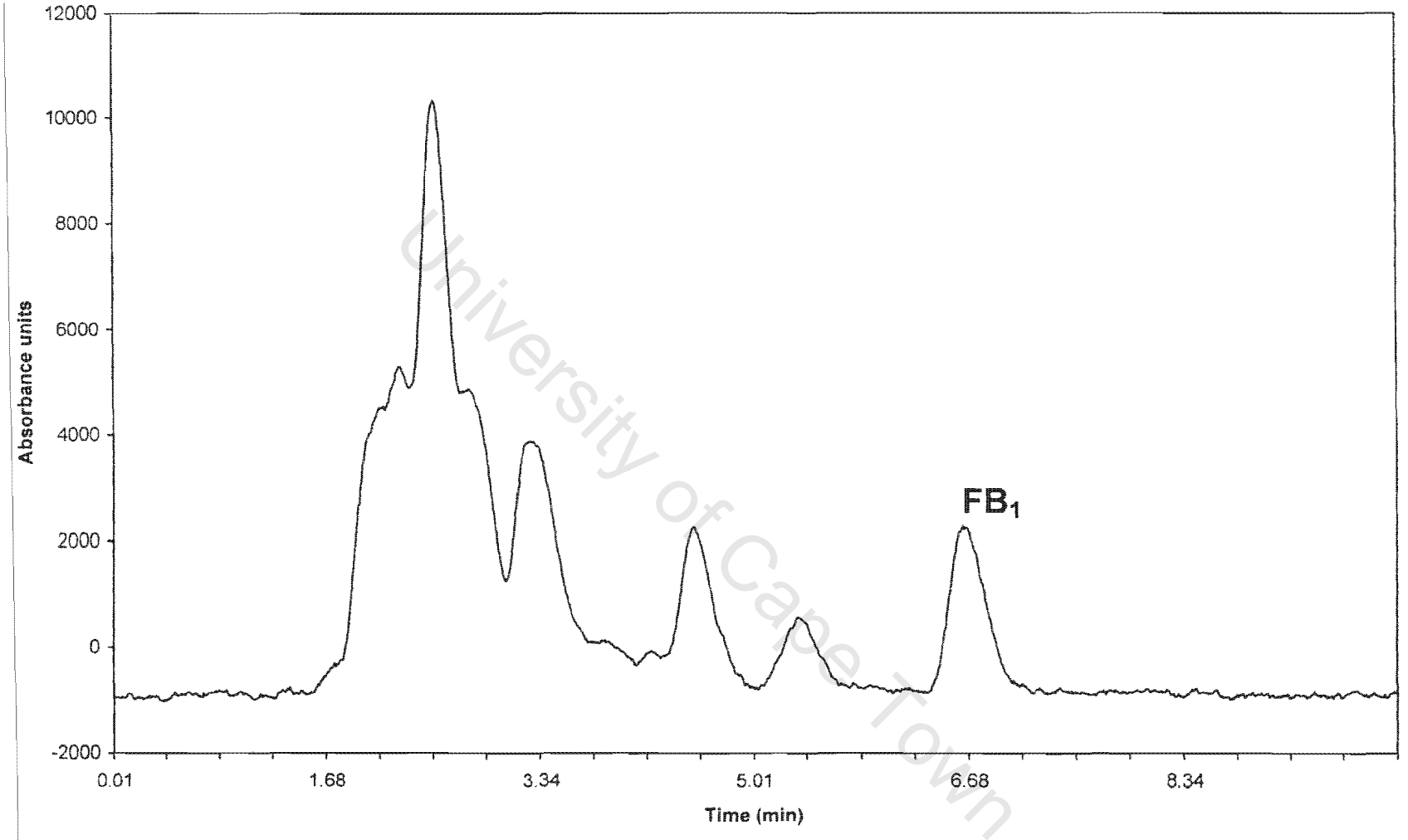


Figure 10.2. Reversed-phase HPLC Chromatogram of *Rumex lanceolatus* extract .

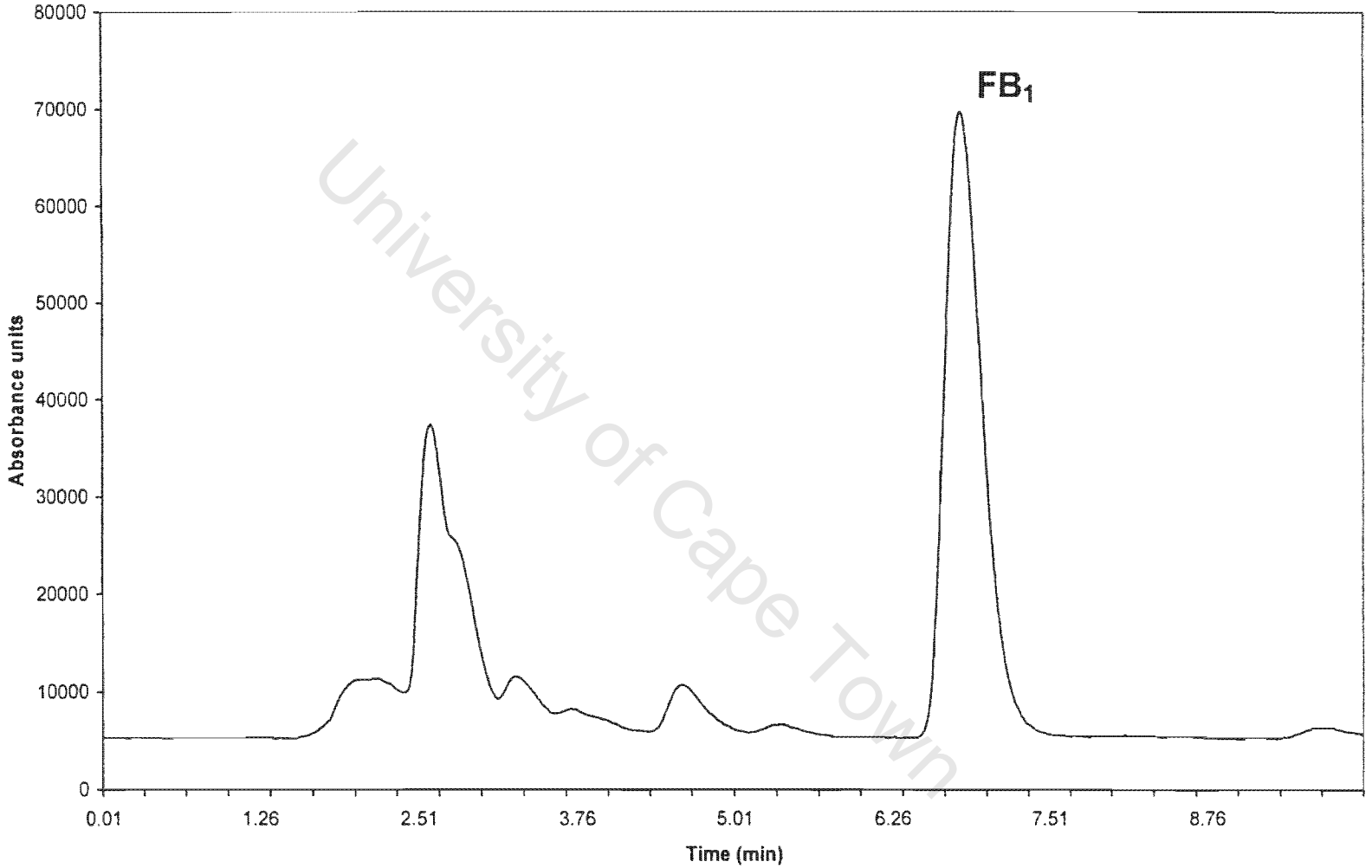


Figure 10.3. Reversed-phase HPLC Chromatogram of *Brunsvigia*. extract.

To confirm the presence of FB_1 in the eight plant samples thata initially tested positive for FB_1, HPLC-MS-MS analysis was undertaken. With the MS-MS mode it was possible to selectively excite the protonated molecular ion of FB_1 resident in the ion trap to produce diagnostic fragment ion spectra and hence obtain unequivocal confirmation of FB_1 presence in these plant samples. The fragmentation pathway for the fumonisins following collision induced dissociation (CID) consisted of sequential losses of water and tricarboxylic acid side chains from the alkyl backbone (Table 10.2). A typical HPLC-MS-MS result is indicated in Figure 7.4.

Table 10.2. Fragment ions observed for FB_1 under MS-MS fragmentation of the protonated molecular ion on the ion trap mass spectrometer.

Product ions	Mass-to-charge ratio *(m/z)*
	FB_1
$[M+H-H_2O]^+$	704
$[M+H-2H_2O]^+$	686
$[M+H-TCA]^+$	546
$[M+H-H_2O-TCA]^+$	528
$[M+H-2TCA]^+$	370
$[M+H-2TCA-H_2O]^+$	352

While the HPLC analytical method was developed and validated in previous studies, the extraction method for AFB_1 and FB_1 was validated prior to the analysis of the plants. This was done to ensure that matrix interferences and analyte losses during extraction were minimal so that the results would reflect the true level of contamination. In this aspect of the study, samples of the plant, *R. lanceolatus*, were each spiked in duplicate with AFB_1 at the 0.1 μg/g and 0.02 μg/g level, while FB_1 was spiked at the 0.5 μg/g and 2 μg/g level. Recoveries of the toxins were calculated after extraction and HPLC analysis, and adjustment for the quantity of toxins present in the background matrix. The results indicated (Table 10.3) that the extraction method yielded acceptable recoveries thus making it suitable for mycotoxin determination in plant matrices.

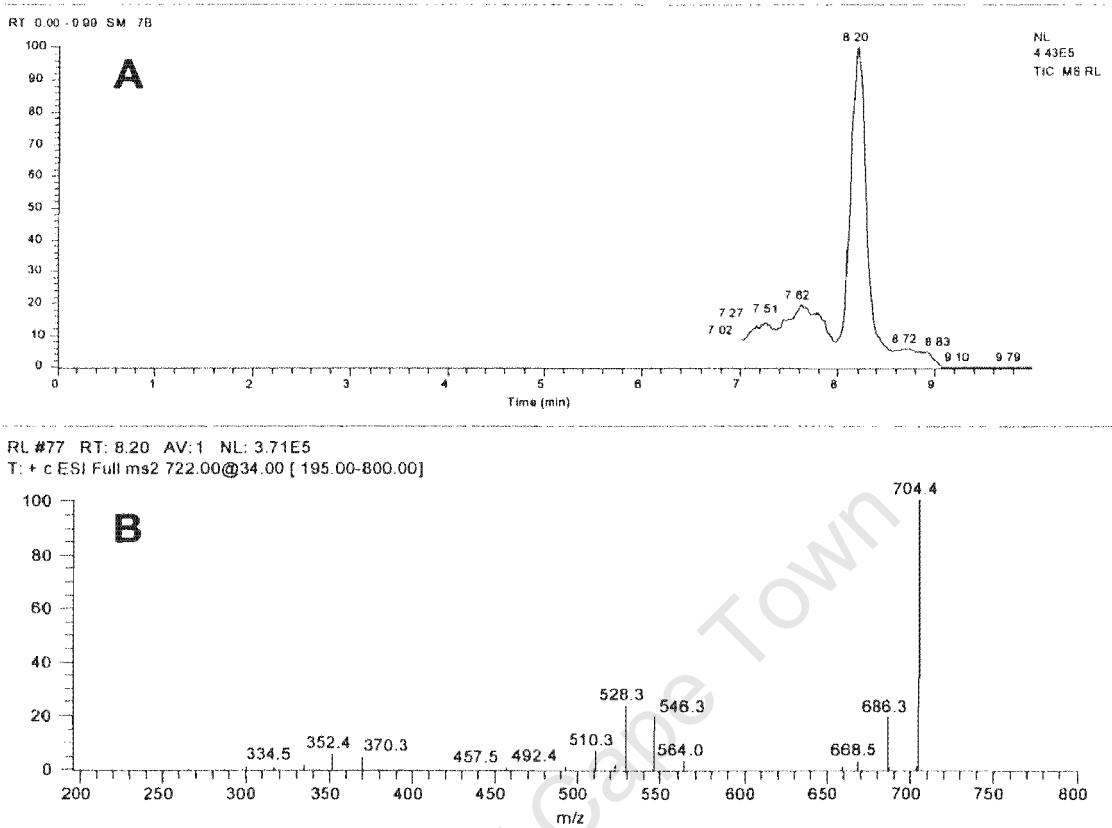


Figure 10.4. (A) Total ion chromatogram of FB_1 in *Rumex lanceolatus* extract an (B) Product ion mass spectra produced by collision induced dissociation of the protonated molecular ions of FB_1 serving as a diagnostic indicator.

Table 10.3. Accuracy of the extraction method for AFB_1 and FB_1 determination in plants matrices

Level of spiking (µg/g)	% Recovery (±SD)
AFB_1	
0.02	93 (7.78)
0.1	85 (5.66)
FB_1	
0.25	97.5 (7.78)
1	73.1 (4.10)

The level of FB_1 found in *R. lanceolatus* is comparable with the mean level of FB_1 found in the leaves of the orange tree (*Citrus sinensis*) (537 μg/kg) harvested in Lisbon, Portugal (Martins *et al.*, 2001) and the 1989 harvest of white corn from South African commercial farms (570 μg/kg) (Shephard *et al.*, 1996). The level is also similar to the 1992 mean levels found in commercial corn-based human foods sampled in retail outlets in Canada and South Africa (corn flour, 550 μg/kg) as well as USA (white maize meal, 550 μg/kg). This indicates that the increased risk of developing OC upon exposure to *R. lanceolatus* is not a result of the FB_1 content. The background levels of FB_1 in other commodities indicate that this toxin in *R. lanceolatus* does not pose a risk hence the increased risk form the plant results from the phytocehmical constituents. This observation is supported by data discussed in Chapter 8, where the extracts of this plant were shown to be mutagenic. The level of FB_1 is however much higher than the levels found in black tea (*Camellia sinensis*), corn silk (*Zea mays*), chamomile (*Matricaria chamomilla*) and the leaves of the linden tree (*Tillia grandifolia*) (Martins *et al.*, 2001). In contrast, the bulb of *Brunsvigia sp.* contained FB_1 at a level comparable to the levels found in naturally contaminated 'good' maize samples from the high oesophageal cancer incidence areas of the Transkei region of South Africa (1600 μg/kg and 1840 μg/kg in the 1985 and 1989 harvest seasons respectively) (Shephard *et al.* 1996). A provisional maximum tolerable daily intake (PMTDI) of 2 μg/kg body weight/day of FB_1 alone or in combination with two other analogues, FB_2 and FB_3, was set by the 56th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)(WHO 2002). Such data only serve to highlight the severity of the problem since rural residents of the Eastern Cape Province and in particular, the former Transkei region are already exposed to high levels (Probable daily intake = 47 μg/kg bw/day for good corn and 355 μg/kg bw/day in the case of mouldy maize) (Marasas, 1999) of this toxin via their consumption of contaminated maize as a dietary staple. Findings of FB_1 presence in dietary and medicinal wild plants raises further concerns with regards to the frequency and magnitude of exposure of these populations to this toxin.

Although FB_1 is not genotoxic and does not respond to any of the tests for mutagenicity with or without microsomal activation (Gelderblom and Snyman, 1989), the available evidence suggests that FB_1 may exert its cancer-promoting and toxic effects by inhibiting key enzymes involved in the *de novo* synthesis and turnover of sphingolipids (Wang *et al.*, 1991), which are important compounds for the structural and regulatory integrity of cells (Merrill, 1991).

The dietary wild plants investigated in this study form part of traditional diets and while some of the plants had low levels of FB_1 contamination, it could be argued that low levels of contamination are not going to be toxicologically significant and remains dependent on the frequency of intake and variation in levels. However the frequency and quantity of intake of these plants can result in prolonged exposures to such levels of toxin which can have a significant impact on the health status of people consuming these plants.

The control of fumonisin production in agricultural commodities is an intrinsically difficult problem. This study indicates that exposure to FB_1 is much more widespread than initially thought and certainly places the residents of the Eastern Cape Province, who are heavily dependent on these indigenous plants for their dietary and medicinal value, into a very high category of exposure to FB_1 as FB_1 is not just present in maize. This study also reports for the first time, the presence of this toxin in dietary and medicinal wild plants of South Africa and in conjunction with the data in Chapters 6 and 7 shows that FB_1 exposure is not a risk factor for OC.

CHAPTER 11: KEY FINDINGS AND STUDY LIMITATIONS

11.1 Tobacco and Alcohol

The results of this study are well in agreement with previous studies that have addressed the risks associated with tobacco and alcohol exposure and show clearly the independent effects of these two exposures. Amongst the male smokers, commercial cigarettes were the most frequently used form of tobacco (76%) followed by hand-rolled cigarettes (51%) and then pipe smoking (38%). A monotonic dose-response was observed across all the categories of the tobacco-related variables. Amongst the female smokers the use of the pipe was most popular (48%) with those smoking 7 or more pipes per day presenting with an almost 6-fold increase in risk compared to non-smokers. Based on total tobacco use, males and females currently smoking more than 14.5 grams of tobacco per day were observed to have approximately 4.5-times a greater risk of developing OC compared to non-smokers. This indicated that there was no gender difference whilst other studies may have been confounded by duration and intensity of dosage. With regards to tobacco chewing 96% and 92% of the male and female respondents indicated that they did not chew tobacco or use snuff respectively.

The type of alcoholic beverage did not appear to be an important factor in OC development but rather the quantity of ethanol consumed. Males and females consuming more than 371 grams ethanol per week had an almost 5-times greater risk in comparison to non-drinkers. Maize beer was the most popular alcoholic beverage contributing to 88% and 93% of overall alcohol consumption in males and females respectively hence in this population, the amount of maize beer consumption would be a better indicator of risk associated with alcohol exposure than a measure of total alcohol consumption. In addition, the effects of tobacco use were observed to be stronger than that of alcohol as has been observed in previous studies.

The joint effects of tobacco usage and alcohol consumption revealed that there was no interaction on the multiplicative scale although higher risks were associated with increased alcohol consumption across all tobacco amount strata. Similarly, increased tobacco use increased the risk estimates across the alcohol amount strata. The population attributable fractions revealed that if smoking and alcohol consumption were removed from the population, the 58% and 68% of OCs respectively would be prevented in this population.

11.2 Nutritional Risk

The consumption of maize did not indicate a statistically significant risk in the developing OC in both males and females and this may have been due to a lack of contrast between the cases and controls. However a protective effect was observed for the consumption of sorghum in both males and females. Daily consumption of sorghum as opposed to 2-4 days/week resulted in a 23% and 51% reduction in OC risk in males and females respectively. Similarly, a distinct inverse trend was observed for wheat-based products where daily consumption in males and females was associated with a 34% and 22% reduction in OC risk respectively. Increased frequency of consumption of green leafy vegetables, fruit and meat was also associated with a statistically significant protective effect in males and females whilst the consumption of *imifino* was positively associated with OC risk. Males and females consuming *imifino* for 2 – 4 days/week had an almost 2- and 2.4-fold increased risk of developing OC respectively. There was no association between OC risk and the consumption of pickled foods in both sexes.

Following principal component analysis, three distinct dietary patterns evolved. Dietary pattern 1, comprising sorghum, green leafy and podded vegetables, fruits and meat was found to be common amongst the more educated population and observed to confer protective effects (males OR = 0.79 & females OR = 0.54, highest category). Adherence to dietary pattern 2, comprising maize, imifino and beans was associated with a 62% greater risk in males and females in the 'moderate' category and was more common amongst the less educated and rural population. Dietary pattern 3, comprising exclusively of wheat-based products was observed to confer protective effects with a 35% reduction in OC risk in both males and females.

11.3 The Mouldy Maize Enigma

In this study, when shown a photograph of mouldy maize, 80% males and 78% females indicated that they consumed such maize. As a result of the high reported prevalence no significant effect of mouldy maize consumption was observed (males OR = 0.98; female OR = 0.86). The duration of mouldy maize consumption also did not show any statistically significant risk of OC development across any of duration strata. It is also known that mouldy maize is used in the preparation of traditional maize beer. In this study the consumption of traditional beer made from mouldy maize did not present with an increased risk of developing OC in both males and females strengthening the evidence that mouldy maize itself is not a risk factor for the development of OC. This observation is consistent with a

previous finding of Sammon (1992). Hence while maize intake may be a risk factor due to the lack of essential vitamins and minerals, in this study, the mould contamination on the maize itself did not seem to pose as a risk factor for the development of OC. Given the high self-reported prevalence of maize intake, a larger study would need to be conducted.

11.4 Dietary and Medicinal Wild Plants

Nineteen dietary wild plants were investigated and six were observed to produce a significant increase in OC risk in males and nine in females. Similarly, of the 29 medicinal plants, 4 were found to increase the risk in both sexes. The results were evaluated together with those from the *Salmonella* reverse mutation assay discussed in Chapter 8. Table 11.1 lists only those plants where the epidemiological results concurred with those for mutagenicity indicating that these plants are indeed high risk, as evidence by mechanistic studies and bolstered by the epidemiological evidence.

Table 11.1 Summary findings for dietary and medicinal wild plants

Dietary Plants	**Odds ratio (95% CI)**		**Positive Ames Assay**	**Mechanism of mutation**
	Males	**Females**		
Rumex lanceolatus	1.86 (1.31-2.63)	1.68 (1.18-2.41)	All strains	Frameshift mutations, Base-pair substitutions, Oxidative DNA damage
Raphanus nasturtio aquatica	1.93 (1.07-3.47)	1.80 (1.06-3.07)	TA100	Base-pair substitution
Raphanus raphinastrum	1.40 (1.05-1.87)	1.49 (1.04-2.12)	TA97A	Frameshift mutation
Sida dregei	3.8 (1.66-8.70)	2.06 (1.06-4.00)	TA97A	Frameshift mutation
Colocasia esculenta	1.78 (1.06-2.97)	2.03 (1.19-3.45)	TA97A TA102	Frameshift mutations, Oxidative DNA damage
Solanum nigrum	1.32 (0.99-1.75)	1.27 (0.91-1.77)	TA97A	Frameshift mutations
Sida rhombifolia	4.54 (1.17-17.60)	6.52 (1.30-32.7)	All strains	Frameshift mutations, Base-pair substitutions, Oxidative DNA damage
Sonchus oleraceus	1.22 (0.86-1.71)	1.84 (1.28-2.65)	TA 100	Base-pair substitution
Medicinal Plants				
Eucomis autumnalis	1.48 (1.10-2.00)	1.19 (0.84-1.68)	TA100	Base-pair substitution
Brunsvigia sp.	1.80 (1.12-2.88)	1.51 (0.70-3.29)	TA100	Base-pair substitution
Artemisia afra	1.09 (0.81-1.46)	1.41 (1.02-1.95)	TA98, TA100	Frameshift mutations, Base-pair substitution
Withania somnifera	1.24 (0.66-2.34)	2.22 (1.14-4.33)	TA102	Oxidative DNA damage

11.5 Trace Elements

Due to their association with OC from previous studies, Mg, Mn and Zn deficiencies have been implicated as a risk factor for OC in South Africa. However the current study indicates that the plants used by residents of the Eastern Cape Province do contain these essential elements hence deficiency of these elements in the population would depend largely on the quantity and frequency of intake. The lack of Zn however can increase oxidant damage to various cell components. Selenium deficiencies were also noted in these plants and has been implicated as a risk factor for OC. The presence of trace elements Co, Cr, Cd, As and V, due to their known toxicities and carcinogenic potential were also investigated and found to be present at higher levels in the plants used for dietary purposes. With regards to the plants used as traditional medicines, the levels were quite low and it is not anticipated that a risk would be imposed. This investigation revealed that exposure to heavy metals which are known carcinogens can occur through wild plant consumption, while beneficial elements such as Se, were severely lacking.

11.6 Mycotoxin Contamination

In addition to the mutagenicity and trace element content, eight of the plants were found to contain fumonisin B_1, a known carcinogen, at levels ranging between 8.6 – 1553 μg/kg of plant material. The plant *Rumex lanceolatus* and Brunsvigia *sp.* were found to be contaminated with FB_1, however the increased risk for OC is more likely to result from carcinogenic phytochemicals as evidenced in the mutagenicity assay. Furthermore the levels found were similar to the background levels found in commercial maize discounting once again the risk associated with FB_1 exposure.

11.7 Study Limitations and Strengths

This study was hospital based and hence may have been subject to various kinds of bias however every effort was made to reduce such biases. Sampling bias may concern cases if they are not representative with respect to all the cases in the population and this is bound to affect the external validity (generalisability) of the results. The three hospitals are the major referral hospitals for the Eastern Cape Province hence the majority of patients residing in this province are likely to visit one of these hospitals. Sampling bias for controls arises if they are not representative of the source population of the cases and would affect the internal validity of the study results. Such bias would be particularly relevant for hospital-based controls if their referral to participating hospitals differs from that of cases. We addressed this source of bias by selecting controls from several diagnostic categories. Lack of response is a

further potential source of sampling bias; however it is not likely to have played an important role since the response rate was high among both cases (98%) and controls (96%).

The study subjects all came from the high risk region for squamous cell carcinoma of the oesophagus in the Eastern Cape Province of South Africa, an ideal place to investigate the causal relationship of social/dietary habits and OC. Furthermore, the case confirmation was excellent because all cases were diagnosed either by histology (36%), radiology (60%) or endoscopy (4%).

Errors in measurement may also be introduced as a result of observer (interviewer) or responder (patient) bias. Although it is rather difficult to exclude observer bias, the interviewers used a structured questionnaire and the same interviewer interviewed a case and two corresponding controls. Responder bias can manifest as a result of differential recall of information by cases and controls; for instance, cases may be more likely to recall past exposure, especially if its association with the disease is widely known. This type of bias can exaggerate the degree of effect associated with the exposure. In this study, this form of bias is unlikely to have generated the difference between cases and controls concerning exposure to wild plants and its association as a risk factor as the patients were interviewed with regards to a variety of plants both dietary and medicinal. Hence it is unlikely that case subjects overestimated their exposure to selected plants since there was no indication that certain plants were considered high-risk. Furthermore the association of wild plant intake with oesophageal cancer is not widely known. Hence if misclassification did occur, it is likely to be non-differential with regard to case-control status. Thus the reported risk estimates might be an underestimation of the real underlying effect of wild plant consumption.

The accuracy of self-reporting may be questionable, however the questionnaire was slightly modified to that used by Pacella-Norman *et al.* (2002) and this allows for national comparability. Furthermore the questionnaire was validated. The results when compared to that obtained by Pacella-Norman *et al.* (2002) displayed similar trends and associations in risk. The categories for tobacco smoking were crude (ex smokers versus current) and it is possible that many smokers may have stopped smoking as a result of symptoms of OC hence some misclassification may have occurred resulting in higher risk for ex-smokers in comparison to current smokers. Althoug the diet is representative of the population and typical of the consumption patterns observed, use of a 24 hour recall as an additional tool to evaluate food consumption would also have reduced recall bias. It would have been

appropriate to conduct biological measurements in subjects consuming medicinal and dietary wild plants however this was not possible given the tight budget. Furthermore, due to the chemical complexity of the various plants, one would need to establish biomarkers for such exposures, an area of research that is severely lacking. Given these limitations, this study was undertaken on a large sample size (largest case-control study of OC in SA) where an excellent response rate was achieved and demonstrates that it is possible to undertake good epidemiological studies in resource poor areas with moderate diagnostic facilities.

11.8 Relevance and potential benefits

This project was highly relevant because through a combination epidemiology and laboratory-based investigations, it attempted to address one of the most prevalent cancers in South Africa and its relationship to wild plant usage which is extremely common given the culture and traditions of the affected communities. The results of this research targeted two main groups, i.e. The scientific community and the residents of the high incidence areas of OC. For the scientific community, the results formed a basis for exploring new directions in OC research as a result of the knowledge generated from this study. The role of phytochemicals as well as heavy metal contamination of plants can now be further explored to elucidate and understand new biochemical pathways to carcinogenesis, which will shed more light about the aetiology of this disease in South Africa.

The results of this study also have short- and long-term impacts on the residents of these areas. The short-term impact is increased awareness of the risks associated with wild plant consumption allowing the residents to make informed choices about their ethnobotanical practices. The public was also made aware of the results of this study through seminars at the local and referral hospitals, and discussion groups/workshops at the community health centres. The hospitals and the local department of health was aware of the study and upon completion, the community nursing staff were also briefed on the results so that they could serve as a constant source of guidance and information concerning the risk factors studied in this project. It was anticipated that these methods of knowledge dissemination would help provide a platform of openness where patients and community members could discuss their concerns, views and perspectives pertaining to the results. It is anticipated that these forms of dialogue and community participation will help in the long-term impact of this project, which is to establish preventative strategies through modulation of lifestyle behaviour in order to reduce the incidence of this disease and thus advance human health protection beyond our current capabilities. The results of this study suggested that lifestyle

modifications and improvements in living conditions would markedly alter the risk of developing OC. It provides the preventive strategies through alteration of lifestyle behaviours for reducing the incidence of this disease. By appropriate transfer of information to the communities, the project would be seen to thus impact on the OC incidence rates (long term) and hence on life expectancy rates in rural communities already well aware of the devastation caused by the currently high OC rates.

11.9 Conclusion

Hence the evidence from this study indicate that the population of the Eastern Cape Province and in particular the former Transkei region indeed fall in the high risk category as a result of the exposures they endure due to their social and dietary habits and therefore are a high-risk subset of the South African population. The evidence suggest that lifestyle modifications including a reduction in tobacco and alcohol use and improvements in living conditions and diet characterised by daily consumption of fruits and vegetables would markedly lower the incidence of OC. The cautious use of certain dietary and medicinal plants will also assist in reducing risk.

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LIST OF APPENDICES

APPENDIX 1: EPIDEMIOLOGICAL STUDY QUESTIONNAIRE

APPENDIX 1

Questionnaire No. ☐☐☐☐

EPIDEMIOLOGY STUDY QUESTIONNAIRE:
RISK FACTORS FOR OESOPHAGEAL CANCER
PROMEC UNIT, MEDICAL RESEARCH COUNCIL,
P.O. BOX 19070, TYGERBERG, 7505

OFFICIAL USE ONLY

P/No.: ☐☐☐☐☐☐ Name: .....

Patient: 1. Case | 2. Control ☐

Age (years) ☐☐

Sex: 1. Male | 2. Female ☐

Name of this hospital/institution: 1. UGH | 2. FRERE | 3. CMH ☐

ICD ☐☐☐☐☐

If patient is **Control Subject**, state reason for admission/visit: .....

If patient is **Case Subject**, indicate the following:

ICD-O ☐☐☐☐☐

Diagnosis: .....

Method of Diagnosis: 1. Histology | 2. Radiology | 3. Scan | 4. Clinical only ☐

Location of tumour: 1. Upper third | 2. Middle third | 3. Lower third | 4. Unspecified ☐

If Histology result available: M ☐☐☐☐☐☐☐☐☐

Name of Interviewer ..... Date of Interview ☐☐/☐☐/☐☐ (dd / mm / yy)

Place of Birth .....

How long have you lived there? (years) ☐☐

Place of current residence/ address .....

Place of usual residence? ☐☐☐☐

How long have you lived there? (years) ☐☐

Telephone no. (if available) .....

1. Have you ever done one of the following? (Please mark with X)

	1. Yes (now)	2. In the past	3. Both (1 & 2)	4. never
(a) smoked commercial cigarettes				
(b) hand-rolled cigarettes				
(c) smoked a pipe				

2. In the past 5-10 years, how many would you usually smoke in a day? (number)

1. commercial	
2. hand-rolled	
3. pipes	

3. Have you ever chewed tobacco? (Please mark with X)

1. Yes (now)	2. In the past	3. Both (1 & 2)	4. never

How long? (years) Years

Unknown

4. Have you ever used snuff? (Please mark with X)

1. Yes (now)	2. In the past	3. Both (1 & 2)	4. never

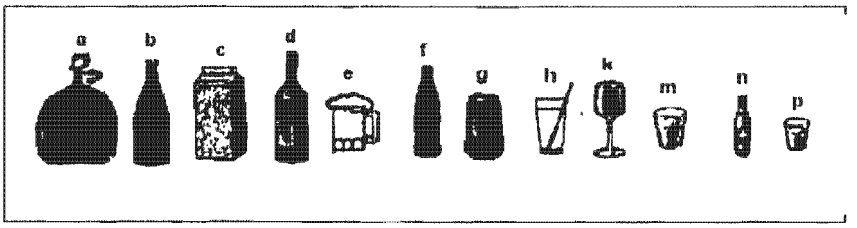
How long? (years) Years

Unknown

In the past 5 - 10 years, how often would you use snuff each day? times per day

5. Before you became ill, how often did you drink either of the following? (Please mark with X)

	1. 5 - 7 days per week	2. 2 - 4 days per week	3. ≤ 1 day per week	4. Never	9. Unknown	amount taken/ day (use diagram below)
maize beer						
Sorghum beer						
Other home-made beer						
Commercial beer						
Home-made spirits						
Commercial spirits						
Wine						
Other (specify)						



a: 2L wine bottle	e: 500ml beer glass	k: 200ml wine glass/nip
b: 1L bottle	f: 375ml half pint	m: 100ml (small glass)
c: 1L carton	g: 340ml beer can	n: 50ml miniature airplane bottle
d: 750ml wine bottle	h: 250 ml glass	p: 25 ml tot

6. In the past 5 - 10 years before you became ill, how often did you eat the following?

	1. 5 - 7 days per week	2. 2 - 4 days per week	3. ≤ 1 day per week	4. Never	9. Unknown
Maize					
Sorghum					
Wheat					
Green, leafy vegetables					
Green pod vegetables					
Imifino (wild/veld greens)					
fruit					
Beans in Umngqusho					
Meat					
Pickled food					
Other:					

7. How do you normally obtain the following?

	1. Buy	2. Home-grown	3. Both (1&2)
Maize			
Sorghum			
Wheat			

8. Did you ever eat maize that looked like this? (Interviewer to show photograph)

1. Yes	2. No

If yes, for how long? ☐☐ years

8. Did your family ever prepare beer from maize that looked like this? (Interviewer to show photograph)

1. Yes	2. No	9. Unknown

PLANTS USED AS DIETARY SUPPLEMENTS											
1. Do you eat wild vegetables/plant foods to supplement your diet?										Y	N
If Yes: go to question 2, 3 and 4											
2a. Which of the following wild vegetables/plant foods do you currently eat? (Please tick block 2)					2b. Which part of the plant do you eat and how often per week?						
		2	3	4	ROOTS	LEAVES	STEM	FRUIT	WHOLE PLANT	UNSURE	
1	Isinama										
2	Itentsi										
3	Umhlabangulo										
4	Iqwaka										
5	Unongotyane										
6	Imbikicane										
7	Idumbe										
8	Ibopha										
9	Umbhanga bhanga										
10	Umsobo										
11	Ikhwane										
12	Itolofiya										
13	Gcamache										
14	Iguzu										
15	Iwatane										
16	Umsobo wehlathi										
17	Irhwabe										
18	Utyuthu										
19	Umbhewe/Umhlashoshana										
20	Unomdlomboyi/ Imbuya/ Umambumbu										
	OTHER										
21											
22											
23											
24											
25											
26											
27											
28											
29											
30											
31											
32											
33											
34											
		1 = Yes 0 = No									

										Y	N
3a. Which of these wild vegetables/plant foods did you eat five years ago? (Please tick block 3)					3b. Which part of the plant did you eat and how often per week?						
		2	3	4	ROOTS	LEAVES	STEM	FRUIT	WHOLE PLANT	UNSURE	
1	Isinama										
2	Itentsi										
3	Umhlabangulo										
4	Iqwaka										
5	Unongotyane										
6	Imbikicane										
7	Idumbe										
8	Ibopha										
9	Umbhanga bhanga										
10	Umsobo										
11	Ikhwane										
12	Itolofiya										
13	Gcamache										
14	Iguzu										
15	Iwatane										
16	Umsobo wehlathi										
17	Imwabe										
18	Utyuthu										
19	Umbhewe/Umhlashoshana										
20	Unomdlomboyi/ Imbuya/ Umambumbu										
	OTHER										
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25											
26											
27											
28											
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33											
34											
* After having asked question 3a, the following question is to be asked: What other plants did you eat five years ago, which you do not eat at the moment? (Write answers below "other")		1 = Yes 0 = No									

PLANTS USED FOR MEDICINAL PURPOSES

1. When you feel sick, do you take plant medicines to get well? Y N

If Yes: go to 2, 3 and 4

	2a. Which of the following plants do you currently use as medicine? (Please tick block 2)	2	3	4	2b. What's your reason for taking the plant? Protective	Illness	2c. Where do you obtain the plant? W	G	B	T	2d. Which part of the plant do you use? R	L	S	F	P	U	2e. How do you use the plant medicine? ROOTS D	E	C	E_M	O	LEAVES D	E	C	E_M	O	STEM D	E	C	E_M	O	FRUIT D	E	C	E_M	O	WHOLE PLANT D	E	C	E_M	O
1	Umbhewe/Umhlashoshana																																							
2	Isinama																																							
3	Itentsi																																							
4	Umhlabangulo																																							
5	Iqwaka																																							
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34																																							

1=Yes; 0 = No

W: wild; G: grow yourself; B: buy; T: traditional healer

R: roots; L: leaves; S: stem; F: fruit; P: whole plant; U: unsure

D: drink; E: eat; C: chew; EM: emetic; O: other

	Y	N

3a. Which of these plants did you take five years ago? (Please tick block 3)					3b. What's your reason for taking the plant?		3c. Where did you obtain the plant?				3d. Which part of the plant did you use?					
		2	3	4	Protective	Illness	W	G	B	T	R	L	S	F	P	U
1	Umbhewe/Umhlashoshana															
2	Isinama															
3	Itentsi															
4	Umhlabangulo															
5	Iqwaka															
6	Unongotyane															
7	Imbikicane															
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15	Iguzu															
16	Iwatane															
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18	Irhwabe															
19	Utyuthu															
20	Unomdlomboyi/ Imbuya/ Umambumbu															
	OTHER															
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* After having asked question 3a, the following question is to be asked: What other plants did you take five years ago, which you do not take at the moment? (Write answers below "other")		1=Yes; 0 = No			W: wild; G: grow yourself; B: buy; T: traditional healer						R: roots; L: leaves; S: stem; F: fruit; P: whole plant; U: unsure					

	3e. How did you use the plant medicine?																								
	ROOTS					LEAVES					STEM					FRUIT					WHOLE PLANT				
	D	E	C	E_M	O	D	E	C	E_M	O	D	E	C	E_M	O	D	E	C	E_M	O	D	E	C	E_M	O
1																									
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	D: drink; E: eat; C: chew; EM: emetic; O: other																								

APPENDIX 2: PROJECT INFORMATION SHEET FOR THE CASES

PROJECT INFORMATION SHEET (CASES)

Hello,

We are from the Medical Research Council and are investigating whether certain aspects of your lifestyle and diet may increase the risk of getting oesophageal cancer.

Why are we doing this?
Research, done previously, has shown that eosophageal cancer occurs at a high rate among black South Africans. This disease is very high in the Transkei region of South Africa when compared to other areas in the country. We are thus very concerned and want to find out why this is so. We know that factors related to lifestyle and diet may contribute to the disease but are not certain of all the risk factors. Furthermore, we know that plants are eaten frequently, but we are not sure whether all the plants that are eaten have been identified and whether the chemicals in these plants are harmful to the body and may play a role in the development of this disease. We would thus appreciate it very much if you would accept our invitation to participate in this study so that we can increase our knowledge in finding out why this disease occurs and prevent it, as much as possible, from occurring in the future.

What do we expect from the participants in the study?
We would like you to answer a few simple questions that relate to your lifestyle and diet. The questions will be asked by a trained interviewer who will also fill in the questionnaire. The answers that you give during the interview will be used to identify potential causes of the disease. These answers will be strictly confidential and will not be shown to other patients or hospital staff not involved in this investigation. We anticipate that the interview will not last longer than 30 minutes.

May I withdraw from the study?
Certainly, you may do this at any time without having to give a reason. Remember that the study is completely voluntary and not taking part in it, or withdrawing from it, will not influence the quality of care that you receive at this hospital/clinic.

If you have any queries concerning this study, more information may be obtained from Doctor Vikash Sewram at the Medical Research Council on the telephone number (021) 938-0272.

If you are happy to take part in the study, please read and sign the attached consent form.

APPENDIX 3: PROJECT INFORMATION SHEET FOR THE CASES (isiXHOSA)

INGCACISO NGESIFUNDO SOPHANDO-NZULU. (KUMTHATHI_NXAXHEBA OGULA NGUMHLAZA WOMQALA)

Molo / bhota

Singamalungu eQumru loPhando kwezo Nzulu - lwazi achophele ukuxilonga izinto ngento ezinokufunyanwa ekutyeni okanye zibe sisiphumo sendlela esiphila ngayo zincedise kunobangela womhlaza womqala.

SIKWENZELANI OKU?

Phambili phaya uphando lufumene ukuba umhlaza womqala ngomnye wonobangela bokufa kuma-Afrika aseMzantsi-Afrika, yaye elona qondo liphezulu lifunyenwe apha eTranskei xa liqhathaniswa nezinye iindawo kweli loMzantsi-Afrika. Yonke loo nto yenze okokuba sizikhathaze sileli qumru, siphande nzulu ngonobangela woku. Nangona singenakuqinisekisa ngonobangela ngqo ngokwangoku, kwezinye zezifundo zophando nzulu sifumene okokuba ukutya kunye nendlela esiphila ngayo kunokuncedisa kunobangela wesi sifo singumhlaza womqala. Kanjalo, siyazi kwakhona okokuba kwizinto ezifana nemifuno yasendle nangona ikwasisidlo singafumanisa ityhefu esenokuba yingozi emizimbeni yethu kanjalo mhlawumbi incedise kunobangela wesifo somhlaza. Ngamafutshane singavuya xa umbono wethu sileli qumru unokwamkeleka sincedisane ngokuphanda nzulu unobangela walombulala zwe, lwazi olo olunokukhokelela ekuthinteleni nokufumana ichiza.

NCEDO LUNI ESILULINDELEYO KUBATHATHI NXAXHEBA?

Ziimpendulo kwimibuzo embalwa, elula ngokutya enikutyayo kunye nendlela eniphila ngayo eniya kuyibuzwa ngoqeqeshelwe ukwenza oko. Iimpendulo zonke ziyakubhalwa phantsi, zibe yimfihlo phakathi kwakho nequmru eli. Yenziwe le mibuzo yamifutshane ukonga ixesha lakho ingathatha imizuzu ekumashumi mathathu.

NGABA UNGARHOXA KWESI SIFUNDO?

Ewe. Nabani na othabatha inxaxheba kwesi sifundo unelungelo lokurhoxa nanini na enganikanga sizathu sakwenza oko. Esi sifundo senziwa ngonomdla wokufaka isandla kuphando, yaye ukuroxa kwakho akusayi kuchaphazela malungelo akho kwiingxaki zempilo kwesi sibhedlele / ikliniki.

Yonke imibuzo yibhekise kuGqira u Vikash Sewram weQumru lwezoPhando - nzulu kule nombolo: (021) 938-0272

Ukuba yonke le ngcaciso iyakwanelisa, uvuma kwanjalo ukuthabatha inxaxhela, sayina igama lakho kwinvume yokuthabatha inxaxheba.

APPENDIX 4: PROJECT INFORMATION SHEET FOR THE CONTROLS

PROJECT INFORMATION SHEET (CONTROLS)

Hello,

We are from the Medical Research Council and we are interested in finding out why many people in this area are getting oesophageal cancer. **You do not have this illness** and we would like to find out the whether certain aspects of your lifestyle and diet might have prevented you from getting this disease.

Why are we doing this?
Research, done previously, has shown that eosophageal cancer occurs at a high rate among black South Africans. This disease is very high in the Transkei region of South Africa when compared to other areas in the country. We are thus very concerned and want to find out why this is so. We know that factors related to lifestyle and diet may contribute to the disease but are not certain of all the risk factors. Furthermore, we know that plants are eaten frequently, but we are not sure whether all the plants that are eaten have been identified and whether the chemicals in these plants are harmful to the body. Since you do not have this disease we would thus appreciate it very much if you would accept our invitation to participate in this study, so that we can increase our knowledge in finding out what can prevent this disease, as much as possible, from occurring in the future.

What do we expect from the participants in the study?
We would like you to answer a few simple questions that relate to your lifestyle and diet. The questions will be asked by a trained interviewer who will also fill in the questionnaire. The answers that you give during the interview will be used to identify potential causes of the disease. These answers will be strictly confidential and will not be shown to other patients or hospital staff not involved in this investigation. We anticipate that the interview will not last longer than 30 minutes.

May you withdraw from the study?
Certainly, you may do this at any time without having to give a reason. Remember that the study is completely voluntary and not taking part in it, or withdrawing from it, will not influence the quality of care that you receive at this hospital/clinic.

If you have any queries concerning this study, more information may be obtained from Doctor Vikash Sewram at the Medical Research Council on the telephone number (021) 938-0272.

If you are happy to take part in the study, please read and sign the attached consent form.

APPENDIX 5: PROJECT INFORMATION SHEET FOR THE CONTROLS (isiXHOSA)

INGCACISO NGESIFUNDO SOPHANDO-NZULU. (KUMTHATHI_NXAXHEBA ONGAGULIYO NGUMHLAZA WOMQALA)

Molo / bhota

Singamalungu eQumru loPhando kwezo Nzulu - lwazi achophele ukuxilonga izinto ngento ezinokufunyanwa ekutyeni okanye zibe sisiphumo sendlela esiphila ngayo zincedise kunobangela womhlaza womqala. **Qaphela, wena akunaso esi sifo** yaye sinomdla wokulandela indlela ophila notya ngayo esenokuba iyakuncedisa ekuthinteleni isifo somhlaza.

SIKWENZELANI OKU?

Phambili phaya uphando lufumene ukuba umhlaza womqala ngomnye wonobangela bokufa kuma-Afrika aseMzantsi-Afrika, yaye elona qondo liphezulu lifunyenwe apha eTranskei xa liqhathaniswa nezinye iindawo kweli loMzantsi-Afrika. Yonke loo nto yenze okokuba sizikhathaze sileli qumru, siphande nzulu ngonobangela woku. Nangona singenakuqinisekisa ngonobangela ngqo ngokwangoku ,kwezinye zezifundo zophando nzulu sifumene okokuba ukutya kunye nendlela esiphila ngayo kungancedisa kunobangela wesi sifo singumhlaza womqala. Kanjalo, siyazi kwakhona okokuba kwizinto ezifana nemifuno yasendle nangona ikwasisidlo singafumanisa ityhefu esenokuba yingozi emizimbeni yethu kanjalo mhlawumbi incedise kunobangela wesifo somhlaza. Nanjengoko ungekabinaso esi sifo, singavuya xa unokuthabatha inxaxheba kwesi sifundo ukuze sandise ulwazi lwethu sizame amacebo okuthintela unobangela kangako sinako ukuze sithintilwe nakwixesha elizayo.

NCEDO LUNI ESILULINDELEYO KUBATHATHI NXAXHEBA?

Ziimpendulo kwimibuzo embalwa, elula ngokutya enikutyayo kunye nendlela eniphila ngayo eniya kuyibuzwa ngoqeqeshelwe ukwenza oko. Iimpendulo zonke ziyakubhalwa phantsi, zibe yimfihlo phakathi kwakho nequmru eli. Yenziwe le mibuzo yamifutshane ukonga ixesha lakho ingathatha imizuzu ekumashumi mathathu.

NGABA UNGARHOXA KWESI SIFUNDO?

Ewe. Nabani na othabatha inxaxheba kwesi sifundo unelungelo lokurhoxa nanini na enganikanga sizathu sakwenza oko. Esi sifundo senziwa ngonomdla wokufaka isandla kuphando, yaye ukuroxa kwakho akusayi kuchaphazela malungelo akho kwiingxaki zempilo kwesi sibhedlele / ikliniki.

Yonke imibuzo yibhekise kuGqira u Vikash Sewram weQumru lwezoPhando - nzulu kule nombolo: (021) 938-0272

Ukuba yonke le ngcaciso iyakwanelisa, uvuma kanjalo ukuthabatha inxaxhela,nceda usayine igama lakho kwimvume yokuthabatha inxaxheba.

APPENDIX 6: PATIENT CONSENT FORMS FOR THE CASES

PATIENT CONSENT FORM (CASES)

CODE:

I, _____, hereby agree to take p
voluntarily in the study being carried out at _____
hospital/clinic, during the period _____.

This study involves me being asked questions concerning my lifestyle and di
The answers that I give during the interview will be used to identify potent
causes of my disease. I also have the choice of withdrawing from the study at a
time and this will not influence the quality of care that I receive at t
hospital/clinic.

I know that this study is to help people and will not harm me. I understand that
the information that I give or that is taken from my hospital files will rem
confidential.

_____ _____

SIGNATURE/THUMPRINT WITNESS

_____

DATE

APPENDIX 7: PATIENT CONSENT FORM FOR THE CASES (isiXHOSA)

PATIENT CONSENT FORM IN XHOSA (CASES)
IMVUME YOKUTHABATHA INXAXHEBA KWISIFUNDO SOPHANDO-NZULU

INOMBOLO:

Mna _____, ndiyavuma ukuthabatha inxaxheba kwesi sifundo siqhubeka apha e _____

ngowe_____.

Esi sifundo siqulathe oku kulandelayo:

Ndizakubuzwa imibuzo ngamagosa ophando-nzulu ngendlela endiphila ngayo nokutya endikutyayo.limpendulo zam ziyakusetyenziswa ekuphandeni nzulu malunga nesifo somhlaza womqala (cancer).

Naku okubalulekileyo:
Ndiyazi ukuba ndingarhoxa nanini na kwesi sifundo, kanjalo ukurhoxa kwam kungasayi kuchaphazela malungelo am kiingxaki zempilo kule kliniki / kwesi sibhedlela.
Ndiyaqinisekiswa ukuba esi sifundo asinabungozi kum.
limpendulo zam ziyakwandisa ulwazi kwiinzame zophicotho nobangela womhlaza womqala.
Zakugcinwa ziyimfihlo phakathi kwam kunye namagosa ophando-nzulu iimpendulo zam kunye nokufunyenwe ezincwadini zesibhedlele ngengcombolo yempilo yam.

_____ _____

INTSAYINO-GAMA/BEKA UBHONTSI INGQINA

_____

UMHLA

APPENDIX 8: PATIENT CONSENT FORM FOR THE CONTROLS

PATIENT CONSENT FORM (CONTROLS)

CODE:

I, _____, hereby agree to take part voluntarily in the study being carried out at _____, hospital/clinic, during the period _____.

This study involves me being asked questions concerning my lifestyle and diet. The answers that I give during the interview will be used to identify potential factors that have prevented me from getting cancer of the oesophagus. I also have the choice of withdrawing from the study at any time and this will not influence the quality of care that I receive at this hospital/clinic.

I know that this study is to help people with the disease and will not harm me. I understand that all the information that I give or that is taken from my hospital files will remain confidential.

_____ _____

SIGNATURE/THUMBPRINT WITNESS

_____

DATE

APPENDIX 9: PATIENT CONSENT FORM FOR THE CONTROLS

IMVUME YOKUTHABATHA INXAXHEBA KWISIFUNDO SOPHANDO-NZULU (UMGULI ONGENAMHLAZA WOMQALA)

INOMBOLO:

Mna _____, ndiyavuma ukuthabatha inxaxheba kwesi sifundo siqhubeka apha e _____ ngowe _____.

Esi sifundo siqulathe oku kulandelayo:

Ndizakubuzwa imibuzo ngamagosa ophando-nzulu ngendlela endiphila ngayo nokutya endikutyayo. Iimpendulo zam ziyakusetyenziswa ekwandiseni ulwazi malunga nenzuzo yohlobo lokuphila kwam ekuthinteleni umhlaza womqala.

Ndiyazi ukuba ndingarhoxa nanini na kwesi sifundo, kanjalo ukurhoxa kwam kungasayi kuchaphazela malungelo am kwiingxaki zempilo kule kliniki / kwesi sibhedlela.
Ndiyaqinisekiswa ukuba esi sifundo asinabungozi kum.
Ndiyazi ukuba esi sifundo sesoku-nceda abo sele benesi sifo yaye asinabungozi kum.
Zakugcinwa ziyimfihlo phakathi kwam kunye namagosa ophando-nzulu iimpendulo zam kunye nokufunyenwe ezincwadini zesibhedlele ngengcombolo yempilo yam.

_____ _____

INTSAYINO-GAMA/BEKA UBHONTSI INGQINA

_____

UMHLA

APPENDIX 10: RESEARCH PERSONNEL CONFIDENTIALITY AGREEMENT

RESEARCH PERSONNEL
CONFIDENTIALITY AGREEMENT

I, _____, hereby agree to uphold the confidentiality between the patients and myself. All data gathered during the interview and from their medical records will only be disclosed to persons involved in this study. The patient has accepted to be a participant in good faith and I will not act irresponsibly, but will uphold the trust that has been placed on me.

_____ _____

SIGNATURE WITNESS

_____

DATE