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GEOPHAGIC PRACTICE AND CHARACTERISATION OF PLANT REMAINS IN GEOPHAGIC SOILS IN SEKHUKHUNE AREA, LIMPOPO PROVINCE, SOUTH AFRICA

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF ECOLOGY AND RESOURCE MANAGEMENT, SCHOOL OF ENVIRONMENTAL SCIENCES, UNIVERSITY OF VENDA, IN FULFILMENT OF THE REQUIREMENTS FOR DEGREE OF **MASTER OF ENVIRONMENTAL SCIENCES (MENVSC)**.

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DECLARATION

Declaration with regard to independent work:

I, Makabudi Valery Phakoago (student number 14015177), do hereby declare that this research project submitted to the University of Venda, Limpopo Province, for the degree Master of Environmental Sciences is my own independent work. This research project was conducted at the University of Venda, Limpopo Province, under the supervision of Prof G.E. Ekosse and co-supervision of Prof J.O. Odiyo.

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I certify that this statement is correct.

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Signature of co-supervisor **Date**
Prof J.O. Odiyo

DEDICATION

I dedicate this work to my beloved parents. My father, Maswi *A*' Kanyana *A'* Morwakoma and my mother, Meta *A*' Monare A' Kanyana as well as my beloved late grandparents Monare *A*' Kanyana *A*' Ngwamorei and his wife Hlapjadi *A*' Phaahla *A*' Phogole.

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ABSTRACT

Certain communities in Sekhukhune area are known to be practicing geophagia. Existing standard questionnaires as adopted to generate data on human geophagia included aspects on demography, socio-economics, cultural, ecological, physico-chemical aspects, indigenous knowledge and health effects of geophagic consumers. These data were gathered through distribution of questionnaires to 200 geophagic participants, of which 135 were from Ga-Nchabeleng Village and 65 from Mphanama Village. Both the Villages were based in the rural settlements in Sekhukhune area. In this study the behaviour of geophagic individuals was investigated and cytotoxicity of plant remains consumed in geophagic soils was evaluated for any toxicity.

A total of 17 different geophagic sites were identified. Six geophagic sites each were from Ga-Nchabeleng Village and six from Mphanama Village. Five other sites were selected from sites where geophagia was not practiced and were recorded as the control sites. All the 17 soil samples were analysed using Munsell Soil Color charts for soil colour classification. Samples of plant remains were collected from the same geophagic sites where soil samples were collected. Due to similar plants and vegetation type from 17 different geophagic sites, a composite study was adopted. Geophagic consumers in Ga-Nchabeleng Village identified four plant species of plant remains consumed in the soil in the area and Mphanama village identified five species different from Ga-Nchabeleng plants.

Samples of plant remains were grouped according to comparable features or characteristics. Sample 1 of plant remains was composed mainly of grasses; *Cynodon dactylon*, *Aristida congesta* and *Eragrostis rigidior* whereas sample 2 of plant remains was composed of Acacia plant; *Vachellia tortilis*. The two samples of plant remains were collected from Ga-Nchabeleng Village. Sample 3 of plant remains was composed of creeping, perennial weed herbs; *Alternanthera pungens* Kunth and *Alternanthera lorentzii.* Sample 4 of plant remains was composed of prominent woody plants; *Combretum apiculutum, Kirkia wilmsii* and *Boscia albitrunca.* Samples of plant remains 3 and 4 were collected from Mphanama Village study sites. Sample 5 of plant remains was the control site and composed mainly of Acacia plants; *Vachellia nilotica*, *Acacia*

mearnsii and *Vachellia tortilis* and were collected from sites not used for geophagic practices. The plant remains parts mostly consumed were roots (50%) in Ga-Nchabeleng, whereas in Mphanama Village they were stems (54.5%) and the control site had leaves at 62.5%. The five samples of plant remains were recovered using physical separation method. The plant remains were washed and dried. Retch Muhle grinding machine was used to ground the samples. Methanol was used in the extraction of all the samples of plant remains.

The result from the administration of the questionnaire revealed that geophagia in this area was practiced by both male and female Sepedi-speaking individuals. Ga-Nchabeleng Village had more female geophagic participants, whereas Mphanama Village had more males who have almost undergone secondary school. However, in general for the study there were more female geophagic consumers. Geophagic consumers ingest soil known locally as *Mobu, Letsopa* or *Leraga* collected mostly from the riverbanks, mountains/hills and valleys with only a few that indicated termite mounds. Geophagic consumers in the study used colour, among other things, to describe their soil of preference.

The study consisted of 200 participants of whom 172 represented the geophagic group and 28 were the control group, aged between 18-65 years analysed using chi-square crosstabulation. There was no significant difference in human health effects associated with geophagia between the geophagic group and the control group. There was also no association established between soil consumption and other non-food substances between geophagic group and control group. Chi-square ($\chi 2$) analyses revealed a significant association of gender with geophagic habits ($p<0.05$), while there was no association of age, educational level, income source and marital status ($p>0.05$) with geophagic habits. Findings of the survey when two villages are combined revealed that more females (75.60%) practice geophagia compared to males (24.40%).

The respondents from both study sites preferred digging technique when collecting the soil. It was established that craving was mainly the reason behind the practice in the study area. Hygiene and environmental conditions were not considered when mining

geophagic soil as the majority of them used dirty utensils, hands for collection and non-sterile bags and tins for packaging. Some of the soils were collected close to waste dumping sites as seen whilst visiting geophagic mining sites. Majority of the consumers had little or no knowledge that the soil could be harmful or if it contained any contaminants. This sample of interviewees provided valuable information on human geophagic practices in Sekhukhune area. It became clear that this practice was entrenched in the cultural behaviour of people in the area and a need for educating them on health related aspects.

The cytotoxicity of methanolic extracts of plant remains on HEK-293T cell line was evaluated using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay for cell viability. The 50% cytotoxic concentration (CC_{50}) was defined as the compound's concentration (500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 µg/ml) required for the reduction of cell viability by 50%. Evaluation of cell viability showed the methanolic extracts of plant remains on HEK-293T cell line ranged in the order of plant remains extract=3>1>4>2>5 according to their cytotoxicity activities. Plant remains extract 3 from Mphanama Village showed high cytotoxicity with a CC_{50} of 13.75 µg/ml, followed by plant remains extract 1 at 16.68 µg/ml, plant remains extract 4 at 58.95 µg/ml, plant remains extract 2 at 92.75 µg/ml and the control at 251.4 µg/ml, respectively. In the study only the methanolic extract was investigated for cytotoxicity using HEK-293T cell line. Further research need to be conducted on the individual plants of each plant remains to be able to have conclusive results on the cytotoxicity profile. This will indicate which specific plant part is toxic or whether they exhibit a higher CC_{50} only when in combination.

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LIST OF ABBREVIATIONS AND UNITS

ATP	Adenosine triphosphate assay
A549 cells	Lung cancer cell line
cm	centimetre
CO_2	Carbon dioxide
CC_{50}	50% of Cytotoxic concentration
EC_{50}	Half maximal effective concentration
IC_{50}	Half maximal inhibitory concentration
df	degree of freedom
DMSO	Dimethyl sulfoxide
DWAF	Department of Water Affairs and Forestry
FBS	Fetal Bovine Serum
FLM	Fetakgomo Local Municipality
Fe	Iron
g	gram
GIT	Gastro-intestinal tract
GPS	Global Positioning System
HEK-293 cells	Human Embryonic Kidney 293 cell line
HEK-293T cells	Modified HEK-293 cell line expressing the SV40 large T antigen
IBM	International Business Machines
IDP	Intergrated Development Plan
IMDM	Iscove's Modified Dulbecco Medium
km	Kilometre
km^2	Square kilometer
K562 cells	Human leukemia cell line
LDH	Lactase Dehydrogenase assay
LD_{50}	50% Lethal dose
LDL	Low Density Lipoprotein
µg	Microgram
mg	Milligrams
ml	Millilitre

nm	Nanomitre
MCF-7 cells	Human breast adenocarcinoma cell line
MDA-MB468	Human breast cancer cell line
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
OD	Optical Density
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus.
PBS	Phosphate Buffered Saline
SKOV3 cells	Human ovarian cancer cell line
SPSS	Statistical Package for the Social Sciences
SV40	Simian Vacuolating virus 40
WHO	World Health Organisation
Y79 cells	Human eye cancer cell line
Zn	Zinc
µl	Microlitre
°C	Degree celsius

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CHAPTER 1
INTRODUCTION

1.1 Background of the study

Geophagia is classified as a form of pica and refers to the habitual consumption of soils and clays by humans and animals (enzootic) (Mahaney *et al.,* 1993; Ekosse *et al.*, 2010). The habit occurs in many countries worldwide, including South Africa (Abrahams, 1997; Geissler *et al*., 1998; Saathoff *et al.,* 2002; Young *et al.,* 2007; Ekosse *et al.,* 2010; Ngole *et al*., 2010). The practice has raised continuous questions to researchers, policy makers and the society at large (Aufreiter *et al.,* 1997; Woywodt and Kiss, 2002). The habit is not limited to age, gender or race (Geissler *et al*., 1998).

Geophagia is common in women of child-bearing age, especially in developing countries (Brand *et al.,* 2009). Cultural beliefs, psychological reasons, nutritional and medicinal values have been advanced to justify the practice (Callahan, 2003). In southern Africa, women believe the consumption of this soil-like substance enhances their beauty and lightens their skin (Woywodt and Kiss, 2002). In Africa, geophagia has been reported in Cameroon, Botswana, Kenya, Ghana, Guinea, Tanzania, Uganda, Nigeria, Swaziland, Senegal, Sierra Leone, Ivory Coast, Malawi, and South Africa (Aufreiter *et al*., 1997; Abrahams and Parsons, 1997; Geissler *et al*., 1998; Smith *et al*., 2000; Kikouama *et al*., 2009; Ekosse *et al*., 2010; Ngole *et al*., 2010).

Geophagic soils are considered as supplements and nutrients, and serve as homeopathic remedies for common illnesses/diseases (Reilly and Henry, 2001; Gomes and Silva, 2007). The relief effects resulting from soil consumption as well as supplementation of minerals and anti-diarrhoeal properties is most common among women indulging in the practice (Brand *et al.,* 2009; Kawai *et al.,* 2009; Bisi-Johnson *et al*., 2010). Geophagic soils are used orally to heal common illnesses of the gastro-intestinal tract (GIT) because of their medicinal properties (Tateo *et al.,* 2001; Carretero, 2002). Soil is also consumed to relieve hunger or added in the preparation of drinks and meals (Brand *et al*., 2009). Ellis and Schnoes (2006), revealed the habit may have serious health consequences. A number of health effects have been associated with

geophagic practice. The effects include anaemia, microbiological infections, heavy metal poisoning, helminthiasis, dental abrasion and intestinal obstruction (Geissler *et al.,* 1998; Callahan, 2003; Hunter, 2003; Ellis and Schnoes, 2006). Reid (1992) and Severance et al. (1988) showed an increased level of anaemia could be due to prolonged soil ingestion. These health effects could also include iron and zinc deficiencies, which were the two health risks debated amongst scholars (Reilly and Henry, 2001; Trivedi *et al*., 2005).

Apart from mineral elements, soils are reservoirs of chemical and biological agents (Bisi-Johnson *et al.,* 2010). The chemical agents are heavy metals, organic chemicals and radioactive gases. With regard to biological agents, soil is the location of numerous microorganisms and basically other higher living organisms found in these habitats (Bisi-Johnson *et al*., 2010).

Plant remains found in geophagic soils from geophagic mines may have possible health benefits to geophagic consumers. However, the remains may also contain poisonous material that could be harmful to those that consume it. Deliberate or unintentional consumption of soil-dwelling plant remains may result in various health effects such as irritable digestive system, fatal symptoms, diarrhoea and nausea (Magid, 1989).

Currently there is an ongoing, large scale investigation in South Africa (Limpopo and Free State Provinces) on geoscience concerns to health impact of human geophagic practices, trends of recent transformations of landscape based on rocks, geochemistry, mineralogy and soil geography. The investigation is led by the supervisor of this study. This work is part of the research titled “Inclusive Innovation of Indigenous Usage and Management of Soils and Clays within Traditional Africa”. The research project is supported by RFBR, N 14-05-93959 (RFBR-SA) and NRF UID 92199 project grants. A need was established to investigate and characterise human geophagic practices, soil colour and thirdly the plant remains content in geophagic soils, including the presence of harmful or beneficial toxins in plant remains consumed in soils. The study was subsequently undertaken as part of the investigation.

1.2 Problem Statement

Geophagia is prevalent in South Africa (Brand *et al*., 2009; Ekosse *et al*., 2010; Ngole *et al*., 2010) and that includes the rural population of Limpopo Province in the country (Ngole and Ekosse, 2012). Sekhukhune area in the Limpopo Province is one of the rural parts of South Africa where certain individuals within the communities in the area are known to be practicing geophagia (Ekosse *et al*., 2010). A reconnaissance survey in Ga-Nchabeleng and Mphanama Villages revealed that people consumed geophagic soils containing plant remains without knowing the constituents of the soils they are consuming. The people lack knowledge of whether the plant remains are harmful or beneficial to their well-being. These plant remains may pose health threats to the consumers. Chronic ingestion of geophagic soils containing plant remains may lead to human health implications. It was therefore, essential to investigate a selection of plant remains samples consumed in geophagic soils in the area for possible toxicity and to ascertain the risks associated with the consumption of such plants.

1.3 Rationale

Geophagic practice is widespread in the Limpopo Province and most of the consumers lack knowledge about the health effects associated with the consumption of geophagic soils. There is concern about the soil in Sekhukhune area, in which the people in certain communities have little or no idea of the health effects associated with the consumption of plant remains in soil. In addition, few studies have been documented on geophagic practices in rural communities of Limpopo Province (Ekosse *et al*., 2010). No studies have been conducted on plant remains in geophagic soils. It is therefore, essential to investigate a selection of soil samples and the plant remains for possible health effects to those who practice geophagia. The study will shed some light on risks associated with consumption of contaminated soils.

1.4 Aims and Objectives

The aim of this study was to obtain a better understanding on geophagic practices and characterisation of plant remains in geophagic soils in Sekhukhune District and infer their possible health effects. The following objectives were devised to address this aim:

a) To determine why humans consume geophagic soils in Sekhukhune area.

b) To classify the soil samples according to soil colour in order to reveal possible soil colour preferences by geophagic consumers.
c) To identify the plant remains in geophagic soil samples.
d) To investigate on possible toxicity of plant remains consumed in geophagic soils.

1.5 Research Question

a) Why do humans in Sekhukhune area consume geophagic soils?
b) Do geophagic consumers prefer soil according to colour?
c) What are the plant remains found in geophagic soils?
d) Do the plant remains in geophagic soils have any toxicity?

1.6 Summary of chapter

The chapter clearly provided the background of the study outlining the general concept of geophagia and the effects of the practice to human consumption. It has also outlined the aims, objectives, research questions and rationale of the research project. The health hazards and health benefits of geophagic practices have also been outlined.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Geophagia is the compulsive and deliberate practice of soil ingestion, and it occurs across the globe (Fallon, 2006; Erick *et al*., 2008; Perridge *et al*., 2011). The practice of geophagia is endemic and is found in many countries across the globe, including South Africa. Previous studies revealed that certain communities in South Africa are known to be practicing geophagia (Ekosse *et al*., 2010; Ngole *et al*., 2010) and the soils they consume are different. It is mostly practiced by females in developing countries with low income (Songca *et al*., 2010). It is not clearly understood why people crave for soil but cultural to religious reasons have been proposed to promote this habit (Ekosse *et al.*, 2010). The reasons for human geophagic practice include detoxification of noxious or unpalatable compounds present in the diet, (Wilson, 2003; Kikouma *et al*., 2009; Momoh *et al*., 2011); alleviation of gastrointestinal such as nausea, diarrhea and still a craving which may be traced back to nutritional deficiencies (Ekosse *et al*., 2008). Geophagia may also lead to different health complications such as constipation, cramping, pain, intestinal obstruction, microbial infections, bacterial contamination and perforation of the intestines.

2.2 Historical and contemporary perspectives of human geophagia

Human geophagia, according to Abrahams (2005), is thought to have been in existence as far back as 3500 BC around India, Egypt, Mesopotamia and China. Previous studies show evidence of when geophagic practice was initiated by humans. One of the prehistoric sites is at Kalambo Falls, the border of Zambia and Tanzania (Root-Bernstein and Root-Bernstein, 2000; Abrahams, 2005), where white powdered clay-like material was discovered adjacent to *Homo habilis* relics (Clark, 1969). The practice is considered to have originated from the African continent then migrated to other continents as humans settled in other areas (Abrahams, 2005; Hooda and Henry, 2009; Perridge *et al*., 2011). According to Woywodt and Kiss (2002), many reports were available from the 16th and 17th centuries, and the term ‘‘pica’’ was initially mentioned in the context of a surgery in that period. Geophagia was often thought as a symptom of chlorosis also

known as *Febris alba* that many girls were prone to have. It then spread and remained common in Europe during the 18th and 19th centuries and is still observed among young girls with chlorosis (malnutritional disorder or green sickness which was frequently seen in young girls and women in the 19th century) (Woywodt and Kiss, 2002).

2.3 Geophagia in Africa

Geophagia is widely spread around the world (Brand *et al*., 2009) and it is practiced mostly by children and women (George and Abiodun, 2012). Though men and children indulge in the habit, it is most common among women particularly of child bearing age in Africa (Brand *et al*., 2009). In many parts of the world women and children consume soil due to mainly craving while others consume when pregnant (Geissler *et al*., 1998). It is also believed that geophagic practice is a response to physiological needs (Shivoga and Mutori, 2008). The habit is widespread and is passed from one generation to another because of cultural beliefs, medicinal value and genuine enjoyment (Geissler *et al*., 1998).

In Africa, geophagia has been reported in 35 countries (Abraham and Parsons, 1966). Some of the countries in Africa where geophagia has been studied are Ghana, Togo (Vermeer, 1971), Cameroon (Von Garnier *et al*., 2008), (DRC) (Hunter-Adams, 2016); Uganda (Abrahams and Parsons, 1997); Botswana (Ekosse and Anyangwe, 2012), Kenya (Geissler *et al*., 1998), Namibia (Thomson, 1997), Guinea (Glickman *et al*., 1999; Luoba *et al*., 2004), Tanzania (Kawai *et al*., 2009; Young *et al*., 2007), South Africa (Saathoff *et al*., 2002; Woywodt and Kiss, 2002; Ekosse *et al*., 2010; Ngole *et al*., 2010; Perridge *et al*., 2010), Swaziland (Ngole *et al*., 2010; Peter, 2011; Ekosse and Ngole, 2012), Uganda (Abrahams, 1997), Zambia (Nchito *et al*., 2004) and Nigeria (Ademuwagun *et al*., 1979; Odewumi, 2011). But geophagia could be practiced in all African countries.

Geophagic soils across the African continent are diverse in origin. The vendors in South Africa in particular, mine their geophagic soils themselves from mountains, valleys and riversides in the wild, whereas geophagic consumers from, for example Kenya and Tanzania mine soils from termitaria (termite mounds) (Geissler *et al*., 1998; Luoba *et al*.,

2004; Young *et al*., 2010). In South Africa, particularly Limpopo Province, geophagic soils are traditionally called *mobu* (Ekosse *et al*., 2010), *munyaka* or *vumba* (Momoh *et al*., 2011) by local consumers whereas geophagic soils from Free State Province are called *sweets*, *dipompong* or *rama* (Perridge *et al*., 2010). Geophagic soils in Kwa-Zulu Natal Province, parts of the District of Mkhanyakude are called *isibomvu* and *isiduli* (Msibi *et al*., 2014). In other countries from the African continent, geophagic soils are traditionally known by different names, including *odowa* (Kenya), *ufue, mchanga* or *udongo* (Tanzania) (Geissler *et al*., 1998; Luoba *et al*., 2004, Young *et al*., 2010) and *calabash chalk* and *nwanra* (Nigeria) (Bisi-Johnson *et al*., 2013). The table below shows summarized traditional names of soils consumed from different areas and countries (Table 2.1).

Table 2.1: Names of local soils consumed.

Country (Area)		Local name	References
South Africa	Sekhukhune District	*Mobu*	Ekosse *et al*., 2010
	Vhembe District	*Munyaka* or *vumba*	Momoh *et al*., 2011
	Thabo Mofutsanyane District.	*Sweets, dipompong* or *rama*	Perridge *et al.,* 2010
	Mkhanyakude District)	*Isibomvu* and *Isiduli*	Msibi *et al*., 2014
Kenya		*Odowa*	Luoba *et al*., 2004
Tanzania		*ufue, mchanga* or *udongo*	Geissler *et al*., 1998; Young *et al*., 2010.
Nigeria (Delta state, Edo state)		*Calabash chalk*, *Nwanra*	Bisi-Johnson *et al*., 2013.

2.4 Aetiology of geophagia

Various studies have described several theoretical approaches to explain nutritional, sensory and physiological, neuropsychiatric and cultural beliefs as aspects of aetiology (Vermeer, 1971; Danford *et al*., 1982; Reid, 1992; Geissler *et al*., 1999). Sometimes geophagia is a result of emotional stress or a developmental problem (Vermeer and

Frate, 1979; Ellis and Schnoes, 2006). Nutritional theories attribute geophagic soils to specific deficiencies of minerals such as zinc and iron. It has been hypothesised that geophagic soil is used because of its high iron content although it has been shown to be an iron chelator and can aggravate the problem (Rose *et al*., 2000). The sensory and physiological finding of the practice is that people do it because they enjoy the smell, taste and texture of geophagic soils (Hunter, 1973). Geophagic soils have been used by individuals who want to lose weight and relieve themselves of nausea (Rose *et al*., 2000). A neuropsychiatric theory is supported by evidence of brain lesions from laboratory analyses showing association with certain brain disorder in humans (Sayetta, 1986).

Generally, geophagic consumers are of the opinion that their soil-eating habit is not detrimental to their health, often consuming soil for treatment of an illness, or in the belief that it will benefit their wellbeing (Knishinsky, 1998; Hunter, 2003; Luoba *et al*., 2004; Kawai *et al*., 2009; Bisi-Johnson *et al*., 2010). The communities in the countries in which geophagic studies have been conducted have advanced various reasons to justify the beliefs that geophagic soils could be harmful. In contrast, constipation and abdominal pains are some of the reasons why some geophagic consumers believe ingested soils could be harmful (Halsted, 1968; Abrahams, 1997; Geissler *et al*., 1998; Glickman *et al*., 1999; Callahan, 2003; Luoba *et al*., 2004; Abrahams, 2005; Luoba *et al*., 2005; Ellis and Schnoes, 2006; Ngozi, 2008; Von Garnier *et al*., 2008; Bisi-Johnson *et al*., 2010; Ekosse *et al*., 2010).

In addition to cultural beliefs, religious and psychological reasons, geophagic soils are believed to increase fertility (Hooda and Henry, 2009). These soils have been eaten as fertility food in European and African countries (Meel, 2012). In Africa, child-bearing women believe that ingesting geophagic soils prevent frequent vomiting in early stages of pregnancy (Diamond, 1998). However, there is no conclusive evidence to substantiate the belief (Hooda and Henry, 2009). According to Woywodt and Kiss (2002), some cultures believe that the consumption of geophagic soils makes women more attractive and improves the colour of their skin and softens it. However, there is no scientific proof that this is true, but these cultural beliefs are still propagated.

2.5 Geophagia in Women, children and men

2.5.1 Geophagia in Women

Women in both urban and rural areas practice geophagia (Ekosse *et al*., 2010), and especially those who are pregnant (Mogongoa *et al*., 2011). Pregnant women in Africa believe that geophagic material is good for fetal development. Women seek for traditional doctors to administer geophagic material to them during pregnancy. Recent studies have indicated that pregnant women in affluent societies engage also in geophagic practice (Ekosse *et al*., 2010). Ngozi (2008), found it is common in developing countries, especially African indigenous communities, and it has been linked to superstition (Halsted, 1968). In urban South Africa, young women believe the consumption of earthly material enhances their beauty and gives them a lighter colour (Woywodt and Kiss, 2002; Ekosse *et al*., 2010). Eventually, mothers who practice geophagia end up transferring the habit to their children and future generations (Vermeer and Frate, 1979; Ellis and Schnoes, 2006).

2.5.2 Geophagia in Children

Geophagia is a natural habit among infants (especially toddlers 18 to 24 months of age) and young children (2 to 6 years) (Ellis and Schnoes, 2006; Bisi-Johnson *et al*., 2010). Children ingest significant quantities of soil due to their tendency to play on the floor either indoors or on the ground outdoors. Children may ingest soil and dust through deliberate hand-to-mouth movements, or unintentionally by eating food that has fallen on the floor (Nwafor, 2008). Geophagic practice is commonly found in metabolically unbalanced, malnourished and inadequately supervised children (Garcia *et al*., 1987), but it may also occur at all levels of society (Kolandaivelu and Balan, 1979). There is little literature documented in teenage children who are involved in the practice even in developed countries (Geissler, 1997; Ellis and Schnoes, 2006). Young children are curious and explore their environment by putting their hands, objects and other materials in their mouths. Most of them try eating dirt, and only some persist with the behaviour.

2.5.3 Geophagia in Men

The practice of geophagia is more common in women than in men (Rose *et al*., 2000). In teenaged and adult males from developed countries geophagia has rarely been

documented (Geissler, 2000; Ellis and Schnoes, 2006). The habit of soil eating is not generally seen in older boys and men, because they possibly feel ashamed by practicing geophagia (Luoba *et al*., 2004). Findings of this study are supported by the study conducted by (Geissler *et al*., 1999), where women reported that men do eat soil but are very secretive about this practice. In the study of Zambian boys they were found eating soil although this decreased as they grew up (Geissler *et al*., 2000). In contrast, studies conducted by Golden *et al*. (2012), have reported that the practice of geophagia was very common amongst men from the Malagasy population. In some African tribes, men have been reported to eat clay prior to travelling long distances to engage in warfare (Schatz *et al*., 1973), for their healing powers, especially for stomach troubles while other believed that geophagic materials would bring good luck for better overall health (Golden *et al*., 2012). A study conducted by Huebl *et al*. (2016), showed men consumed soil because of craving, hypersalivation and natural stimulants.

2.6 Physical properties of geophagic soils

Geophagic soils are obtained from very specific locations such as riverbanks, termite mounds and pit areas (Reilly and Henry, 2001; Brand *et al*., 2009) and the preparation of the clays differs among the consumers as well. Once the soil is obtained it can be dried in the sun, shaped or put in an oven (Reilly and Henry, 2001). Some prefer wet clay and cold clay that is kept in the refrigerator. According to Knishinsky (1998), others mix the geophagic soils with plant materials serving as ingredients in certain drinks and dishes.

The physical properties of geophagic soils are important, according to the consumers (Brand *et al*., 2009). The texture, structure, colour, smell and taste are considered during soil collection (Young *et al*., 2007; Brand *et al*., 2009; Ekosse *et al*., 2010). Studies conducted in South Africa show geophagic consumers prefer geophagic soils that are soft, smooth, and powdery (Ekosse *et al*., 2010). Areas in the Free State (Qwa-qwa and Mangaung) where geophagic research has been conducted showed geophagic consumers preferred silky soils and in Limpopo Province (Sekhukhune and Polokwane) preference was for gritty and powdery geophagic soils (Ekosse *et al*., 2010).

Soil texture is important physico-chemical parameter which influences choice of geophagic soils (Ekosse *et al*., 2011). Soil texture refers to the size of the soil particles that make up the soil matrix that is represented by the coarseness or fineness of the soil (Olson, 1981). The soil particles differ according to sizes namely; large particles (gravel), smaller ones (sand), further smaller ones (silt) and the microscopic (clay) (Olson, 1981; Singer and Munns, 2006). Fine earth fraction has been classified as follows: sandy loam, loamy sand and sand (0.05-2.0 mm); silty soils are silty loam and silt (0.002-0.05mm); and clayey soils are clay, silty clay, silty clay loam, sandy clay, sandy clay loam and clay loam (<0.002mm) within the soil matrix (Singer and Munns, 2006; Ekosse *et al*., 2011).

Geophagic consumers in South Africa use textural feel such as grittiness, silkiness, soapiness and powdery nature of soils to decide on their palatability (Ekosse *et al*., 2010; Ekosse *et al*., 2011). Soil texture plays an important role in nutrient management because it influences nutrient and water retention capacity (Olson, 1981). Clay soil contains high water retention capacity meaning that it holds water for a longer period compared to other soil particles and has high cation exchange capacity (Poesen and Lavee, 1994). It retains cations like Ca^{2+}, Mg^{2+}, and NH^{4+} meaning that clay soil can hold negatively charged surfaces which enable it to hold nutrients for plants and microorganisms. Therefore good soil structure allows the free movement for biota and penetration of plants enables microbial activities (Bardgett, 2005).

Soil structure refers to the arrangement of soil particles into units called aggregates (Singer and Munns, 2006). An aggregate possesses solids and pore space. Aggregates are separated by planes of weakness and dominated by clay particles. Silt and fine sand particles may also be part of an aggregate. Soil structure is important because it reflects the manner in which soil was formed and other aspects of soil like infiltration rate (Olson, 1981). According to Oades (1993), soil particles usually cling together to form large aggregates. Cracks separate this aggregates and that phenomenon gives the structure of soil. Structures are differentiated in soils according to their types namely; Crumb, Granular, Sub angular blocky, Angular blocky, Columnar, Prismatic and Platy structures (Singer and Munns, 2006).

Geophagic soils are being collected by consumers from various geophagic mining sites. As a result of this, there are vast differences between the colours of the respective soils mined by consumers and the sites mined by the geophagic consumers as well as those sold by vendors. Colour is the most important criteria used by the consumers during soil collection (Nchito *et al*., 2004; Ekosse *et al*., 2010; de Jager *et al*., 2010). Colour of geophagic soil gives an outline of the chemical and mineralogical contents of the specific soil or clay (Brand *et al*., 2009). The Munsell Soil Color Book was developed to assist in the classification of soil samples. Soil classified according to this book gives a uniform interpretation of soil colour (Munsell, 2000). Geophagic consumers are very much specific about colour when selecting clay for mining (Ekosse *et al*., 2010). Different characteristics of soil colours are noted when using a Munsell color system. There is reference of colour to the *hue* referring to the colour of the sample related to red, yellow, green, blue and purple; the *value* portraying the lightness of the colour, while the *chroma* portrays colour strength (Munsell Soil Color Charts, 2000) (Figure 2.1).

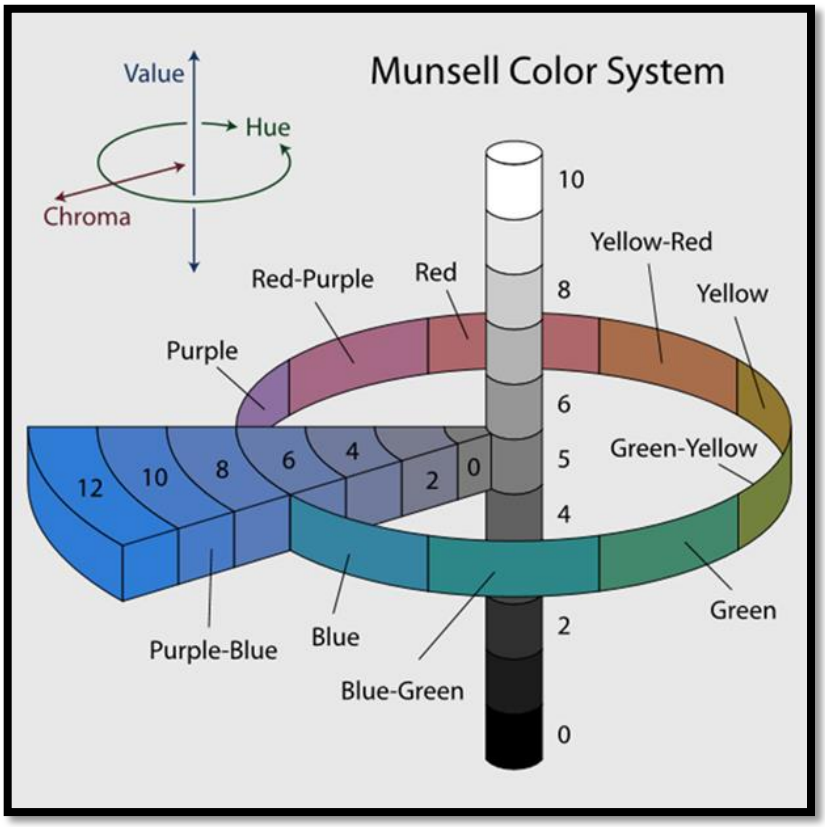

Figure 2.1: The Munsell colour system showing the positions of *hue*, *chroma* and *value* (Cleland, 2005).

The soil is furthermore dependent upon iron oxides/hydroxides and water drainage (Strydom *et al*., 2009). White clay largely consists of kaolin (Knishinsky, 1998), which has the ability to absorb toxins and bacteria and is therefore commercially processed and sold as remedy against diarrhoea (Knishinsky, 1998; Brand *et al*., 2009). Red clay is rich in oxides of iron and aluminium colour (Knishinsky, 1998). According to Knishinsky (1998), when iron is in the ferrous-oxide state it is poorly absorbed by the human body. The yellowish coloured soil contains goethite (Young *et al*., 2008; Strydom *et al*., 2009). The craving for geophagic soils could be attributed to deficiency of nutrients or minerals such as iron, zinc or calcium. It may serve as a meal replacement (Young *et al*., 2008; Mogongoa *et al*., 2011). In addition other whitish geophagic soils contain quartz and kaolinite. The other soils contain smectite and calcite in smaller quantities (Ekosse *et al*., 2010).

Geophagic consumers prefer mostly the white and dark grayish brown soils that geophagic consumers buy. Most geophagic soils from South Africa are whitish, grayish or khaki because of Kaolin, smectite, and calcite; and others from Swaziland are reddish or yellowish due to hematite and goethite contained in them (Ekosse *et al*., 2010; Ngole *et al*., 2010). The colours obtained for geophagic soils from Botswana are similar to those reported for samples studied in South Africa and Swaziland which were greyish to yellowish and those from Swaziland were greyish to reddish. South African geophagic soil is generally classified as greyish to yellowish in colour (Ngole *et al*., 2010). Studies conducted in Zambia, brown earth and white clay were mostly favoured (Nchito *et al*., 2004). In Tanzania, white, brown to reddish geophagic soils are mostly consumed (Young *et al*., 2010). In Uganda, other soil colour found was dark brown (Hooda *et al*., 2002); while pregnant women in Kenya preferred yellowish geophagic material dug from excavation sites (Ngozi, 2008).

Soil consumers are very choosy when collecting soil for consumption as they use criteria such as smell and taste (palatability) of the soil (Reilly and Henry, 2001). Among other common reasons forwarded by soil consumers, the attractiveness or smell of the soil has been the reason for the practice (Young *et al*., 2007). It has also been noted that some women practice geophagia simply because they like the smell and taste of soil (Geissler

et al., 1999; Reilly and Henry, 2001; Simpson *et al*., 2000; Msibi *et al*., 2014). Some of geophagic consumers prefer the smell and taste of soil after rainy days. Desire for soil is claimed to be more intense and informants noted that the smell of soil after rain heightens the desire (Vermeer and Frate, 1979). Others prefer their soil when heat treatment is done through boiling, baking and burning to improve the smell and taste of geophagic soils (Ekosse *et al.,* 2010).

2.7 Mining

In Limpopo and the Free State Provinces of South Africa, geophagia is widely practiced. Other vendors that prefer not to mine their own soils obtain soils from traditional miners who mine and live in the country side in the two provinces. The consumers obtain geophagic soils from river beds, termitaria, valleys and excavation sites. Different techniques are used to mine the soils, namely; digging, hand grabbing, scraping, and selective hand picking (Ekosse *et al*., 2010). Grinding, sieving and slurrying are other methods of processing particular geophagic soils. Geophagic consumers generally prefer geophagic soils that are soft, sticky, silky, soapy or powdery (Ekosse *et al*., 2010).

Geophagic consumers are very selective when digging soils. They tend to avoid the topsoil which might have contaminants and mine soil around 25-75 cm below the surface area (Vermeer and Frate, 1979; Hunter 2003). Through low scale selective traditional mining techniques, miners seek for whitish, khaki and yellowish geophagic soils with goethite, smectite and/or with kaolinite contents (Ekosse *et al*., 2010). However, that is contrary to young children, as they consume any contaminated topsoil, which may result in health implications such as infectious diseases (Callahan, 2003; Young *et al*., 2008; Ekosse, 2010).

There are certain tools that miners use when digging geophagic soils. They use various utensils such as hoes, spades, shovels, forks, pickaxes, machetes, crowbars and cutlasses (Ekosse *et al*., 2010). The tools are used according to the location where the digging is occurring and the type of mining on the specific area (Ekosse *et al*., 2010). The tools are also used by traditional potters in sub-Sahara Africa (Gosselain, 1999) and traditional healers in South Africa. Some of the miners use very unusual tools such as

dry sharpened sticks, broken bottles and edges of used cans. Forks, pickaxes and crowbars are used for digging while hoes, spades and cutlasses are used for scrapping. Shovels are used to gather the dug and scrapped geophagic soils after mining (Ekosse *et al*., 2010). Where there are visible impurities and visually undesirable earthly material, alternative hand grabbing is used to remove unwanted material on geophagic soils (Ekosse *et al*., 2010). The mining of the geophagic soils is achieved through surface collection, pit extraction or gallery and these techniques are similar to those used by potters in Africa (Gosselain and Smith, 2005).

After mining geophagic soil, it goes through numerous processes before consumption. Pounding, sieving, slurrying and grinding processes are some of the procedures conducted before consumption (Ekosse *et al*., 2010). Geophagic consumers also perform heat treatment by boiling, baking and burning unwanted materials that may be on geophagic soils (Reilly and Henry, 2001; Ekosse *et al*., 2010; Young *et al*., 2010). Heat treatment improves the palatability of geophagic soils, reduces the moisture content, enhances the desirable colours and may completely remove potential pathogens such as bacteria from these soils (Hunter 2003; Ekosse *et al*., 2010).

2.8 Plant remains in soils

Plants have been used for decades for health benefits and to treat several different diseases (Lukhele, 2016). The use and consumption of plants could be traced as far back as the beginning of human civilisation (Gupta *et al*., 2010). About 80% of the world's population relies on plants for different reasons (Jamison *et al*., 2006; Koduru *et al*., 2007; Mainardi, 2009). Many communities are still dependent on plants for human consumption, as medicine and to maintain their mental and physical health (Jamison *et al.,* 2006; Mainardi, 2009; Street and Prinsloo, 2012). However, some of the plants used by these communities are not known to be beneficial and their safety is still unclear.

Plants and plant remains extracts have been widely explored for their therapeutic activities against most cell lines (Patel and Tikoo, 2006; Nawab *et al*., 2011). But virtually research has not been done on plant remains in geophagic soils consumed by humans around the globe. Therefore, it is important to scientifically evaluate and validate their

effectiveness and safety specifically to those consuming them. Plant remains are any or all pieces, parts or fragments of dead plant materials that have been excavated from the soil. These remains are often in two main categories: namely, macrobotanical and microbotanical remains (Magid, 1989). The remains are usually found in arid regions with dry clay or soils. Although the study was primarily on plant remains in geophagic soils, the main focus was specifically on macrobotanical plant remains.

2.8.1 Macrobotanical remains

The macrobotanical remains are those which can be seen by the naked eye and their identification usually requires no more than low power microscope. The remains consist of large fragments or pieces of dead plant parts such as stems, roots, wood, leaves, and flowers (Ford 1979; Pearsall, 2000; Fritz, 2005).

Stems

Stems are the basic organs, as they give rise to other plant organs, such as roots, leaves, and flowers and some of these stems are specialized underground stalks (Jones and Luchsinger, 1986). Stems have nodes, buds, leaves and underground stems retain many of these features. According to Hather, (1994) stems are known by a variety of names that designate specific forms. Thus rhizomes are horizontal underground stems and tubers are swollen storage organs as the ends of some rhizomes (Jones and Luchsinger, 1986). Corms are short, upright, solid bulb-like underground stems covered by thin, dry leaves, whereas bulbs are short, underground stems with thick, fleshy leaves. Bulbils are small bulbs that arise from the base of a larger bulb and bublets are small bulbs borne above ground (Harris and Harris, 2001). Stolons form runners that grow horizontally and root at the nodes or at the tip.

Roots

Roots are vegetative storage organs consisting largely of parenchyma cells, whose primary functions are to anchor the plant, absorb water and store organic products (Gifford and Foster, 1989). The concentration of starch in these tissues makes them important sources of food for many organisms. Roots are used by people as sources of

food and also for medicinal purposes. Some distinctions among roots reflect differences between monocotyledons and dicotyledons (Gifford and Foster, 1989).

Roots are the most underground extensions of the main axis of the plant that are recovered from geophagic soils (Pearshall, 2000). These include tap roots, which are vertical roots that may produce many smaller roots (rootlets). The roots have no leaves, buds, or eyes (nodes), though rootlets leave secondary root scars (Hather, 1994). Roots are most likely to be recovered from permanent dry sites where the entire root was discarded (Hather, 1994). In some cases it is the outer layer or peel that survives, instead of the entire storage organ.

Wood

Wood tends to be the most ubiquitous of the macrobotanical remains. Wood is the secondary xylem of woody plants constituting the major permanent tissue of stems and roots (Gifford and Foster, 1989). Remains of seeds, fruits, nutshell and tubers are typically used to infer diet and subsistence strategies (Dark, 2004).

Leaves

A leaf is an organ of a vascular plant and is the principal lateral appendage of the stem. The leaves and stem together form the shoot. Foliage is a mass noun that refers to leaves collectively (Howard, 1974). Leaves are the primary photosynthetic organs of plants, and are borne only at the nodes of a stem (Pallardy, 2010). Mature leaves of monocots usually broadly consist of a linear lamina (blade) and flat and is supported by a petiole (except when sessile), and may be either simple or compound (Pallardy, 2010). Some plant species possess compound leaves called leaflets that are borne either on a central stem-like axis (Howard, 1974; Rudall, 2007).

On either side of the petiole or leaf base, there are often stipules subtending the leaf; these may be leaflike or modified as bristles or sheaths. In the axil, or angle between each simple or compound leaf and stem, there is an axillary bud that will develop into a shoot system (branch) if not inhibited by plant hormones (auxins). Leaves that persist on a plant for several years or growing seasons are called evergreen, whilst those that fall after one growing season are deciduous. Leaves vary in shape, margin, apex, base, and

vestiture (refers to covering of glands or hairs. It is the surface is smooth, without hairs or glands, the condition is called glabrous) (Rudall, 2007).

Flowers

The flower is a modified shoot structure concerned with sexual reproduction (van Wyk and Malan, 1998). The flowers are arranged in different types of inflorescences and they exhibit enormous variation in structure, symmetry, position of ovary in relation to other parts. After fertilization the ovary is converted into fruits and ovules into seeds (Pallardy, 2010). Flowers may be borne on leaves as single flowers or in inflorescences and have been described as epipetiolous (Howard, 1974, Pallardy, 2010).

2.8.2 Microbotanical remains

The microbotanical remains are those that cannot be seen by the naked eye and their identification requires high power microscope. The remains consist of fragments of pollen, phytoliths and starch grains (Pearsall, 2000; Fritz, 2005).

Pollen

Pollen is one of several kinds of very small plants (Bryant and Holloway, 1983). Pollen refers to actual pollen that has been preserved due to its extremely small size and tough silica shells that encase most pollen (Pearsall, 2000; Fritz 2005). It forms in the anther or what comprises the male organ of reproduction in seed-bearing plants (Bryant and Holloway, 1983; Pearsall, 2000). Spores, the asexual reproductive cells of fungi, ferns, and some algae are traditionally included in pollen analysis. Pollen collected from middens often indicates the types of plants collected and utilised for food or other economic purpose.

Phytoliths

Phytoliths is another type of microbotanical remains that are produced when certain higher plants absorb silica in a soluble state from ground water, which is then deposited in intracellular and extracellular locations in the epidermal tissues of stems, leaves and roots (Esau, 1965; Pearsall, 2000; Piperno, 2006). The silica solidifies as phytoliths or discrete, microscopic particles of varying sizes and shapes that are consistent with a family, genus, or species of plant. After the death and decay of the plant the phytoliths

are deposited into soils and sediments. Phytoliths can be common in hearths and ash layers but they can also be found in clay. Phytoliths are inorganic, thus they survive in a well-preserved state over long periods of time. Piperno (2006) the phytoliths are the most durable terrestrial plant fossil known to science. It is precisely their ability to withstand many of the rigors of nature that affords knowledge about plant use in regions where the recovery of macro-remains has been poor.

Starch grains

Starch is the major food reserve of higher plants in the geophagic soils, although it is also found in fungi, algae and other organisms (Badenhuizen, 1965; Banks and Greenwood, 1975; French 1975 and 1984; Shannon and Garwood, 1984). Two main forms of starch are important for geophagic analyses, classified by their function and location within the plant: transitory starch, which is found chiefly in leaves and acts as an ongoing plant energy source, and storage starch. Storage starch is formed in granules within specialised plastids known as amyloplasts, which are found in seeds, roots, tubers, corms, fruits and rhizomes. In these parts of the plant, starch acts as a long-term energy storage device, a source of nutrients that allows a plant to survive during unfavourable conditions and a carbon source during such processes as germination (Haslam, 2004).

Apart from minor non-carbohydrate components, all starch is composed of glucose molecules (as is cellulose, the structural component of wood) and intact granules are insoluble in cold water. The glucose molecules are formed into two different chains within starch, a linear chain (amylose) and a highly branched chain (amylopectin), with amylopectin typically forming 70-80% of storage starch (Haslam, 2004). This ubiquity of plant remains in geophagic soils reflects the importance of starchy plants in the human geophagic consumers' diet throughout human history (Banks and Greenwood, 1975; Hather, 1994; Wang *et al*., 1998). The nutritional value of the sugars in starch, along with the presence of large quantities of starch in the seeds, roots, corms, rhizomes and tubers of plants such as potatoes, maize, rice, and yams has contributed to the dominance of starches in many past and present diets (Haslam, 2004).

2.9 Positive health effects of plants consumption

Plants are irreplaceable food resources for humans diet and therefore, their nutritive value is important (Indrayan *et al*., 2005; Bennett, 2010). Plants contain almost all of the mineral and organic nutrients established as essential for human nutrition, as well as a number of unique organic phytochemicals that have been linked to the promotion of good health (Grusak and DellaPenna, 1999). The plants in geophagic soils consumed may have health benefits to the consumers. The benefits could be an indication of the contribution of food to the nutrient content of the diet (Mtunzi *et al*., 2012). Phytochemicals are non-nutritive substances in plants that possess health protective benefits. The main phytochemicals in plants are called flavonoids, and they have extensive biological properties that promote human health.

The phytochemicals in grains reduce the risk of cardiovascular diseases and cancer (Winston, 1997). They extend the activity of vitamin C, act as antioxidants and protect Low Density Lipoprotein (LDL) cholesterol from oxidation to unsafe cholesterol oxides (Winston, 1997). They also contain anti-inflammatory and antitumor properties. Most of these flavonoids are found in the color pigmented part of the plant; therefore, different colored plants will provide protection in different ways. Carotenoids act by fighting against free radicals in the body that enter in on a daily basis. Free radicals cause our skin to wrinkle and cause slow damage to many other parts of the body. Phytochemical also provides protection against oxidative damage and stimulates immune function. Oxidative damage is what we know as the slow aging process of our body. The consumption of plants high in phytochemicals thus has a significant benefit to our health (Winston, 1997).

Plants contain chemical compounds that may or may not contain minerals such as calcium, iron, magnesium and zinc (Katzmarzyk and Waist, 2004). Minerals are naturally-occurring chemical elements that the body uses to help perform certain chemical reactions. Minerals form an integral part of functionally important organic compounds such as iron (Fe) in hemoglobin and zinc (Zn) in insulin. They are essential for the normal functioning of muscles, the heart, nerves and in the maintenance of body fluid

composition among others, as well as for building strong bones (Chaney, 2006; Mtunzi *et al*., 2012).

2.10 Negative health effects of plants consumption

Although plant consumption is an important part of human life, there are various risk factors associated with it. Humans have a long history of consuming the harmful products of environmentally-tolerant plants with toxic potential (Spencer, 1994). Toxic plants can adversely affect every organ system and pose a risk to human health (Diaz, 2011). Considering how soil is the reason we are able to sustain ourselves, the contamination of it has major consequences on our health. Plants grown on polluted soil absorb much of the pollution and then pass these on to individuals that consume them. This could explain the sudden surge in small and terminal illnesses. Long term exposure to such soil can affect the genetic make-up of the body, causing congenital illnesses and chronic health problems that cannot be cured easily. Plants with extensive acute toxicity are in general already recognized as dangerous because of the historical incidents of poisonings (Kristanc and Kreft, 2016). The most harmful plant substances are neurotoxins, which are followed by cytotoxins and metabolic poisons that disturb the structural integrity and functions of the internal organs, such as the liver, heart, kidneys, gastrointestinal system and lungs (Vandenberg *et al*., 2012). This study investigated cytotoxic effects of plant remains in geophagic soils.

2.11 Cell line

A cell line is defined as a permanently established cell culture that will proliferate indefinitely in appropriate fresh medium and space (Harrison *et al*., 1907; Allen *et al*., 2005; Lucey *et al*., 2009). Cell lines differ from cell strains in that they have absconded the Hayflick limit and become immortalised (Hayflick and Moorhead, 1961; Hu *et al*., 2015). The Hayflick limit (or Hayflick Phenomenon) is the number of times a normal cell population will divide before it stops, presumably because the telomeres reach a critical length (Hayflick and Moorhead, 1961). A cell line arises from a primary culture at the time of the first successful subculture (Ormos, 2015). The terms finite or continuous are used as prefixes if the status of the culture is known (Hayflick and Moorhead, 1961; Chatterjee, 2007; Skloot, 2013; Freshney, 2010).

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favourable artificial environment (Ormos, 2015). The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established. Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology of cells, the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis (Gey *et al*., 1952; Freshney, 2010; Ormos, 2015). It is also used in drug screening and development, and large scale manufacturing of biological compounds. The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells (Allen *et al*., 2005; Freshney, 2010).

2.11.1 Cell line as in vitro models

In vitro model are studies conducted using components of an organism isolated from their usual biological surroundings such as cells (Allen *et al*., 2005; Patel and Patel, 2011; Swiatek *et al*., 2013). These cells are studied in culture media using test tubes, flasks, petri dishes and other similar test tube experiments. Examples of *in vitro* studies include the isolation, growth and identification of cells derived from multicellular organisms in cell or tissue culture (Jacobson and Piper, 1986; Young *et al*., 2004; Allen *et al*., 2005; Braydich-Stolle *et al*., 2005).

2.11.2 History of cell culture

Cell culture can be traced back to the 16th century (Harvey, 2001). A scientist Sir William Harvey, observed that a piece of myocardium kept in the palm of his hand covered in his own saliva, could remain contractile for extended periods of time (Harvey, 2001). Sir Roux in the late 1885 showed that embryonic chick cells can be maintained alive in a saline solution outside the animal body (Rensberger, 1998). Another scientist named Ross Harrison back in 1907 also discovered that neuronal cells could be cultured *in vitro* (Harrison *et al*., 1907; Phillips, 2014). The discovery by Harrison was among two types of cells used within cell culture that included primary and established cell lines (Harrison *et al*., 1907; Ahmadi *et al*., 2016). Sir Harrison later on in 1910 showed that amphibian

spinal cord could be cultured in a lymph clot, demonstrating that axons are produced as extensions of single nerve cells (Harrison, 1910).

In 1910 another scientist named Rous observed for the first time that indications of cell transformation by successfully inducing a tumor using a filtered extracts of chicken tumor cells, which later showed to contain an RNA virus (Rous sarcoma virus) (Rous, 1910; Allen *et al*., 2005). This landmark of cell culture discovery prompts the notion of *in vitro* immortalisation, and demonstrated the concept of enhanced tissue survival in culture, and the possibility of producing cell models. By the middle of the 20th century, Earle and colleagues isolated single cells of the L cell line and showed that they form clones of cells in tissue culture (Earle, 1943). Later, Gey and colleagues established probably the most commonly continuous cell line derived from a human cervical carcinoma, which later become the well-known HeLa cell line (Jones *et al*., 1971).

2.11.3 Types of used cell lines as in vitro models

HeLa cell line

HeLa was the first human cell line established (Gey, 1952) and has since become the most widely used human cell line in biological research. Its application as a model of living organism has contributed to the characterisation of important biological processes described in more than 80 000 publications (Lee *et al*., 2015; Fabbri, 2015). This cell line originated from a cervical cancer tumour (adenocarcinoma of the cervix) of a patient named Henrietta Lacks, who died of cancer in 1951 (Lasso, 2011). The cells were named ‘‘HeLa’’ after the initial two letters of Henrietta Lacks’ first and last names (Culliton, 1974; Gold, 1986; Lucey *et al*., 2009). One of the earliest uses of the cells was to develop the vaccine against the polio virus conducted by Sir Enders and colleagues (Scherer *et al*., 1953; Harvey and Hopkins, 1976).

Since then, more efforts to grow other normal cervical epithelium or cervical carcinoma in culture proved unsuccessful (Jones *et al*., 1971; Lucey *et al*., 2009) however; efforts to grow cells from the aggressive adenocarcinoma of the cervix that had affected Henrietta Lacks were successful. These cells have since contributed to many fundamental scientific breakthroughs till today and have been employed to investigate cancer, AIDS

mechanisms (Berg, 1991) and the effects of drugs, toxins and radiation (Murray *et al*., 2004). The HeLa cell line has been discovered to survive in culture after countless passages, cell divisions, and viral infections (Lucey *et al*., 2009). The widespread use of this cell line is mainly due to the easy handling and manipulation in different conditions. Figure 2.2 below shows HeLa cell line when viewed on a microscope slide.

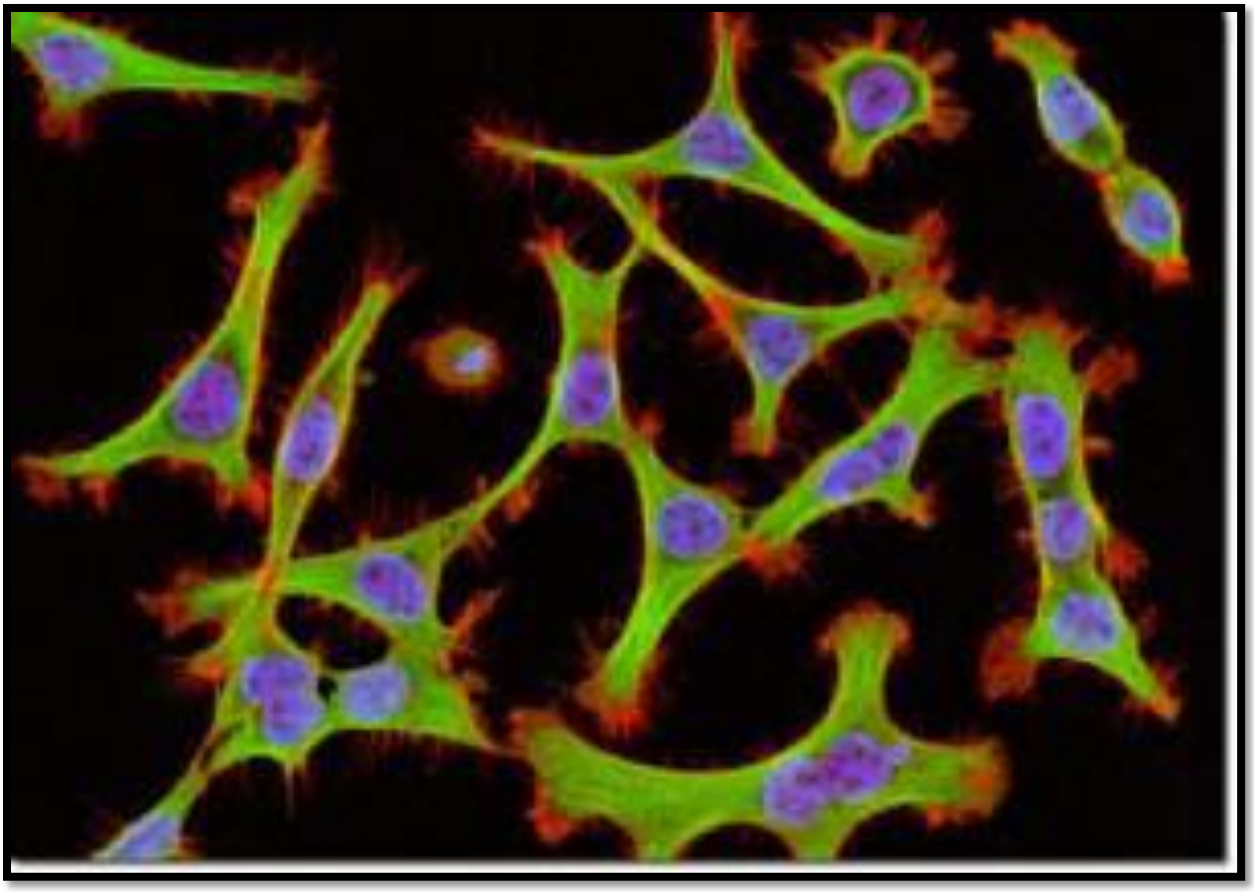

Figure 2.2: Microscopic view of HeLa cell line on a slide.

Human breast adenocarcinoma cell line (MCF-7)

Human breast adenocarcinoma cell line well known as MCF-7 is another type of cancer cell line commonly used and it serves as an excellent *in vitro* model (Ashidi *et al*., 2010). The name Human breast adenocarcinoma cell line was derived from Helen Marion who in 1963 had a mastectomy for a benign tumor in her breast and in 1967 underwent a radical mastectomy for an adenocarcinoma in her left breast (Soule *et al*., 1973). In 1970 Marion developed metastatic disease to the pleura and chest wall and researcher Herbert Soule from Machigan Cancer Foundation developed a cell line from an excision

of chest wall nodule and pleural effusion of Marion (Soule *et al*., 1973). The cells from chest wall nodules overgrown but the cells from pleural effusion grew in suspension and ultimately formed a monolayer and it grew as a continuous culture till today (Soule *et al*., 1973). Thus, the resulting cell line is presently referred to as MCF-7, named after the Michigan Cancer Foundation, and represents Soule's seventh attempt at generating a cancer cell line (Lee *et al*., 2015).

To date there have been approximately 25 000 published reports on this cell line. It is mostly used for studying cytotoxicity, the mechanism of tumour response as well as complex relationships between binding and biological actions of hormones (Ashidi *et al*., 2010; Ali *et al*., 2014; Kumaran *et al*., 2014; Cheshomi *et al*., 2016). MCF-7 cells have the ability to process estrogen, in the form of estradiol, through estrogen receptors in the cell cytoplasm makes the MCF-7 cell line an estrogens receptor (ER) positive control cell line (Brooks *et al*., 1973; Novaro *et al*., 2003; Dillon *et al*., 2010). Figure 2.3 below shows the MCF-7 cell line when viewed under an electronic microscope.

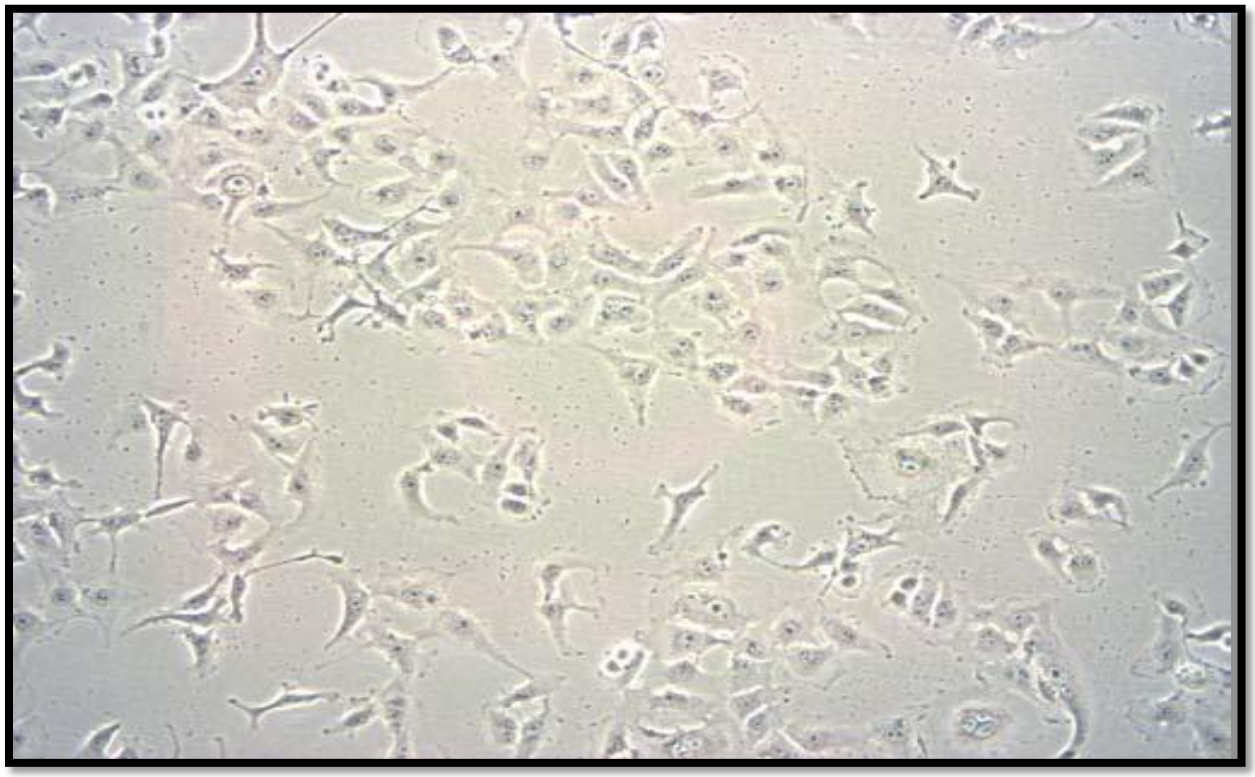

Figure 2.3: Microscopic view of a MCF-7 cell line under an electron microscope (https://www.mybiosource.com/prods/Cell-Line/MCF-7).

U87 cell line

The cell line U87 was established in 1966 at Uppsala University in Sweden using tissue from a 44 year-old woman with an aggressive brain cancer known as glioblastoma. It is a human primary gliblastoma cell line formerly known as U-87MG abbreviated for Uppsala 87 Malignant Glioma (Allen *et al*., 2016). The U87 cell line has since been used in countless investigations that have yielded around 2,000 scientific papers (Pastore *et al*., 2004; Princen *et al*., 2004; Yu *et al*., 2008; Edo-Matas *et al*., 2011; Dolgin, 2016; Oh *et al*., 2017). However, there is no clear origin of the U87 cell line; it remains a mystery and is still under investigation (Clark *et al*., 2010). Figure 2.4 below shows the U87MG when viewed under an electron microscope.

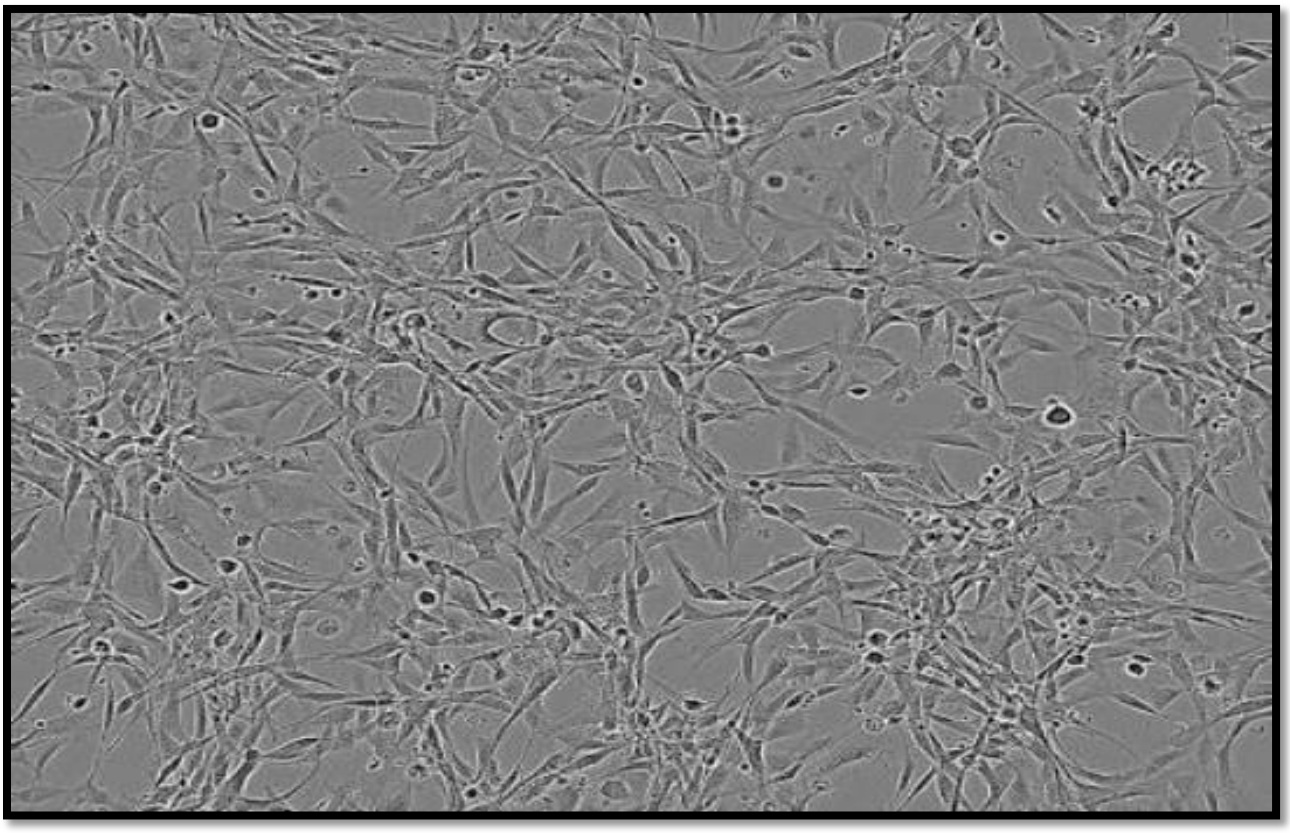

Figure 2.4: Microscopic view of U87MG under an electron microscope (Allen *et al*., 2016).

SKOV-3 cell line

A human ovarian cancer cell line commonly known as SKOV-3 was derived from the ascites of a 64-year-old Caucasian female with adenocarcinoma of the ovary in 1973

(Hua *et al*., 1995). These cells are resistant to tumor necrosis factor and to other cytotoxic drugs including diphtheria toxin, cisplatin, and adriamycin. The SKOV-3 cell line is also able to grow in soft agar, an indicator of transformation and tumorigenicity, and displays a relatively high colony forming efficiency. In vivo, SKOV-3 cells can form moderately well-differentiated adenocarcinoma consistent with ovarian primary cells. The SKOV-3/Luc cell line stably expresses firefly luciferase gene and Neomycin resistant gene (Hua *et al*., 1995). Figure 2.5 below shows the SKOV-3 cell line when viewed under an electronic microscope.

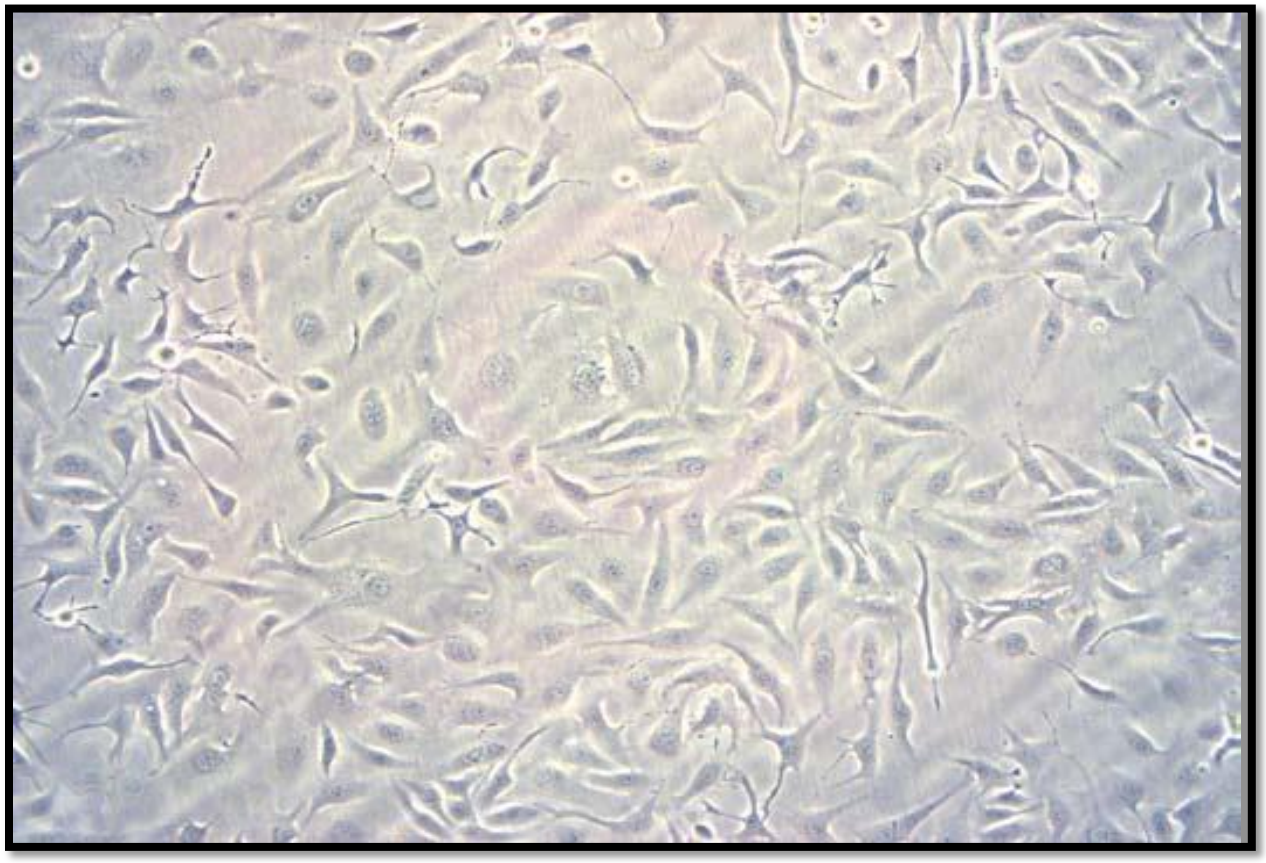

Figure 2.5: Microscopic view of SKOV-3 cell line under an electron microscope (https://www.mybiosource.com/prods/Cell-Line/SKOV-3).

Vero cell line

The Vero cell line was derived from the kidney of an African green monkey (*Cercopithecus aethiops*) in 1962, by scientists Y. Yasumura and Y. Kawakita at the Chiba University in Japan (Yasumura and Kawakita, 1963). This cell line is one of the

most common mammalian continuous cell lines used in research (Ammerman *et al*., 2008). It has also been used extensively for virus replication studies and plaque assays and also been used in many other applications including intracellular bacteria, parasites, assessments of the effects of chemicals, toxins and other substances on mammalian cells (Govorkova *et al*., 1996; Zamboni *et al*., 2001; Masoko *et al*., 2007; Ammerma *et al*., 2008; Hegde *et al*., 2008; Swiatek *et al*., 2013; Senthilraja and Kathiresan, 2015). Vero cells are sensitive to infection with SV-40, SV-5, measles, arboviruses, reoviruses, rubella, simian adenoviruses, polioviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses and vaccinia (Senthilraja and Kathiresan, 2015). There are also other several lines of Vero cells available namely; Vero 76, Vero E6, Vero C1008, and CCL-81 Vero but they derive from the same source Vero (Ammerma *et al*., 2008). Figure 2.6 below shows the Vero cell line when viewed under an electronic microscope.

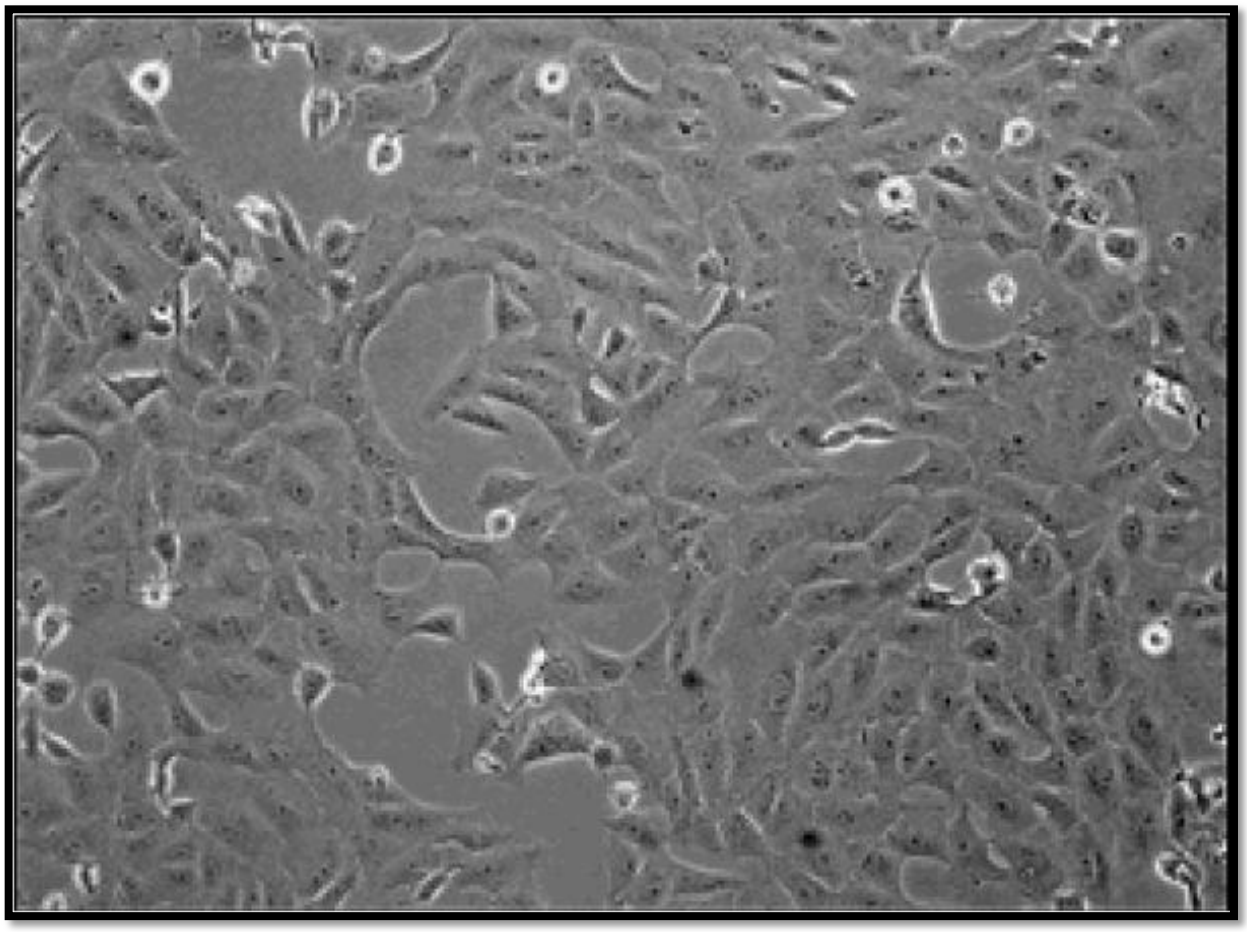

Figure 2.6: Microscopic view of Vero cells under an electron microscope (Ammerma *et al*., 2008).

A549 cell line

The Human lung adenocarcinoma cell line commonly known as A549 from the American Type Culture Collection (ATCC, Rockville, MD) is another cell line used as *in vitro* model and it was established in 1972 (Swain *et al*., 2010). The cell line was derived from a Type II pneumocyte lung tumor a pulmonary adenocarcinoma in a study attempting to establish continuous cell lines from 200 different tumours (Giard *et al*., 1973; Swain *et al*., 2010). It has characteristic features of Type II cells of the pulmonary epithelium, cytoplasmic lamellar bodies and apical microvilli (Lieber *et al*., 1976). Since then, A549 cells have been used for *in vitro* studies of surfactant production, cytotoxicity and regulation of surfactant systems (Salmona *et al*., 1992; Lestari *et al*., 2005; Ashidi *et al*., 2010; Patel and Patel, 2011). This cell line has been extensively used in cell biology as a model and it had been referenced in a number of research papers (Swain *et al*., 2010; Patel and Patel, 2011; Sypniewski *et al*., 2013). Figure 2.7 below shows the A549 cell line when viewed under an electronic microscope.

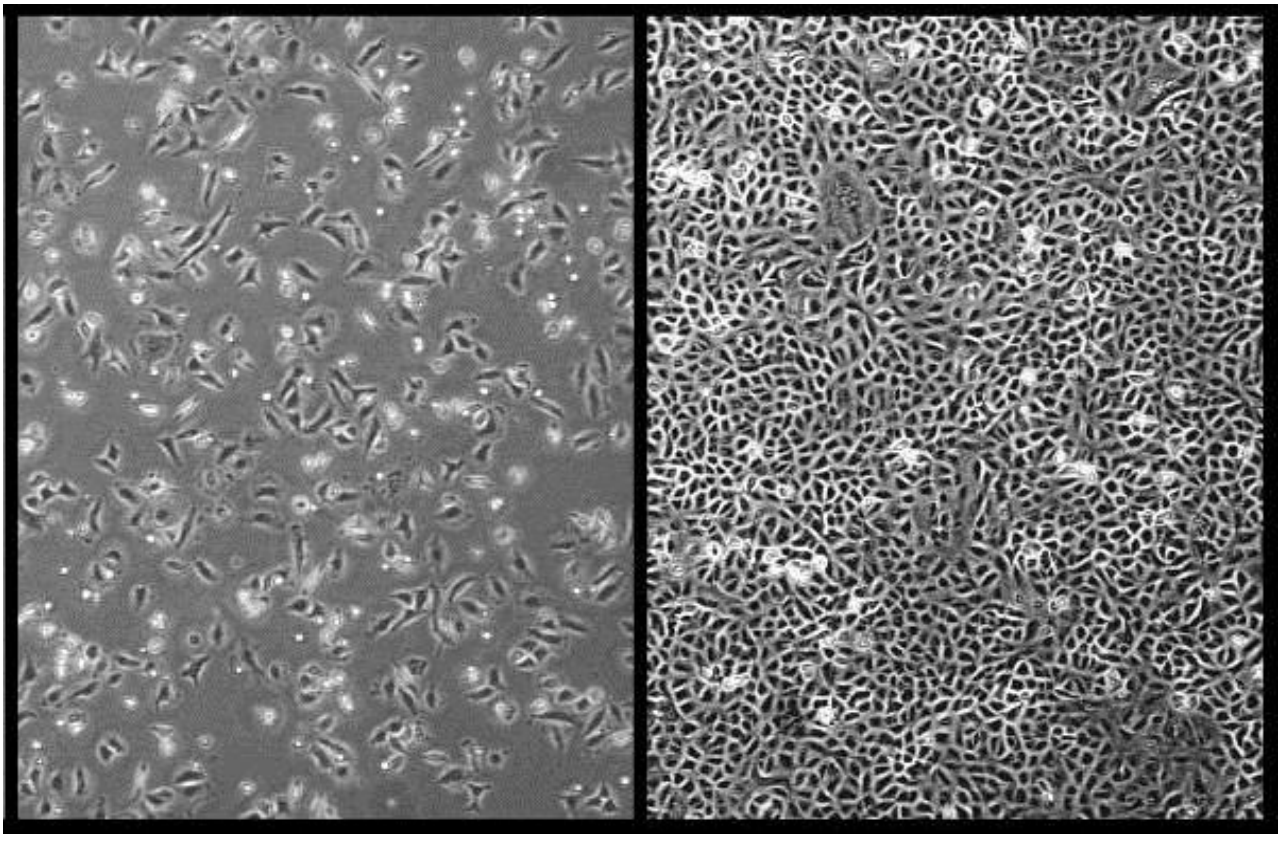

Figure 2.7: Microscopic view of A549 cell line under an electron microscope (Honma *et al*., 1996).

HEK-293T Cell line

The cell line Human Embryonic Kidney (HEK-293) was cultured by a scientist Alex van der Eb in the early 1970s at his laboratory in Leiden and published in 1977 (Graham *et al*., 1977). The source of the cells was a healthy legally aborted fetus under the Dutch law (Graham *et al*., 1977). The name HEK-293 is thusly named because it was Frank Graham's 293rd experiment (Graham *et al*., 1977). Exposure of Human Embryonic Kidney cells to sheared Adenovirus Type 5 fragments yielded the immortalised HEK-293 cell line (Graham *et al.*, 1977), which contains the adenoviral transcription units' early region (Louis *et al.*, 1997). The transformation resulted in the incorporation of approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells (Graham *et al*., 1977). HEK-293 cells are the parental line for HEK-293T. Though HEK-293 and HEK-293T cells have not been thoroughly characterised, they have been widely used in many areas of research in the technique of cell culture (Philips, 2014). Figure 2.8 below shows the HEK-293 cell line when viewed under an electron microscope.

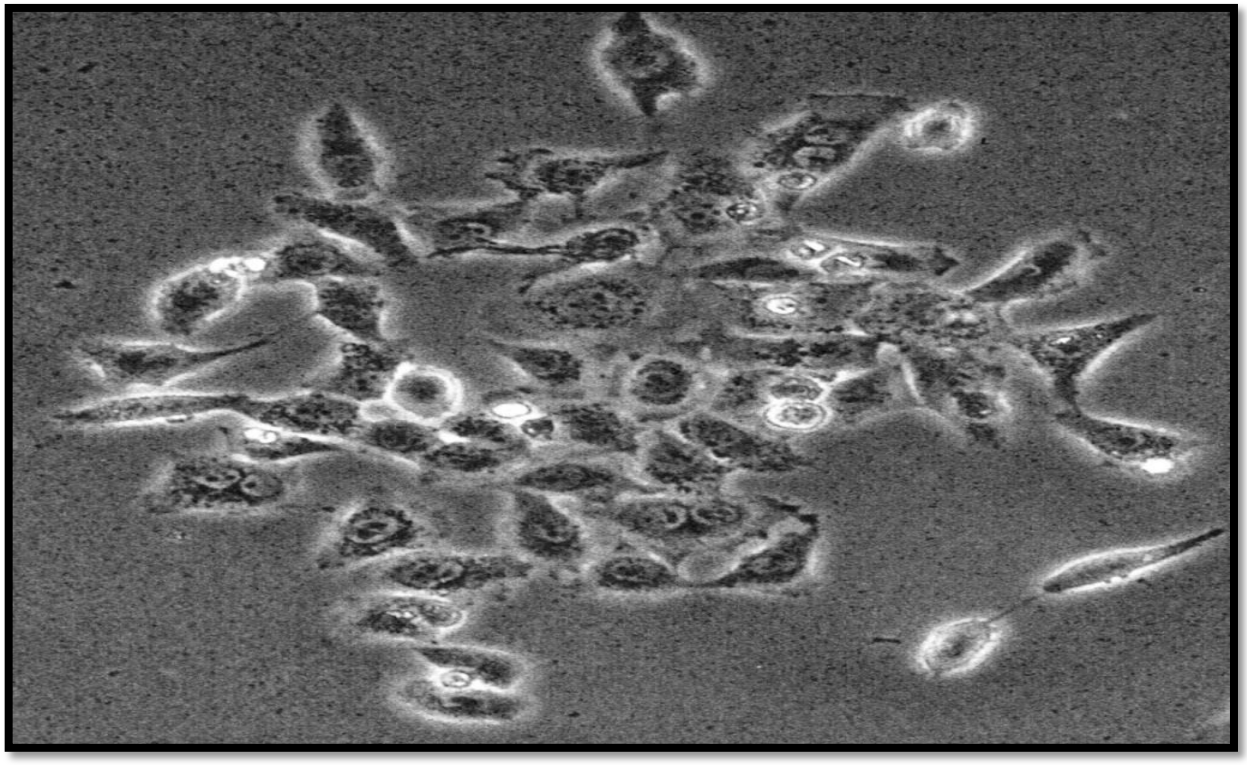

Figure 2.8: Microscopic view of HEK-293 cells under an electron microscope (http://www.wikiwand.com/en/HEK_293_cells).

HEK-293T is an HEK-293 derived cell line that expresses a temperature-sensitive allele of the Simian Vacuolating virus 40 (SV40) large T-antigen, which is important for replicating to the high copy number in the transfection cell (Rio *et al*., 1985; DuBridge *et al*., 1987). Expression of the T-antigen in the HEK-293 as compared to HEK-293 has been reported to improve titres by around 4 to 10 fold (Merten *et al*., 2010; Ausubel *et al*., 2012), and can enhance proliferation of cultured human cells (Bednarz *et al*., 2000; Lin *et al*., 2014).

The SV40 origin of replication is present on our lentiviral packaging and vector constructs and therefore should be transfected into HEK-293T cells for virus production. Parental 293 cells do not contain the T antigen and would not produce sufficient titers (Lin *et al*., 2014). HEK-293 cells have been widely used because of their reliable growth and propensity for transfection (Ahmadi *et al*., 2016). HEK-293T is the most commonly used cell derivative (Cockrell and Kafri, 2007; Schweizer and Merten, 2010; Gama-Norton *et al.*, 2011). They are also used in the biotechnology industry to produce therapeutic proteins and viruses for gene therapy (Jaluria *et al.*, 2008; Siqueira-Silva *et al*., 2009).

Plant consumption and cytotoxicity

Plants consumption is often regarded as low risk since they have been used by humans throughout for centuries. However, some of them may reveal a very strong and even toxic activity in humans, specifically the extracts, concentrates or pure compounds obtained from plants (Swiatek *et al*., 2013). An *in vitro* cell-based cytotoxicity assay is an easy and cost effective tool for hit ranking and lead optimization at the early stage for research purposes or drug discovery (Swiatek *et al*., 2013).

A study conducted by Sukhramani *et al*. (2011), on novel bis-benzimidazole derivatives were screened for cytotoxicity against HEK-293T with the use of short term cytotoxicity MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay protocol. The results of the study exhibited significant cytotoxic activity after 48 hours at various concentrations (0.005-100µg/ml), on HEK-293T cell line with half maximal inhibitory concentration (IC_{50}) of 70.23µg/ml, comparable with standard drug doxorubicin and were considered to be the best candidate of the series that could be a good starting point to

develop new lead compounds in the fight against cancer (Sukhramani *et al*., 2011). Another study conducted by Sukhramani and Patel. (2013) revealed that methanolic extract of *A.marina* plant showed negligible cytotoxicity on normal cell line (HEK-293T).

Patel and Patel. (2011), conducted a study on cytotoxic activity of methanolic extracts of *A. heterophyllus* plant by various *in vitro* cytotoxic assays against different cell lines including (HEK-293) normal cells. The IC_{50} at 45.671µg/ml values of methanolic extract of *A. heterophyllus* were found to be non-toxic to HEK-293 normal cells at a concentration range between 5.0-100000n gm/ml, which proved that the methanolic extract exhibited significant anti-cancer potential with no toxicity on normal cell line (Patel and Patel, 2011).

Another study was conducted in Iran on cytotoxicity using HEK-293 cell line, to perform cytotoxicity assays using methanolic extract of *K.odoratissima* tested on a panel of cell lines including MDA-MB468 (human breast cancer cell line), K562 (human leukemia cell line), SKOV3 (human ovarian cancer cell line), Y79 (human eye cancer cell line), A549 (lung cancer cell line), and HEK 293 (normal human embryonic kidney cell line) (Momtazi *et al*, 2017). The results suggested a direct cytotoxic activity of *K. odoratissima* leaf extract showed low toxicity (IC_{50} > 400mg/mL) on human embryonic cell line (HEK 293), but showed high selective cytotoxicity on MDA-MB468, K562, SKOV3, Y79, and A549 cancer cell lines with IC_{50} values of 85mg/mL, 70mg/mL, 120mg/mL, 82mg/mL, and 145mg/mL, respectively (Momtazi *et al*., 2017).

Cheshomi *et al*. (2016) conducted a study to evaluate the cytotoxicity of the methanol extract of *Datura innorxia* petals on HEK-293 cell lines. The cytotoxic effects of the extract on the cell lines, extracts at concentrations between 5µg/mL and 100µg/mL were prepared at 48 and 72 hours after treatment, where the viability percentage of the cells was evaluated by the MTT assay. The results showed no cytotoxic effects on the HEK-293 cell lines (Cheshomi *et al*., 2016). The effect on the cells was dose-dependent and therefore, significantly increased with a rising concentration. With an increasing time from 48 hours to 72 hours, there was significant change in IC_{50} values. However, with this time

change, the toxicity effect on normal cells greatly increased and was derived from the toxicity of the plant (Cheshomi *et al*., 2016).

Another study conducted on HEK-293 cells were exposed to SiO_2 nanoparticles (20 and 50nm) at 2, 20, 100, 200, 500, and 1000µg/ml for 24 hours (Wang *et al*., 2009). Cell viability decreased as a function of dosage levels, significant differences were seen from the 20nm group treated at the concentrations of 100, 200, and 500µg/ml. Both 20 and 50nm nanoparticles showed significantly cytotoxicity at concentrations above 20µg/ml (Wang *et al*., 2009).

From the above described cell lines as *in vitro* models, HEK-293T cell line was the only cell line used for the study. HEK-293T cell line as compared to other cell line is that they are easy to grow in tissue culture and transfection kits are commercially available, second to HeLa cells (Philips, 2014). HEK-293T cell line has also been used extensively with MTT assay protocol which was used for cytotoxicity in the study. Thirdly the study on plant remains consumed in geophagic soils for cytotoxicity evaluation using HEK-293T cell line has not been adequately conducted and as a preliminary study it was significant to use HEK-293T cell line without any means of manipulation as compared to cancer cell lines. However, for further studies other cell lines could be evaluated.

2.11.4 Determination of appropriate cell viability assays

Introduction

Cell viability refers to the number of healthy or live cells in a sample compared to unhealthy or dead cells (Stoddart, 2011). The same assays used to determine viability are used repeatedly over a period of time to investigate cell proliferation within a population (Mosmann, 1983; Cory *et al*., 1991; Stoddart, 2011). One of the earliest methods for assessing cell viability was trypan blue dye exclusion assay, which is still widely used today. It is based on the principle that viable cells have an intact cell membrane which can therefore exclude the trypan blue dye (Louis and Siegel, 2011).

Dead cells take up trypan blue, and appear blue as a consequence, as their membrane is no longer able to control the passage of macromolecules (Louis and Siegel, 2011).

The assay requires the cells to be in a single cell suspension and they are then visualised and counted under a microscope using a haemocytometer (Louis and Sigel, 2011; Stodart, 2011). From these counts, it is relatively simple to calculate the total number of cells and the percentage of viable cells within a population (Stoddart, 2011).

Cytotoxicity

Cytotoxicity is the cell killing property of a mediator cell independent from the mechanisms of death (Horvath, 1980). It is the failure of the cell to attach to surfaces and by the changes in the rate of cell growth (Horvath, 1980). Most current assays for measuring cytotoxicity are based on alterations of plasma membrane permeability and the consequent release (leakage) of components into the supernatant or the uptake of dyes, normally excluded by viable cells. The cells are determined for cytotoxicity in a number of ways for metabolic activity usually measured for populations of cells with a tetrazolium salt (Tennant, 1964; Louis and Siegel, 2011).

Cytotoxicity evaluation using cell cultures is a rapid, standardised, sensitive and inexpensive means to determine whether a compound or extract contains significant quantities of biologically harmful chemicals (Masoko *et al*, 2007). Cytotoxicity test methods are useful for screening as they serve to separate toxic from nontoxic materials, providing predictive evidence of compound safety (Slater, 2001). Some commonly used methods in cytotoxicity evaluation include the lactate dehydrogenase (LDH) assay, the adenosine triphosphate (ATP) assay and MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay cleaved into a coloured formazan product by metabolic activity (Eguchi *et al*., 1997; O'Brien *et al*., 2000; Abid *et al*., 2012).

Lactase Dehydrogenase (LDH) assay

This is an assay modified to evaluate cytotoxicity (O'Brien *et al*., 2000). It is found in different types of healthy cells, located exclusively within the cytosolic enzyme (Thomas, 2014). The assay measures the number of cell death or damaged cells causing the plasma membrane to rupture and leads to LDH leakage into the surrounding media (Kroll *et al*., 2009; Kroll *et al*., 2011). By measuring the amount of LDH that has been released from cells, the LDH assay reveals the extent of plasma membrane rupture and as such a

measure of late stage toxicity and cell death (Koh and Choi, 1987). In practice, LDH presence in the media is determined through the reaction based on enzymatic conversion of tetrazolium salt to formazan salt in the presence of LDH and diaphorase (Olgun, 2004).

LDH catalyses the conversion of lactate to pyruvate, producing NADH molecule as a result. NADH is then used by diaphorase, supplied with the assay kit to reduce a tetrazolium salt into a red formazan product. The conversion is quantified through the measurement of absorbance at 490 nm. Thus, the production of formazan is directly proportional on LDH activity and, therefore, gives a quantitative measure of end-stage cytotoxicity (Thomas, 2014; Koh and Choi, 1987). Figure 2.9 below shows an illustration of the relationship between LDH activity and formazan production in the LDH assay. NADH produced through LDH activity is used by diaphrose to convert the colourless iodonitrotetrazolium into red formazan (Figure 2.9).

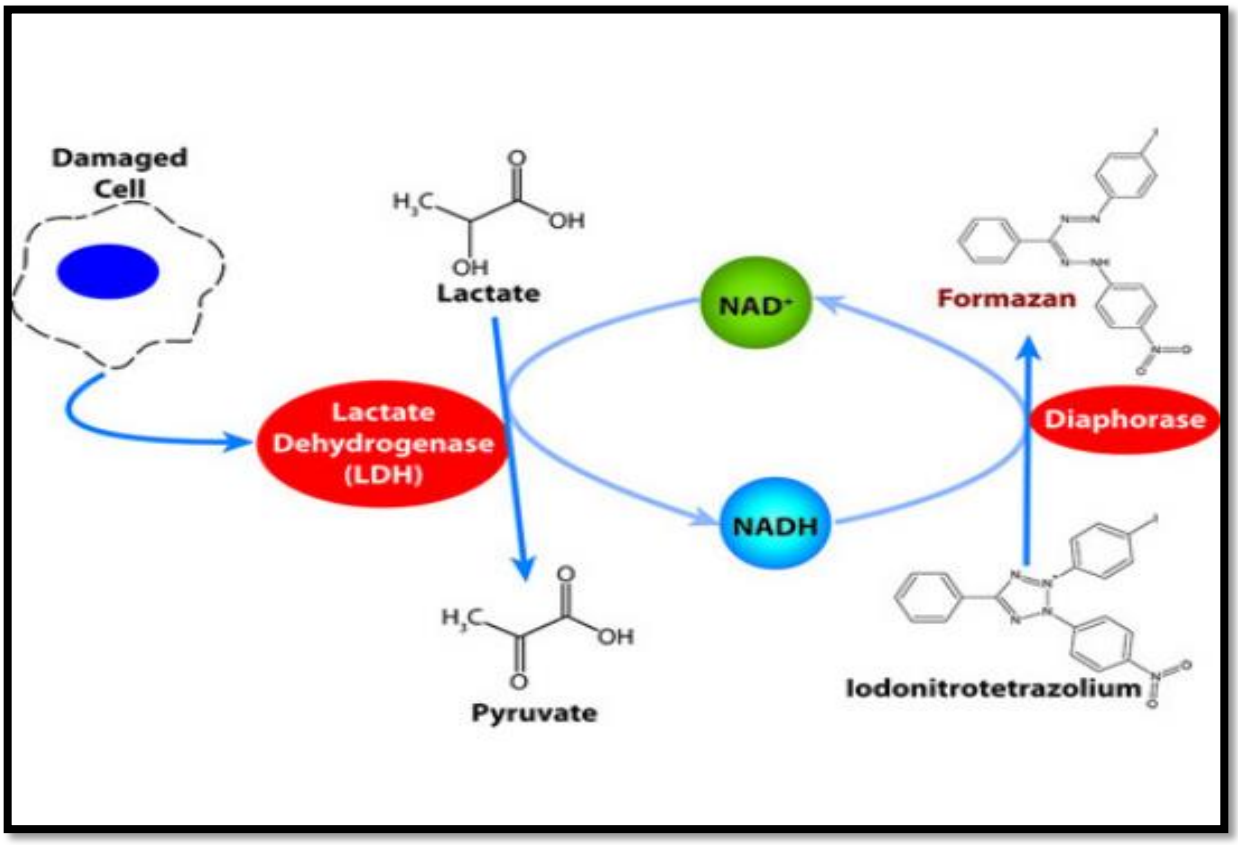

Figure 2.9: Schematic representation of the relationship between LDH activity and formazan production (http://caymanchem.com/pdfs/1008883.pdf).

Adenosine triphosphate (ATP) assay

The ATP assay is another commonly used assay for cellular toxicity (Thomas, 2014). It is the measurement of intracellular ATP concentration, given its role as the principal energy store within the cell. The assay involves the addition of a D-luciferin and firefly luciferase containing solution to cells (Thomas, 2014). This lyses the cells and provides firefly luciferase with intracellular ATP to drive the light-producing oxidation of D-luciferin. Thus, the amount of light produced is directly proportional to the amount of ATP contained within the cells. Viability studies involving the ATP assay can, therefore, give an indication of toxicity occurring through the depletion of ATP stores. In addition and similar to its MTT-based counterpart, the ATP assay can give an indication of toxicity prior to cell death, as the assay's high sensitivity allows it to rapidly detect changes in ATP content (Crouch *et al*, 1993; Mueller *et al*., 2004). Figure 2.10 below shows a schematic representation of the ATP luciferase assay.

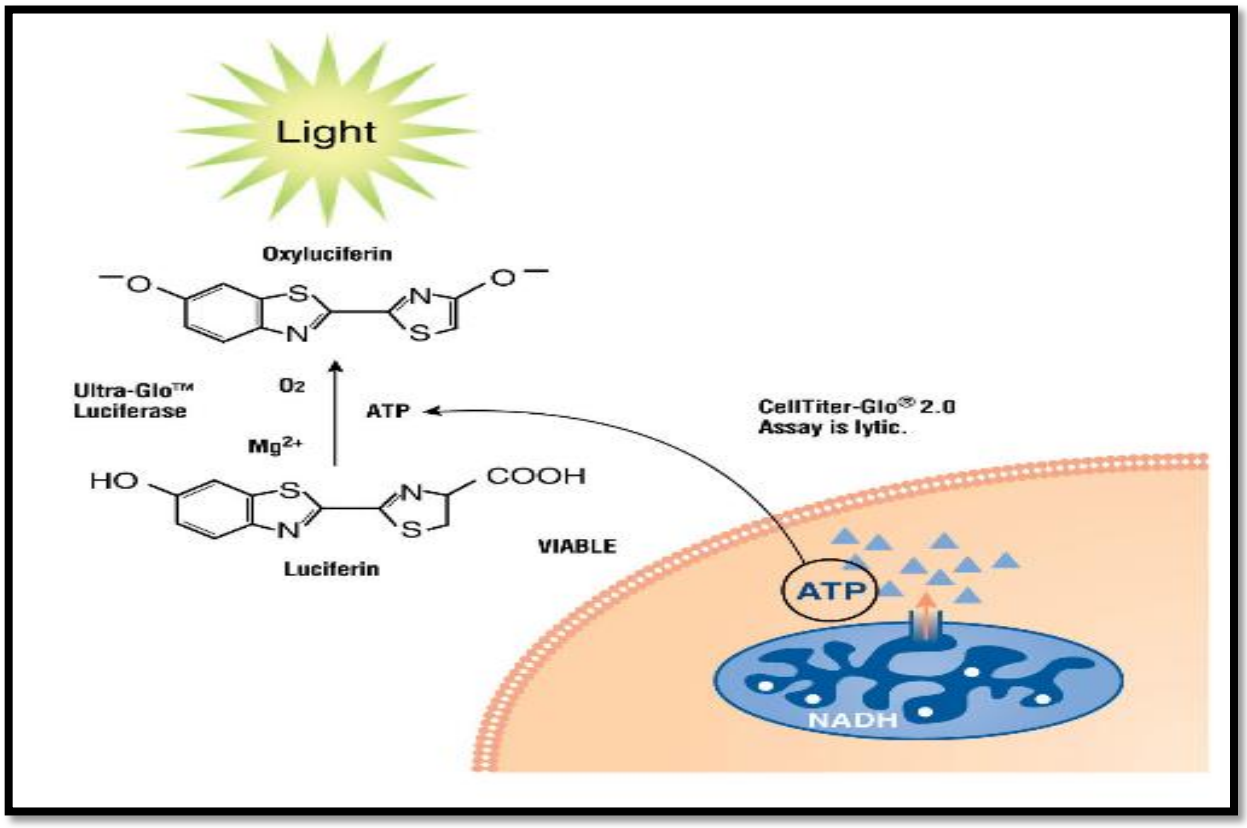

Figure 2.10: Illustration of ATP assay principle (https://worldwide.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-2_0-assay/).

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] Assay

This is a non-radioactive quantitative colorimetric assay used for *in vitro* measurement of the cytotoxicity, cell proliferation, viability, and cell attachment following treatment of cells with a toxic substance (Abid *et al*., 2012). The assay was developed by Mosmann, (1983) with modification based on the reduction of a yellow coloured 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), a water soluble tetrazolium salt, which is converted into insoluble formazan crystals. Only viable cells with an active metabolism are able to be taken by the dye (Fotakis and Timbrell, 2006; Senthilraja and Kathiresan, 2015). This event occurs in the mitochondria wherein the active mitochondrial succinate dehydrogenized enzyme cleaves the tetrazolium ring and reduced the MTT reagent into purple formazan (Mosmann, 1983; Lukhele *et al*., 2016) (Figure 2.11).

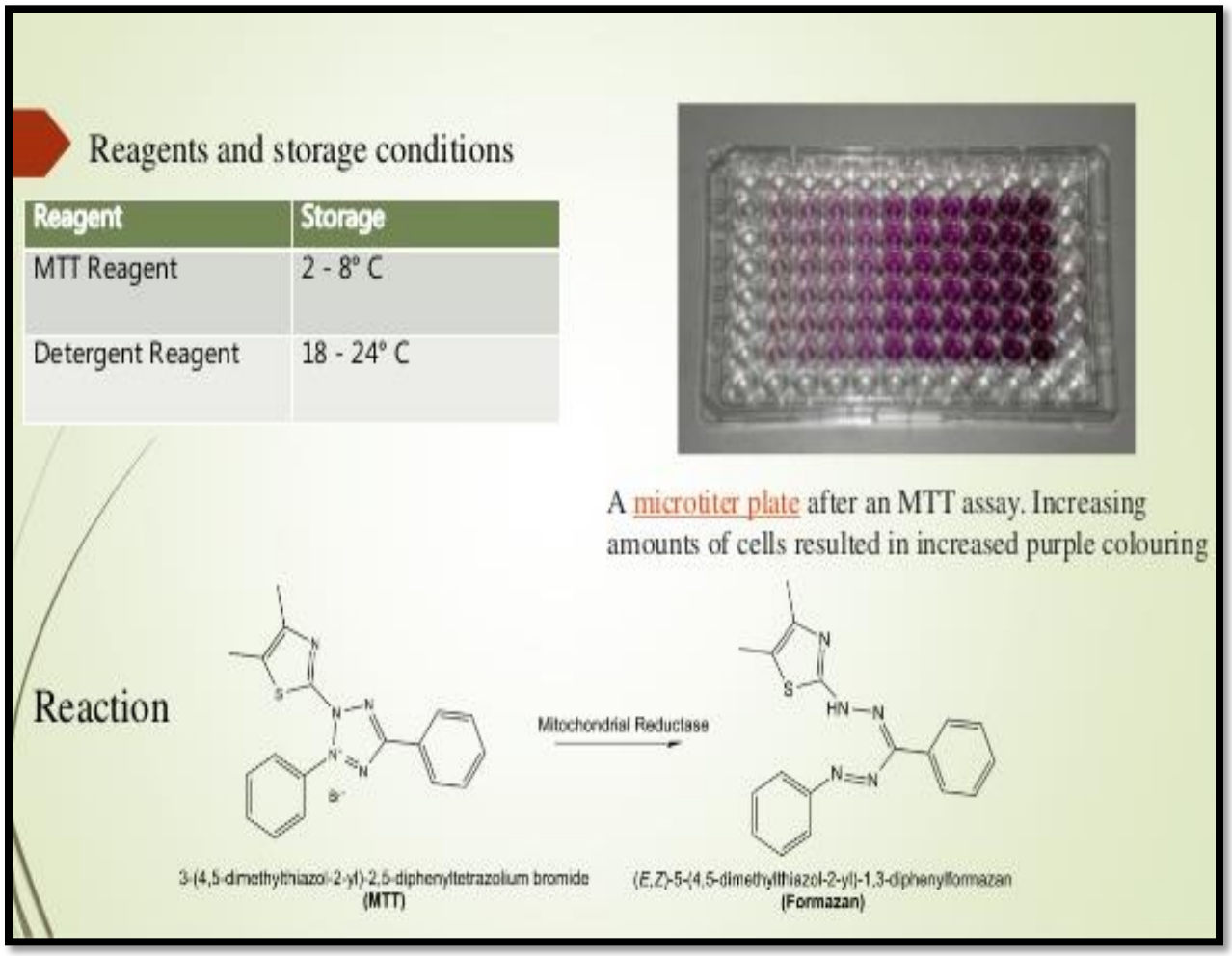

Figure 2.11: Illustration of the MTT assay principle (Berridge *et al*., 2005)

From the above mentioned assays, although all the assays are used for cellular toxicity testing, MTT was the only assay used. Reason being the main focus of the study of plant remains extracts was to evaluate for cell viability and MTT assay is used for cell viability. MTT assay is an easy, comfortable colometric and easy for cell proliferation tool to use for cytotoxicity evaluation using HEK-293T cell lines (Masoko *et al*, 2007; Selvaraj *et al*., 2014; Cheshomi *et al*., 2016; Momtazi *et al*., 2017).

2.12 Summary of the chapter

The chapter reviewed the historical and relevant perspectives of human geophagia, physical properties, mining and the possible health impact of plant remains in geophagic soils. A number of studies on geophagia have shown the practice has negative and positive influence to those that practice it. Geophagia is a common phenomenon in the world today and also plays an important role in consumers who ingests it (Halsted, 1968; Callahan, 2003; Wilson, 2003; Ellis and Schnoes, 2006; Ekosse *et al*., 2010). Geophagia is widely practiced by women and children and cherished for various reasons.

Geophagic soils contain decomposed vegetation and roots of dead plants that may pose possible health threats. Therefore, more focused research is necessary in this area to ascertain the exact health implications of geophagic practice. There are different kinds of plant remains that are recovered from geophagic soils consumed and include stems, roots, wood, seeds, fruits, tubers and nutshell as well as fibers that have been woven into fabric or stems (Fritz, 2005). Their recovery from geophagic mines may involve hand collecting, screening, or flotation (Fritz 2005; Pearsall, 2000). Plant remains form a major portion of the diet and therefore, their nutrition content is important. However a high concentration of the cytotoxicity in plant remains could be harmful to human health. The graphical analysis of these data can indicate the plant remains present, the parts that are most often collected, the season of collection and the reasons for such preferences.

Cell lines as *in vitro* models are simple, cost-effective and rapid cell lines used to assess efficacy and performance for variety of cytotoxicity evaluations and similar analysis in cell biology. Cell lines such as HeLa, MCF-7, U87, A549, SKOV-3, Vero and HEK-293T are some of the most commonly used cell lines in research purposes. HEK-293T cells are

the adherent *in vitro* model cells which are widely used, easy to grow in tissue culture and used in cell biology research. The cell line has been used to evaluate the cytotoxicity of plants to investigate how toxic or nontoxic a plant can be using MTT assay protocol. MTT is used for evaluation of cytotoxicity to determine cell viability. This assay is the colorimetric assay measuring the activity of enzymes that reduces MTT to formazan dyes, giving a purple colour. MTT assay determines cytotoxicity of potential toxic materials since those agents would stimulate cell viability and growth. Some of the commonly used methods include the (LDH) assay, and the (ATP) assay. LDH is used for cell death or damaged cell whereas ATP is used to give an indication of toxicity prior to cell death.

CHAPTER 3
MATERIALS AND METHODS

3.1 Study area

The study was carried out in Sekhukhune area (Figure 3.1), which is a District Municipality situated in the Limpopo Province, in the northern part of South Africa. Sekhukhune covers an area of approximately 13 264 km^2, most of which is rural and it lies to the North West of Mpumalanga and the South of Limpopo (Aird and Archer, 2004). Sekhukhune area is one of the five Districts in the Limpopo Province with five local Districts in which Fetakgomo Local Municipality (FLM) falls. The two Villages namely Ga-Nchabeleng and Mphanama fall within Fetakgomo Local Municipality in the Sekhukhune area. The Villages are serviced by a few major rivers- the Olifants River, Tubatse River and Elands River, all of which supply a number of large dams.

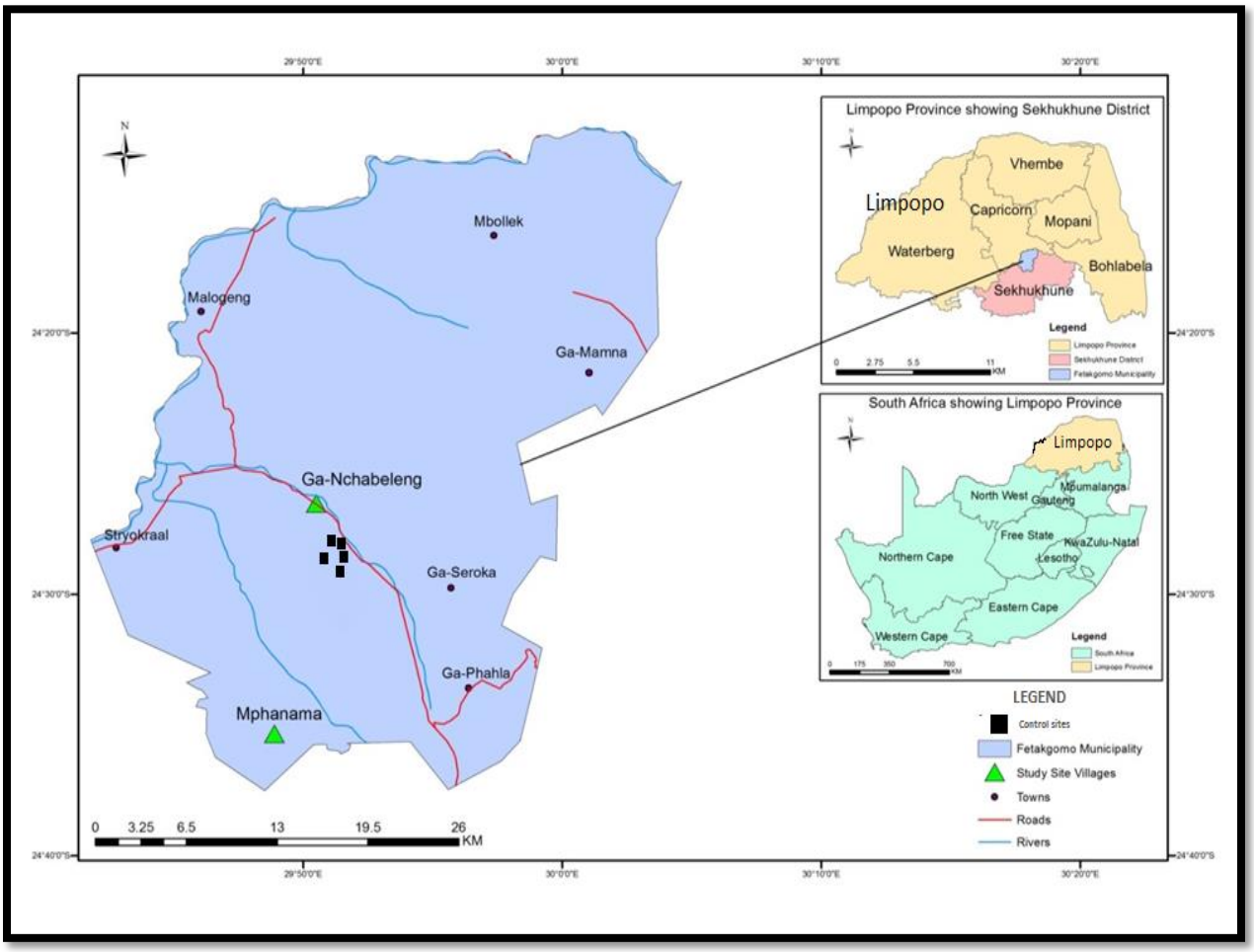

Figure 3.1: Ga-Nchabeleng and Mphanama study sites.

3.1.1 Ga-Nchabeleng Village

Ga-Nchabeleng Village is approximately 94 km south-east of Polokwane on the R37 tarred road. The geographic grid of Ga-Nchabeleng Village study site was 24º26'0'' S latitude and 29º51'0'' E longitude. A wide variety of soils were found in the study site area of Ga-Nchabeleng Village ranging from deep brownish black to reddish brown loams. The mountainous area was dominated by shallow, poorly developed soils and the substrate was often completely dominated by bedrock. The site was not underlain by dolomitic rock and as far as could be determined no mining activities occur close to the study area (von Well, 2013). The vegetation in Ga-Nchabeleng Village was dominated by thick, thorny *Acacia* bushes on sparse grasslands (Anteneh *et al*., 2004). On dip slopes the village was dominated by *Combretum apiculatum* or *Diplorhynchus* species. The scarp slopes and Pediments were occupied by *Kirkia vilmsii, Acacia nilotica, Acacia nigrescence* and *Commiphora spp* (Anteneh *et al*., 2004).

3.1.2 Mphanama Village

Mphanama Village is approximately 108 km south-east of Polokwane on the R37 tarred road. The geographic grid of Mphanama Village study site was 24º36'0'' S latitude and 29º49'0'' E longitude. The area was prominent with eroded dongas of clay rich soils (Mucina and Rutherford, 2006). The plains within this land type were deemed to be covered predominantly by red-yellow apedal soils, with highly localized pockets of red-coloured, weakly structured clayey soils, and highly localized pockets of moderately structured clayey soils (von Well, 2013). The vegetation in Mphanama Village consisted of generally open valleys between chains of hills, semi-arid plains and small mountains that ran parallel to the escarpment. The vegetation was predominantly short, open to close thornveld and abundant *Aloe* species and other succulents (Mucina and Rutherford, 2006). Some areas were heavily degraded and overexploited by man's activities due to cultivation (Low and Rebello, 1996).

According to Mucina and Rutherford (2006) some of the vegetation types found in the two villages were *Acacia* sp., combination of tall trees (*Acacia erioloba, Philenoptera violacea*), short trees (*Acacia mellifera subsp. detinens, Acacia nilotica, Acacia tortilis* subsp. heteracantha, *Boscia foetida subsp. rehmanniana, Acacia grandicornuta,*

Commiphora grandulosa), succulent trees of *Euphorbia tirucalli*, tall shrubs (*Rhus engleri, Dichrostachys cinerea*, *Ehretia rigida* subsp.rigida, *Grewia bicolour*) and short shrubs *(Felicia clavipilosa subsp. transvaalensis, Seddera suffruticosa, Lantana rugosa*), succulent shrubs of *Aloe* species and *Euphorbia enormis*, Graminoids (Grasses and sedges) and herbs (forbs).

Two local geophagic consumers and interpreters from the Villages in Sekhukhune area; Manaso Morwaswi from Ga-Nchabeleng Village and Magabjane Kgaphola from Mphanama Village were selected, respectively. The two interpreters guided the suggested selection of the sites, because of the existence of prior knowledge of geophagic mines and the consumers from their respective communities. Soil samples were thus collected in areas known for human geophagic practices. The soil samples were collected from 17 different geophagic sites of the studied sites in Sekhukhune area and included 12 well known geophagic sites and five non-geophagic sites as control.

3.1.3 Climate of the geophagic area

The soil samples were collected during September-October 2014 just after the first seasonal rains. The annual rainfall in the Sekhukhune area varied between 600 and 800 mm while the northern part was drier, between 500 and 600 mm, receiving more than 80% between November and March (DWAF, 2005). During September-October 2014 the average mid-day temperatures were about 27 ºC with a humidity of 16% (Figure 3.2) (Fetakgomo IDP, 2014).

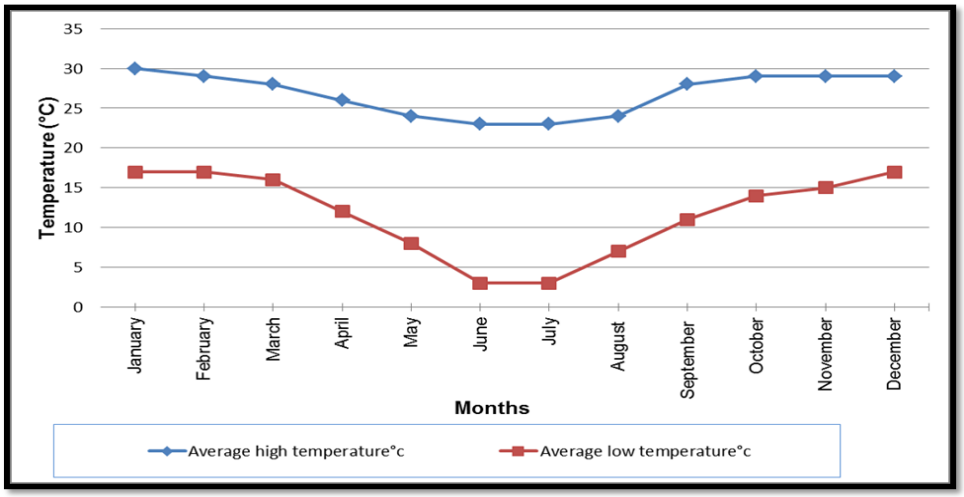

Figure 3.2: Average monthly temperatures for Sekhukhune area

3.2 Geophagic soil sampling sites

Geophagic sites were located with the help of the consumers and interpreters from both studied areas. The consumers were able to supply information on different locations and the type of soils preferred and consumed according to their own individual preferences. Generally, all of the geophagic mining sites were well known and had been extensively mined by consumers from both studied areas and soil samples were collected from loose-lying soil within each of the mines as well as 10 cm deeper depending on the type of mine. The different mines were located in areas that were easily accessible.

Geophagic soils from Ga-Nchabeleng Village were collected from quite a number of different geophagic sites. Consumers collected their soils from public gravel road as illustrated by a young girl (Figure 3.3). The public gravel road was adjacent to houses and the surrounding area was covered with natural grasses and the presents of *Acacia* plants (Figure 3.3). Whereas the young girl preferred gravel road, older women preferred soil under the *Acacia* plant where the area was neat and the women used sticks for digging when collecting the soil (Figure 3.4). Figure 3.5 simply shows how much extensively used were these mines and how close a mine could be to houses. Some of the geophagic consumers in Ga-Nchabeleng Village preferred soil from the valley and underneath the stones (Figure 3.6).

The surrounding area of the valley was neat with no rubbish near the sites. Although others preferred a neat valley, some of the consumers preferred collecting from a dumping site close to the valley under small *Acacia* plants (Figure 3.7). Other geophagic site preferred by consumers in Ga-Nchabeleng Village was soil from riverbanks of the *Mohwetse* River (Figure 3.8).

The weather during the discovery of sites and collection of these soil samples was sunny although it was evident that the area received rainfall during the past few days as was noted from the moistness of some soil samples, as well as a few water puddles near some of the mining sites. The moistness of the soil also contributed as some of the consumers preferred the moist and smell of the soil after rainy days when it was still wet.

Figure 3.3: Geophagic soil collected on public gravel road in Ga-Nchabeleng Village.

Figure 3.4: Geophagic soil collected under the *Acacia* plant in Ga-Nchabeleng Village.

Figure 3.5: An extensive geophagic mining site in Ga-Nchabeleng Village.

Figure 3.6: Geophagic soil collected on a valley in Ga-Nchabeleng Village.

Figure 3.7: Geophagic soil collected around a dumping site in Ga-Nchabeleng Village.

Figure 3.8: Geophagic soil collected on the riverbanks in Ga-Nchabeleng Village.

Geophagic sites from Mphanama Village also varied according to individual preferences. Different sites were discovered. Unlike Ga-Nchabeleng Village where they collect soil at a river or valley or road, Mphanama Village consumers preferred their soils right outside their own houses and even inside the yard of their houses as illustrated in Figure 3.9 and Figure 3.10, respectively. The sites were fairly neat with open grassland and vegetation. Some geophagic women from Mphanama Village collected their soil from the wild on the termite mound (Figure 3.11). Males in the Village of Mphanama showed their sites as well. A young boy illustrated his site and preferred to collect soil from decaying woods of Red bushwillow (*Combretum apiculatum*), Shepherd's tree (*Boscia albitrunca*) and Mountain Kirkia trees (*Kirkia wilmsii*) (Figure 3.12). Other women collected soil from termiterian trees (Figure 3.13). It was interesting to discover that male geophagic consumers from Mphanama Village preferred to collect soils from soft rocks on a mountain close to houses (Figure 3.14).

Figure 3.9: Geophagic soil collected outside the yard in Mphanama Village.

Figure 3.10: Geophagic soil collected inside the yard in Mphanama Village.

Figure 3.11: Geophagic soil collected from the wild on the termite mound in Mphanama Village.

Figure 3.12: Geophagic soil collected from decayed woods in Mphanama Village.

Figure 3.13: Geophagic soil collected on a termiterium tree in Mphanama Village.

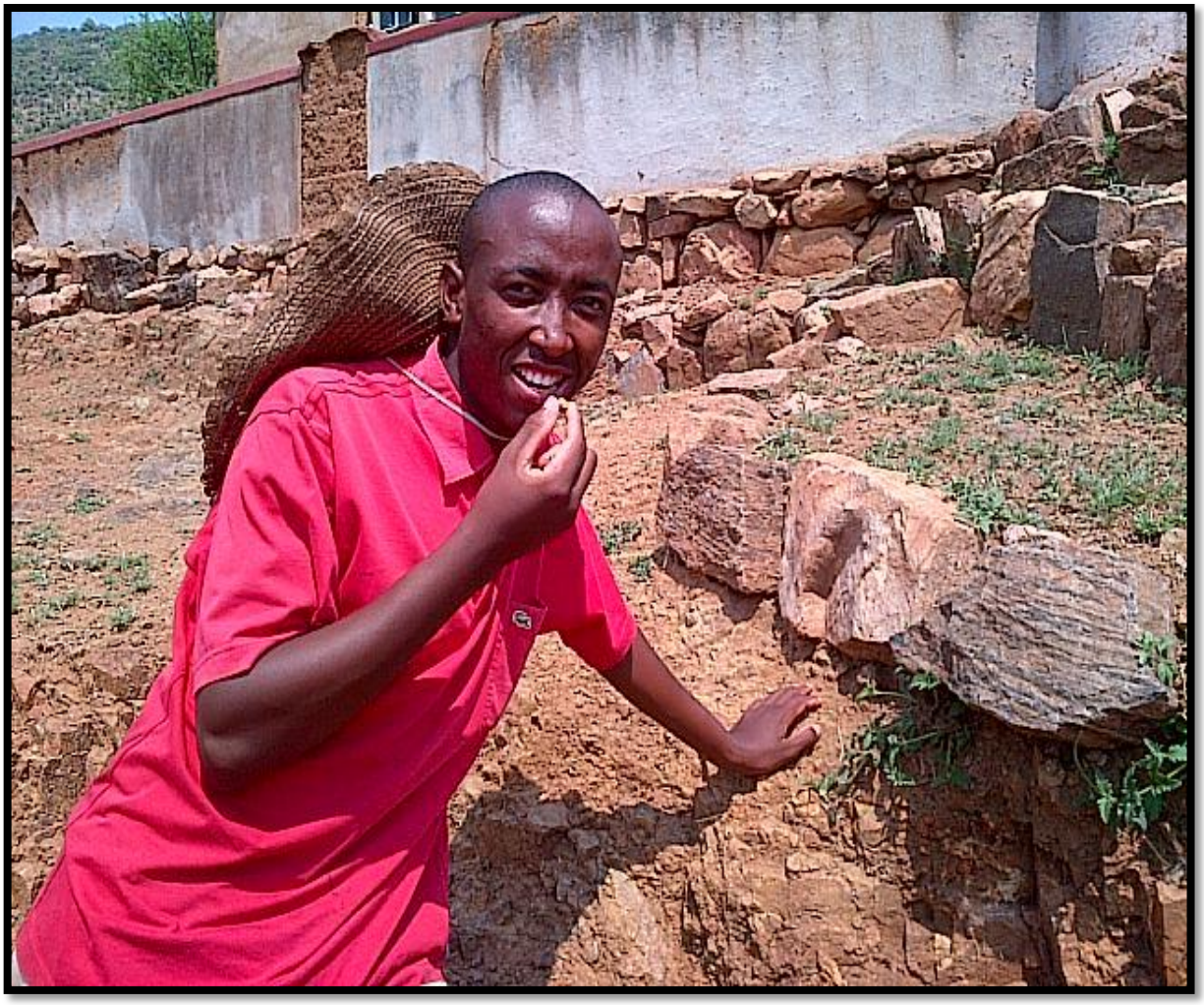

Figure 3.14: Geophagic soil collected on soft rocks on a mountain in Mphanama Village.

The sites varied from relatively neat to very dirty with pieces of glasses, plastics, and other rubbish tools. Most of the sites were found to be located close to footpaths, houses, dumping sites, under the trees and even on roads, which raised a concern about the health aspects of these soils. Some consumers in the study preferred the soils of the decaying wood of Red bushwillow, Shepherd and Mountain Kirkia trees while other consumers preferred soils from the mounds**.** In addition to this, the collection and preparation practices of geophagic soils may play a role in the cytotoxic effects and microbial infections to their health, as the majority used hands for collection and non-sterile bags for packaging.

3.3 Development and administration of questionnaires and Ethical considerations

3.3.1 Questionnaires

A questionnaire titled Human and Enzootic Geophagia developed by Ekosse (2007) attached as Appendix 1 was part of the tool used for investigation to obtain information about the practice of human geophagia in Sekhukhune area. The questionnaire was used to gather data about the demographic, socio-economic, medicinal and cultural beliefs of geophagic consumers who practice geophagia. A combination of purposive and snowball sampling techniques were used to identify geophagic individuals from each of the studied villages in Sekhukhune area to whom questionnaires were administered (Ekosse *et al*., 2010). Snowball technique was employed because of the reluctance of individuals to reveal their geophagic habits. Purposive sampling was then used to identify another initial subject who formed the initial respondent through whom others were identified (Ekosse *et al*., 2010).

3.3.2 Ethical considerations

To achieve this, a survey was conducted to investigate the importance of the practice of geophagia in Sekhukhune area and the consumer's knowledge pertaining to the practice. The study proposal including the administration of questionnaires was ethically approved by the Ethical Committee of the University of Venda (Appendix 2; ethnic number: SES/15/ERM/04/2511). All the participants were given informed consent (Appendix 2) before administering the questionnaire. The content in the informed consent was properly explained to the participants and signed prior to the study. Statistical data of the overall results is attached as Appendix 3.

3.3.3 Questionnaire sampling

The number of participants was chosen based on proportionality and size of the population in each of the Villages. According to Fetakgomo Local Municipality (FLM) Intergrated Development Plan (IDP) (2008/9), population in Ga-Nchabeleng Village was approximately 12 798 and Mphanama Village about 9798, respectively. A total of 135

participants from Ga-Nchabeleng Village volunteered for this study and 65 were from Mphanama Village, making a total of 200 participants.

The first group consisted of 172 participants who practiced geophagia and as a result represented the geophagic group while the second group consisted of 28 participants who did not practice geophagia thus they represented the control group. The questionnaire clarified information on specific areas where geophagic soils were mined and was completed with the assistance of the interpreters who originated from the two villages studied.

The criteria for geophagic group were as follows:

a) Participants practicing geophagia
b) Participants between the ages of 18-65 years
c) Participants reside permanently in Ga-Nchabeleng and Mphanama Villages.

The criteria for control group were as follows:

a) Participants not practicing geophagia
b) Participants between the ages of 18-65 years
c) Participants reside permanently in Ga-Nchabeleng and Mphanama Villages.

3.4 Soil sampling

The consumers in this study area practice geophagia extensively and without condemnation. It was also emphasized during this study that soil samples collected should be from the sites representative of those consumed by geophagic consumers (Young *et al*., 2008). Approximately 200 g of each soil samples were collected using the polyethylene bags. It was also important to collect control samples from the same area as the geophagic samples, however from soils not consumed. The collection of soil samples was done as described by Young *et al*. (2008) for geophagic samples.

The control soil sample group was collected from sites not used by geophagic consumers located within the rural settlements of Sekhukhune area. The collection of the control soil samples took place in November of 2014. The soil samples were collected, labeled and also documented using the same method of collection as used in the

geophagic sites group, packed in transparent Ziploc bags. In total, soil samples for laboratory analyses were collected from 17 sites for colour classification.

The coordinates of each geophagic site were noted with a Garmin GPS (Global Positioning System) device. GPS is a satellite-based navigation and surveying system used for determination of precise position and time, using radio signals from the satellites, in realtime or in post-processing mode. It is being used all over the world for numerous navigational and positioning applications, including navigation on land, in air and on sea. It determines the precise coordinates of important geographical features as an essential input to mapping. The device offers very high accuracy coordinates in most surveying and navigational applications at very low cost and with high efficiency (Anderle, 1988; Colombo and Watkins, 1991).

The size measurements of each mine were recorded with a measuring tape. The amount of soil collected from each mining site varied and was determined by the size of the mine. Some geophagic sites were small and others were large. The soil samples were collected from the topsoil up to 10 cm deep with a steel shovel and placed into a clean transparent Ziploc bag. The shovels were cleaned prior to collection, by spraying it with 70% ethanol by allowing it to air dry completely before the sample was taken.

3.5 Soil colour classification

The soil sample colours were identified following method described by Young et al. (2008). A plastic spatula was used to mount the samples on white cardboard sheets. It provided the use of Munsell Soil Color classification charts as a comparative standard to maintain objectivity (Young *et al*., 2008). A portion of each sample in its natural state was spread onto a white paper and, under white light, compared to the Munsell Soil Color Charts which selected the closest resembling colour chip.

3.6 Plant remains

3.6.1 Plant remains collection and identification

Plant remains in geophagic soils were obtained from geophagic sites in Sekhukhune area (Ga-Nchabeleng and Mphanama Villages), respectively. Plant remains were

washed with sterile water, dried in shade and stored in covered dried papers. After the recording of the samples, identification of plant remains from geophagic soils was classified into two, namely:

Macrobotanical remains (roots, leaves, stems, leaves and wood) in the form of hand picking method; and microbotanical remains (seeds) in the form of sieving method. Since the study was focused mainly on macrobotanical remains once dry, the macrobotanical samples were packaged for laboratory analysis (Hastorf *et al*., 1989; Pearsall, 2000; Fritz, 2005).

3.6.2 Recovery of plant remains

Plant remains provide an extremely important source of evidence on past environments and use of plants as food or medicinal source. Macrobotanical and microbotanical remains are studied in different laboratories because of the distinct methods, comparative collections and skills required to study them. For the purpose of this study, macrobotanical remains were the ones specifically focused on and studied.

Physical separation was used in the recovery of plant remains (macrobotanical remains). Six geophagic sites were discovered from Ga-Nchabeleng Village and six from Mphanama Village recorded as geophagic sites group. The remaining five sites were discovered from non-geophagic sites and were recorded as the control site. It was discovered that soils in the area were different; however the plants and vegetation type within the 17 sites were similar. The use of a composite study in plants has become novel, rapid and inexpensive method and less time consuming (Collier *et al*., 2005). Previous studies have been done on composite plants using families, similarities and characteristics/features (Collier *et al*., 2005; Ashraf *et al*., 2011; Nethengwe *et al*., 2012). For this study, plant remains were grouped according to characteristics/features. Thus a composite study of five plant remains samples for the study collection in the recovery was adapted (Ashraf *et al*., 2011).

Plant remains sample one collected from Ga-Nchabeleng Village study sites composed of grasses. These plant remains were collected from three different geophagic sites. The

grasses were identified as Couch grass (*Cynodon dactylon*), Tassel three-awn (*Aristida congesta*) and Broad-leaved curly leaf (*Eragostis rigidior*) (Figure 3:15).

Figure 3.15: Plant remains sample one collected from Ga-Nchabeleng Village study sites.

Plant remains sample two composed of White thorn *Acacia* (*Vachellia tortilis*), which were also collected from three different geophagic sites in Ga-Nchabeleng study sites (Figure 3.16).

Figure 3.16: Plant remains sample two collected from Ga-Nchabeleng Village study sites.

Plant remains sample three composed mainly of Rhodesian weeds (*Alternanthera pungens* kunth) from two different sites and Khaki weed (*Alternanthera lorentzii)* from

another site. These weeds plant remains were collected from three different geophagic sites in Mphanama Village (Figure 3.17).

Figure 3.17: Plant remains sample three collected from Mphanama Village study sites.

Plant remains sample four composed of woody plants. They were plants of the Red bushwillow (*Combretum apiculutum*), Mountain Kirkia (*Kirkia wilmsii*) and Shepherds tree (*Boscia albitrunca*) and were collected from three different geophagic sites in Mphanama Village (Figure 3.18).

Figure 3.18: Plant remains sample four collected from Mphanama Village study sites.

The control plant remains samples composed of *Acacia* plants; Knob thorn (*Acacia nigrescens*), Black wattle (*Acacia meansii*) and White thorn *Acacia* (*Vachellia tortilis*).

The plant remains were collected from sites not used for geophagic practices (Figure 3.19).

Figure 3.19: Plant remains five control sites collected from Ga-Nchabeleng study sites

3.6.3 Preparation of plant remains samples for analysis

Five composite plant remains consisted of roots, leaves and stems were ground using Retsch Muhle grinding machine and sieved through 0.75 micro metre meshes. The machine was firstly cleaned inside before use (Figure 3.20).

Figure 3.20: Retsch Muhle grinding machine used to ground the plant remains

Extraction of plant remains

A 20 g of five composite samples were weighed and placed into 1000 ml transparent bottles. Finely ground powder of five samples of the plant remains were each immersed in 300 ml of methanol solution mixed and left for 48 hours. The ground materials were allowed to soak for two days to allow efficient isolation. Following soaking, crude extracts were filtered using a funnel and filter paper and five sample solvents remained and were recorded (Figure 3.21). Five solvents were evaporated using the Rotavapor Rll reflux extraction machine to remove the solvent and remained with the crude extracts only at a temperature of 45°C. The extracts were put to dry then dissolved with methanol to evaporate. The plant remains extracts were stored in a dark cool place until used.

Figure 3.21: Filtering of solutions using a funnel and filter paper.

Reconstitution of plant extracts

Approximately 0.36 g of each plant extracts were reconstituted in 100% Dimethyl sulfoxide (DMSO). Extracted stock solutions were sterilized using a 0.2 µM filter (Corning Incorporated, Germany). The prepared stock solutions of 100 mg/ml were then serially diluted according to each assay. The final concentration of DMSO in the test assays was not more than 5%.

3.7 Cytotoxicity of plant remains

Cytotoxicity of plant remains were evaluated in Human Embryonic Kidney (HEK-293T) cells using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay protocol (Promega). The HEK-293T cells obtained from Dharmacon (USA) was used in this study because the cells are easy to grow; are easily transfectable and have been widely used in cytotoxic research for years.

MTT assay which is a non-radioactive quantitative colorimetric assay used for *in vitro* measurement was used for cytotoxicity evaluation of the plant remains extracts. The assay was used to modify the reduction of yellow coloured water-soluble tetrazolium salt into insoluble purple formazan crystals. These insoluble purple formazan was dissolved with a suitable solvent DMSO using the spectrophotometer to measure the absorbance at a wavelength of 570 nm and was proportional to the number of live cells present. In this study, MTT assay was used to test the cytotoxic effect of plant remains on the HEK-293T cells and also account for CC_{50}, which represented the half maximal percentage of 50% cytotoxic concentration.

Propagation of the HEK-293T cells

The HEK-293T cells were maintained in a complete 500 ml Iscove's Modified Dulbecco Medium (IMDM) supplemented with 50ml of Fetal Bovine Serum (FBS) and 550 µl of 50 mg/ml of Gentamycin (Thermofisher Scientific). Cells arrived frozen in 1 ml aliquots and stored at -80 freezer until use.

Thawing

The frozen HEK-293T ampoule was removed from the freezer and quickly thawed by shaking it in a 37°C water bath. The outside part was wiped with an alcohol soaked paper

towel. The cells were quickly aspirated using a pipette and added to a 10 cm flask that had 9 ml of complete medium. The cell suspension in the 10 ml flask was gently mixed. The flask was then incubated at 37°C in a humidified 5% CO_2 incubator. Within four to eight hours some of the cells had already attached at the bottom of the flask; however they were left overnight to attach and proliferate. When most of the cells had attached, the flask was removed from the incubator and the media was aspirated and replaced with fresh medium. The cells were then incubated at 37°C in a humidified 5% CO_2 incubator to allow 50 to 80% confluency for splitting.

Splitting or passaging of HEK-293T cells

After incubation overnight, the flask was removed from the 37°C in a humidified 5% CO_2 incubator and the cells were approximately 80% confluent, which meant 80% of the flask surface was covered by cell monolayer and they were still in the log phase of growth and required sub-culturing.

When the cells were over 80% confluent, splitting, passaging or sub-culturing, which refers to transferring of some cells from previous culture to fresh growth medium was done. The flask was removed from the incubator and medium was discarded. Cells were rinsed three times with 10 ml Phosphate Buffered Saline (PBS) to remove any debris or contaminants in the residual culture media. After discarding the PBS, 1 ml of 0.025% trypsin/EDTA was added and quickly aspirated to detach the cells from the flask. The flask was placed in the incubator for two to three minutes. Cells were re-suspended with 3 ml medium and 1ml of cell suspension was added to two new flasks with 9 ml IMDM. The flasks were placed in the incubator at 37°C in a humidified 5% CO_2 for overnight. The cells were passaged when 80% confluent after two days continuously until ready for the assay.

Seeding cells for cytotoxicity assay

When cells were at the 5^{th} passage, they were seeded for the cytotoxicity assay as follows: The two flasks were removed from the incubator and cells were evaluated under an inverted microscope for contamination and if they were 80% confluent. The media was aspirated from each flasks and were washed three times with PBS. After discarding

the PBS, cells were detached from the bottom of the plates as described above. From the cell suspension, cells were counted using a hematocytometer by adding 50 µl of trypan blue stain with 50 µl of cell suspension, mixed and placed on a hemacytometer, the cells were viewed under the microscope and counted. The cells were diluted with growth medium and seeded in a 96 well plate per assay; in each well we seeded 20 000 cells/100µl. The plates were incubated at 37°C overnight in a humidified 5% CO_2 incubator. The extracts of each plant remains were diluted in growth media in a two-fold dilution from 500-7.8125 µg/ml concentrations. Each of the plant remains extracts were done in triplicate. After overnight incubation, the plates were removed from the incubator and added to 100 µl serial dilution of each plant remains. Wells for control had cells only without any plant remains. The plates were incubated for 48 hours at 37°C in a 5% CO_2 humidified incubator.

Cytotoxicity assay

After 48 hours of incubation 15 µl of the yellow dye solution (Promega) was added to each well and incubated for 4 hours at 37°C in a 5% CO_2. The plates were covered with foil to avoid light exposure. After 4 hours of incubation, supernatant was aspirated from the wells and 100 µl of Stop solution/Solubilization (Promega) was added to dissolve the formazan. Optical density (OD) was measured at 570 nm using a Spectrophotometer reader (Versa max).

3.8 Data analysis

Data from geophagic consumers together with questionnaires and soil colour samples were captured electronically in Microsoft Excel 2010. Further analysis was done using the International Business Machines, Statistical Package for the Social Sciences 23 (IBM SPSS 23). Descriptive statistics and graphical analysis of the data were captured and explained. Statistical methods, namely frequencies and percentages, were calculated for categorical data. Means and standard deviations, medians and percentiles were calculated for numerical data. Analytical statistics, namely the Chi-Square statistic or Fisher's exact test for differences between categorical data, was used to calculate significant differences amongst the different geophagic soil samples and the control groups. A significance level of 0.05 was used throughout the study. Data was presented

in a form of tables and graphs. If p-value was >0.05, it indicated that there was no significant difference between the mean or median values of the control group and the geophagic group. In addition, if the p-value was <0.05, then there was a significant difference between the mean or median values of the control group and the geophagic group existed.

Data for cytotoxicity analyses of plant remains were obtained from triplicate wells. Cell viability was expressed with respect to the absorbance of the control wells (untreated cells), which were considered as 100% viable cells. The percentage of cytotoxicity effects was calculated as %Cell Viability=OD Sample/OD Control x 100, where OD represented optical density sample of the mean value of each concentration of plant remains extracts with cells divided by OD control of the mean value of cells only, multiplied by 100. The 50% of the CC_{50} was obtained with the use of Microsoft Excel 2010.

3.9 Summary of the chapter

The chapter summarises the materials and methods used in collection and analyses of data for the study. The field work comprised of field survey, questionnaire distribution, soil and plant sampling and sample preparation from suitable sites where geophagia was prevalent in Sekhukhune area. The data for questionnaire analyses that included aspects on demography information, socio-economic and cultural aspects, indigenous knowledge, physico-chemical, mineralogical, geological and chemical aspects, ecological aspects and human health elements associated with geophagic practices have been described in full. The map of the study site illustrating the locations where geophagia occurs in Sekhukhune area has been provided. The study sites from the two Villages (Ga-Nchabeleng and Mphanama) in Sekhukhune area were also described in detail. The soil colour analyses for colour classification from the 17 studied sites were also identified and explained. Plant remains characterisation which included collection and identification, grinding and extraction as well as cytotoxicity assay protocol of plant remains extracts were explained in full. The results obtained from the field and laboratory analyses were presented in the subsequent chapter.

CHAPTER 4
RESULTS AND DISCUSSION

The phenomenon of human geophagia, pica, the consumption of soils is widely practiced not only in Africa, but across the globe (Aufreiter *et al*.,1997; Young *et al*., 2007; Ekosse *et al*., 2010; Ngole *et al*., 2010), in every continent in the world including North America (Vermeer and Frate, 1979; Aufreiter *et al*., 1997; Grigsby *et al*., 1999), Central America (Hunter and De Kleine, 1984), South America (Abrahams and Parsons, 1997), Asia (Aufreiter *et al*., 1997), Europe and the Middle East (Höllriegel *et al*., 2007). In Africa, geophagia is more common among communities with poor socio-economic status particularly in the sub-Saharan Africa (Abrahams, 2002; 2005; Wilson, 2003). Some South African communities are also known to practice geophagia (Saathoff *et al*., 2002; Ekosse *et al*., 2010; Ngole *et al*., 2010), including certain communities from the poorer rural settlements within the area of Sekhukhune, Limpopo Province. The latter area was the subject of the study in particular the two Villages within the area (Ga-Nchabeleng and Mphanama).

4.1 Data from questionnaire: Human geophagia

In this section of the chapter, data was obtained through the administration of 200 questionnaires to geophagic participants in Sekhukhune area. Ga-Nchabeleng Village had 135 volunteers and Mphanama Village had 65 volunteers who participated in the questionnaire survey. Questionnaires were set on aspects of demographic information, socio-economic and cultural aspects, indigenous knowledge, physico-chemical, mineralogical, geological and chemical aspects, ecological aspects and human health elements associated with geophagic practices (Appendix 1).

4.1.1 Demographic information of participants from Ga-Nchabeleng Village.

A section on demographic information associated with the consumption of geophagic soils in Ga-Nchabeleng Village showed 100% were from the rural settlement of the area. Ninety four percent of the participants were females and 5.2% were males. The majority of the respondents (77.1%) were single while 21.5% were married. The majority of the respondents (83%) had no source of income and (73.3%) were unemployed (Table 4.1).

Table 4.1: Demographic information of the respondents from Ga-Nchabeleng Village n=135

Location	Rural	Suburban	Urban		
%	100	0	0		
Sex	Male	Female			
%	5.2	94.8			
Age (Years)	≤20	21-30	31-40	41-50	≥ 50
%	10.4	30.4	43.0	14.1	2.1
Marital status	Married	Divorced	Single	Living together	
%	21.5	0.7	77.1	0.7	
Income source	Wage employment	None wage employment	Other		
%	16.3	83.0	0.7		
Occupation	Cleaner	Unemployed	Student		
%	16.3	73.3	10.4		
Income	≤ R2000	> R2000	None		
%	15.6	0.7	83.7		

4.1.2 Demographic information of participants from Mphanama Village.

The demographic information associated with the consumption of geophagic soils from 65 participants in Mphanama Village was also 100% rural. However, Unlike Ga-Nchabeleng Village which had more female geophagic participants, Mphanama Village had more male geophagic participants with 55.4% and females with 44.6%. The highest percentage of geophagic participants from Mphanama Village was between the ages of 41-50 with 29.3% (Table 4.2), different to respondents from Ga-Nchabeleng Village. Findings of this study were in contrast with the study conducted by Geissler et al. (1999) where women reported that men do eat soil but are very secretive about this practice. The statement was also supported by a study conducted by (Luoba *et al*., 2004) revealing that older boys and men felt ashamed by the practice. However, for this study especially in Mphanama Village, men were not secretive neither ashamed to reveal to soil consumption. In the study boys consumed soil even when they grew up and the

findings were in contrast to Zambian boys, who indicated to stop soil consumption as they grew up (Geissler *et al*., 1999).

Table 4.2: Demographic information of the respondents from Mphanama Village n=65

Location	Rural	Suburban	Urban		
%	100	0	0		
Sex	Male	Female			
%	55.4	44.6			
Age (Years)	≤20	21-30	31-40	41-50	≥ 50
%	29.2	13.8	10.8	29.3	16.9
Marital status	Married	Divorced	Single	Living together	
%	21.5	1.2	60.0	17.3	
Income source	Wage employment	None wage employment	Other		
%	1.5	98.5			
Occupation	Cleaner	Unemployed	Student		
%	1.5	70.8	24.6	3.1	
Income	≤ R2000	> R2000	None		
%	1.5	0.0	98.5		

Figure 4.1 illustrates the educational level of geophagic consumers from both study sites. Ga-Nchabeleng Village had 90% of consumers at secondary level while Mphanama Village had 28% of consumers at primary level. Mphanama Village also showed 6% of no schooling and 2% of tertiary level. The results from the studied Villages are concomitant with those reported in another study by Halsted (1968). From 225 geophagic consumers, 60% had secondary education, whereas 31% had only reached tertiary education

(Halsted, 1968). Studies conducted by Peter (2011) reported that the educational level was low as the majority had only secondary education (60.38%), primary (22.68%), tertiary (7.55%) and 6.60% did not have any formal education (Peter, 2011). Furthermore, studies conducted by Msibi *et al*. (2014) also reported results relating to the study. The results indicated that out of 94 participants 60.7% had secondary education level, 8.5% had never attended school and only 6.3% had tertiary education (Msibi *et al*., 2014). Another study conducted by Perridge *et al*. (2010) also reported that 63.6% had secondary education, 18.2% primary education and 18.2% no schooling. All these results indicate that majority of geophagic consumers only undergo degree of secondary level. Low level of education can have a negative impact on nutritional education and even on employment opportunities due to lack of knowledge (de Jager *et al*., 2013; Msibi *et al*., 2014) (Figure 4.1).

The level of education of geophagic consumers in the two Villages studied indicated that these consumers were sufficiently literate to understand the consequences of geophagic practices.

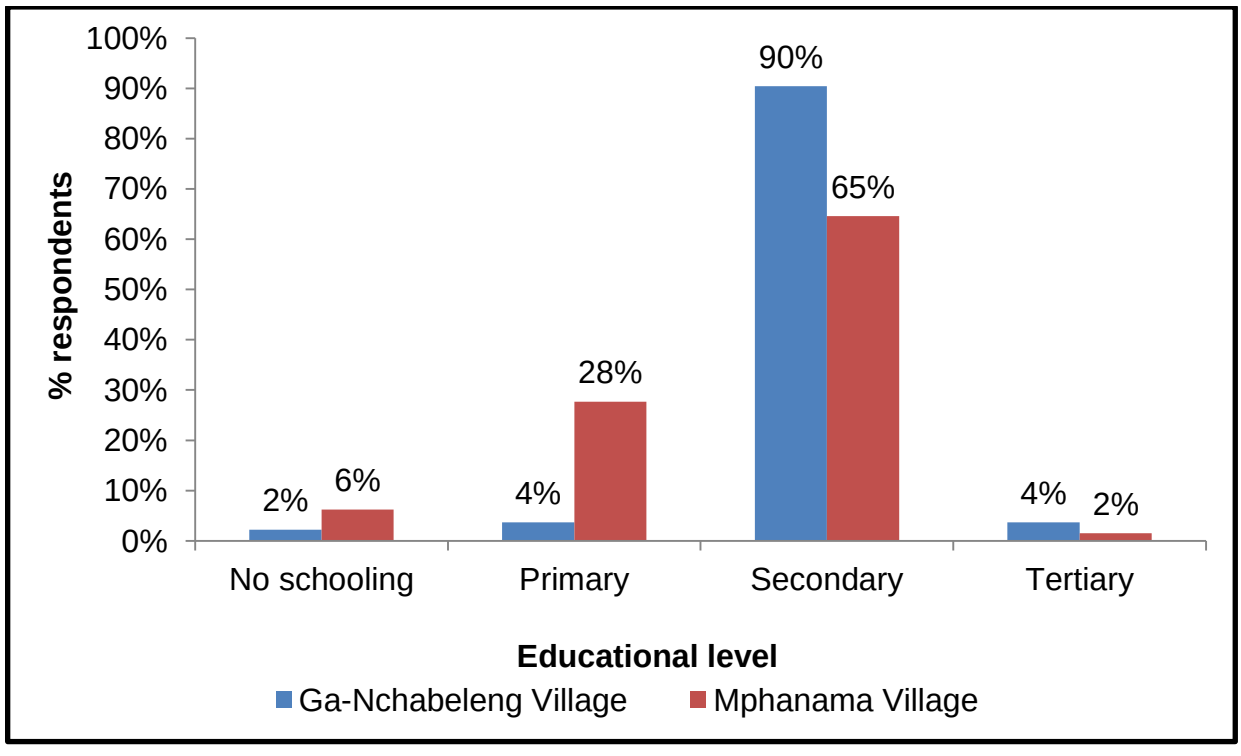

Figure 4.1: Educational level of geophagic consumers from the study sites.

4.1.3 Socio-economic and cultural aspects of geophagia.

Geophagic consumers from Ga-Nchabeleng Village showed 82.2% consumed soils. About 66.4% indicated that they have consumed soil for more than five years. Seventy eight percent craved the soil daily and 90.4% of the females indicated to have craved the soil when pregnant. This suggests that most of respondents were addicted to soil eating. Family members (73.9%) indicated to be aware of the practice and 87.2% of the respondents perceived the practice negatively (Table 4.3).

Table 4.3: Socio-economic and cultural aspects of geophagia from Ga-Nchabeleng Village (n=135)

Are you presently consuming soil?	Yes	No		
% (n=135)	82.2	17.8		
If yes, for how long have you been consuming soil?	≤ 2years	3-5 years	>5years	
% (n=110)	11.8	21.8	66.4	
Do you crave to eat soil?	Daily	Once a week	Once a month	Only when pregnant
% (n=127)	78.0	3.1	2.4	16.5
When do you crave for the soil?	Pregnant	Both pregnant & lactating	Experiencing sleeplessness	Feeling weak
% (n=71)	85.9	9.9	2.8	1.4
When pregnant how often do you consume the soil?	Daily	Weekly	Once a month	
% (n=73)	90.4	5.5	4.1	
Do other people know that you consume clay?	Yes	No	Don't know	
% (n=129)	87.6	9.3	3.1	
If yes who are aware?	Family members	Friends	Others	
% (n=115)	73.9	25.1	1	
How do people perceive this habit of eating non-food substances	Positive	Negative	Don't know	Indifferent
% (n=133)	1.5	87.2	1.5	9.8
Is this practice more common among certain members of the community?	Yes	Don't know		
% (n=133)	99.2%	0.8%		
If yes, specify	Classmates	Friends	Neighbours	
% (n=77)	1.3	11.7	87.0	

Results obtained from Mphanama Village showed that 93.8% consumed soil and 78.6% consumed soil for more than five years. About 91.7% of the respondents craved the soil daily and 84.6% craved soils when pregnant. The respondents also indicated the practice is perceived negatively at 63.1% as asked in the questionnaire (Table 4.4).

Table 4.4: Socio-economic and cultural aspects of geophagia from Mphanama Village (n=65).

Are you presently consuming soil?	Yes	No	
% (n=65)	93.8	6.2	
If yes, for how long have you been consuming soil?	≤ 2yrs	3-5yrs	>5yrs
% (n=61)	6.6	14.8	78.6
Do you crave to eat soil?	Daily	Once a month	Only when pregnant
% (n=60)	91.7	1.6	6.7
When do you crave for the soil?	Pregnant	Lactating	Both pregnant & lactating
% (n=13)	84.6	7.7	7.7
When pregnant how often do you consume the soil?	Daily		
% (n=20)	100.0		
Do other people know that you consume clay?	Yes	No	Don't know
% (n=60)	91.6	6.7	1.7
If yes who are aware?	Family members	Friends	Others
% (n=57)	49.1	49.1	1.8
How do people perceive this habit of eating non-food substances?	Positive	Negative	Indifferent
% (n=65)	1.5	63.1	35.4
Is this practice more common among certain members of the community?	Yes		
% (n=64)	100.0		
If yes, specify	Classmates	Friends	Neighbours
% (n=24)	37.5	4.2	58.3

The participants were further asked why they consumed the soil. For most of the respondents, craving was the main reason for soil consumption with 61% in Ga-Nchabeleng Village and 36% in Mphanama Village. Additionally, craving was also

reported by interviewees in the study conducted by Songca et al. (2010) in Free State and Limpopo. However, this was in contrast with a study conducted by Msibi et al. (2014) that revealed consumers ingested soil because of pregnancy. Other studies elsewhere have also indicated taste to be one of the reasons for soil consumption (Geissler *et al*., 1999; Perridge *et al*., 2010; Peter, 2011; Msibi *et al*., 2014). About 33% of the respondents from Ga-Nchabeleng Village consumed the soil when pregnant compared to the Mphanama Village where only 11% consumed soil when pregnant. The respondents from Mphanama Village used to take soil as standard practice (21%) and Ga-Nchabeleng Village with only two percent. Mphanama Village respondents consumed soil as a complementary diet and for ritualistic with 12% each and Ga-Nchabeleng respondents with only two and one percent respectively (Figure 4.2).

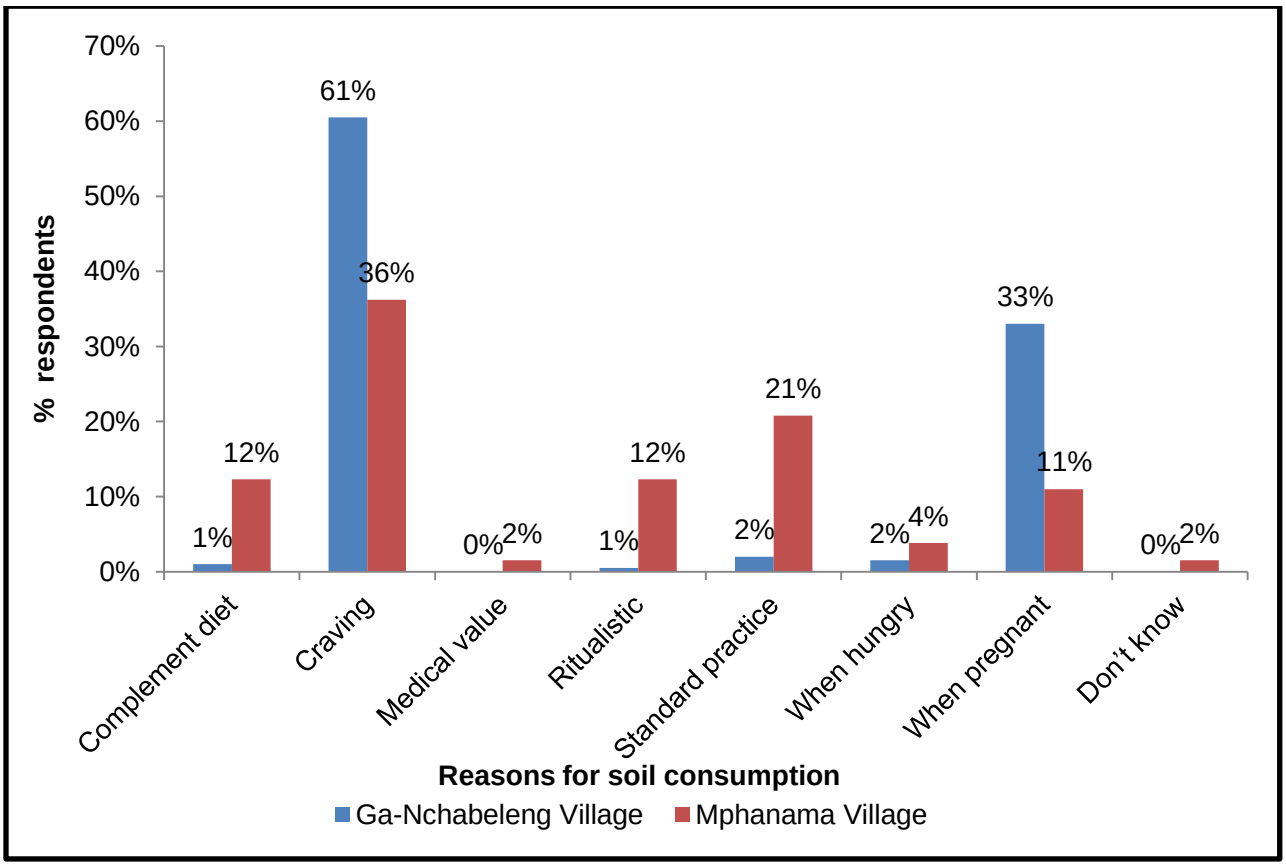

Figure 4.2: Health reasons advanced by geophagic consumers from the study sites.

Since the above results showed majority of the respondents craved for soil, it was important to establish the situation under which that happened. The results showed that

majorities craved more than once a day (76%) in Ga-Nchabeleng Village and 74% in Mphanama Village. The respondents also showed 26% of the soil was consumed once a day in Mphanama Village and 17% in Ga-Nchabeleng Village. These results on craved soils showed how much geophagic the consumers in the two study sites either pregnant or not (Figure 4.3). This revelation opposed what usually happens and is well known regarding geophagia because the finding in Ghana was that 63% of women were pregnant during the study (Vermeer, 1971). Even in Kenya the majority (56%) of women consumed soil when pregnant (Geissler *et al*., 1999). Furthermore, in another study of Bondo District, western Kenya, 54% of women were also pregnant (Luoba *et al*., 2004).

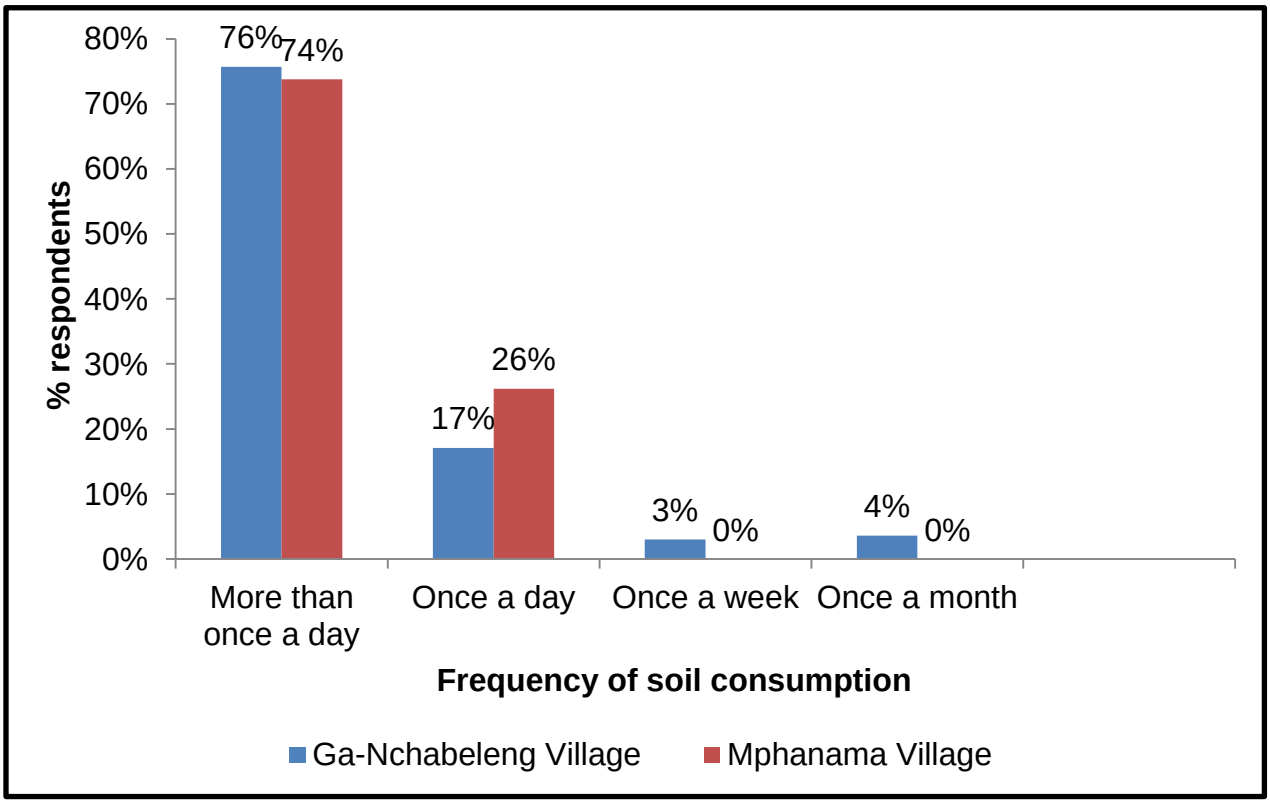

Figure 4.3: Frequency of soil consumption from study sites.

Respondents were also asked about eating other non-food items. Findings reflected that 67% of the respondents from Mphanama Village consumed chalk and 50% at Ga-Nchabeleng Village. Respondents from Ga-Nchabeleng Village consumed ice (18%) while Mphanama Village consumed a medicinal herb called *serokolo* (5%). Ashes, pencil lead and cow dung were also some of consumed non-food substances in the study sites

(Figure 4.4). Soil consumers are normally driven by different situations. The consumers mentioned to have started the habit on their own without any influence, whereas others said they were influenced by peer group. The rest was influenced by the following: saw somebody else in the family consuming and neighbours.

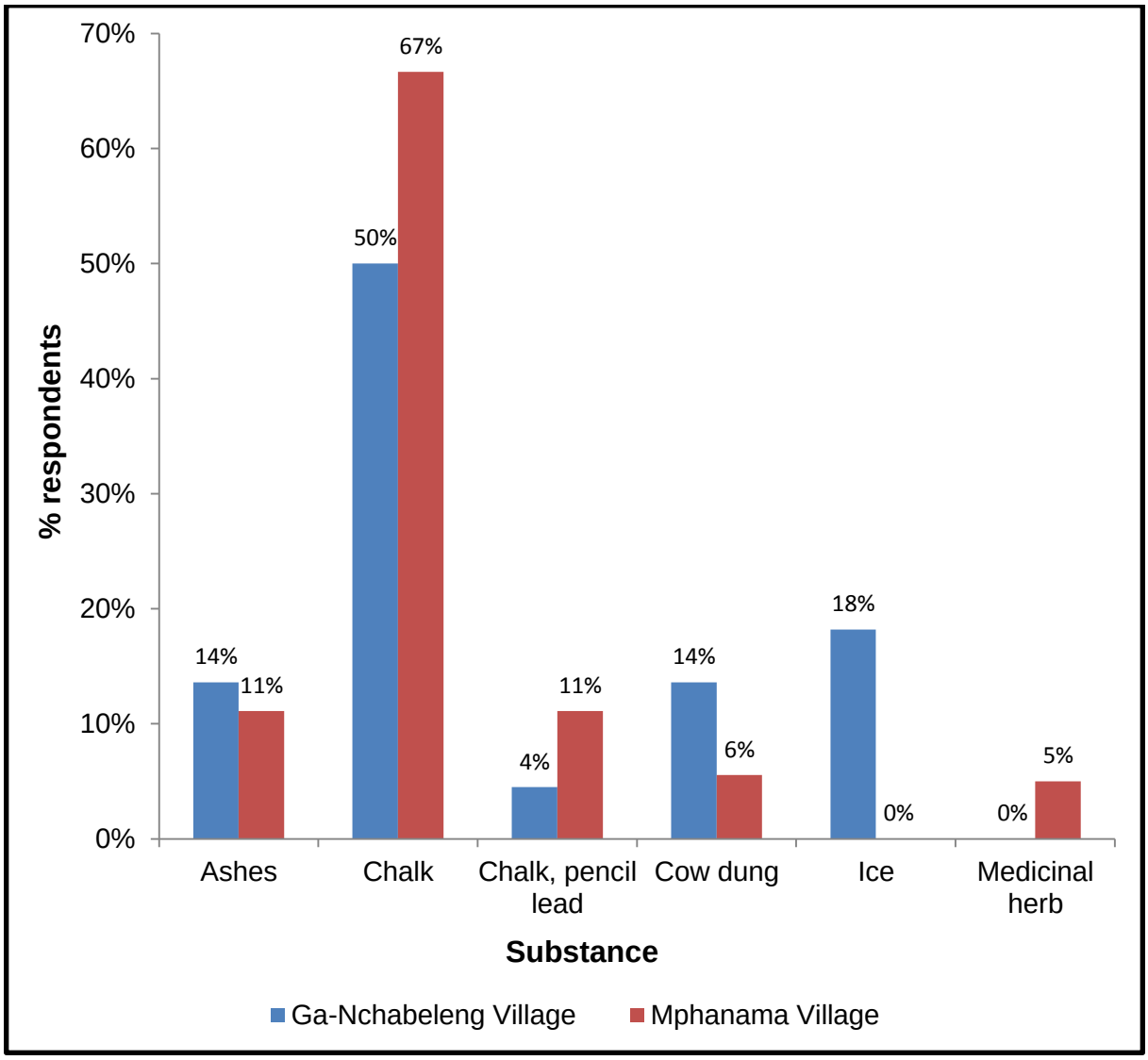

Figure 4.4: Other non-food substances consumed from the study sites.

4.1.4 Indigenous Knowledge

Respondents from Ga-Nchabeleng Village preferred soil (93.3%) more than clay (5.2%). As indicated in Table 4.5 total of 90.4% of respondents consumed soil locally called *mobu* and 96.2% of respondents were collecting the soil from the wild. The main reason

why some preferred to purchase soil was because it allows them to taste (84.2%) it. This was similar to findings in other parts of Southern African region (Ekosse *et al*., 2008). Other local names of soils that were consumed were *leraga* and *letsopa* as shown in (Table 4.5).

Table 4.5: Indigenous knowledge of the respondents from Ga-Nchabeleng Village (n=135)

What substances are consumed?	Clay	Soil	Soil from termite mounds	
% (n=135)	5.2	93.3	1.5	
How are substances consumed?	Wet	Dry	With other food	
% (n=135)	0.0	100.0	0.0	
What are the traditional names of substances consumed?	*Leraga*	*Letsopa*	*Mobu*	*Mobu & letsopa*
% (n=135)	0.7	5.2	90.4	3.7
Where do you obtain the substance?	Buy	From the wild	Given	
% (n=111)	2.3	96.2	1.5	
If you buy indicate the price per handful?	50C	R1	R2	R3
% (n=37)	33.3	40.7	22.3	3.7
Why do you prefer to consume the specific colour type?	Easily accessible	Taste	Tradition/belief	
% (n=24)	10.5	84.2	5.3	
Length of storage	1 day	2 days	one week	
% (n=4)	25.0	50.0	25.0	

Similar to the respondents in Ga-Nchabeleng Village, Mphanama respondents also preferred to consume soil (90.8%) compared to clay (9.2%) collected from the wild (96.8%). Even those that purchase soil, 100% of the respondents indicated that taste was the reason for buying the particular soil. The habit by the consumers in the area were also not very secretive and this was similar to what has been observed elsewhere (Nchito *et al*., 2004; Peter, 2011) and this suggested it might be as a result of copied behavior as they thought other people also practiced it. Local names were also the same as Ga-Nchabeleng Village (Table 4.6).

Table 4.6: Indigenous knowledge of the respondents from Mphanama Village (n=65)

What substances are consumed?	Clay	Soil	
% (n=65)	9.2	90.8	
How are substances consumed?	Dry	Wet	With other food
% (n=65)	95.4	3.1	1.5
What are the traditional names of substances consumed?	*Letsopa*	*mobu*	
% (n=65)	10.8	89.2	
Where do you obtain the substance?	From the wild	Given	
% (n=63)	96.8	3.2	
If you buy indicate the price per handful	50c	R1	R2
% (n=8)	25.0	62.5	12.5
Why do you prefer to consume the specific colour type?	Taste		
% (n=5)	100		
Length of storage	3 days		
% (n=1)	100		

As indicated from above; repondents stated that they purchase also other type of soils. About 60% of respondents preferred to buy soil with yellowish colour in Mphanama Village than Ga-Nchabeleng with 16%. It was followed by whitish soil (37%) in Ga-Nchabeleng Village and Mphanama Village (20%). Respondents from Ga-Nchabeleng Village preferred blackish soil (21%) and Mphanama Village did not prefer blackish soil. Khakhi soil was also preferred in both sites (Figure 4.5). Geophagic soils across the African continent are diverse in origin. An investigation carried by Ekosse *et al*. (2010) revealed that yellowish, whitish, khaki and black geophagic soil consumed in communities within South Africa were mined from hills and mountains, riverbeds, valleys, excavated sites and termiteria using selective digging, hand grabbing and picking techniques.

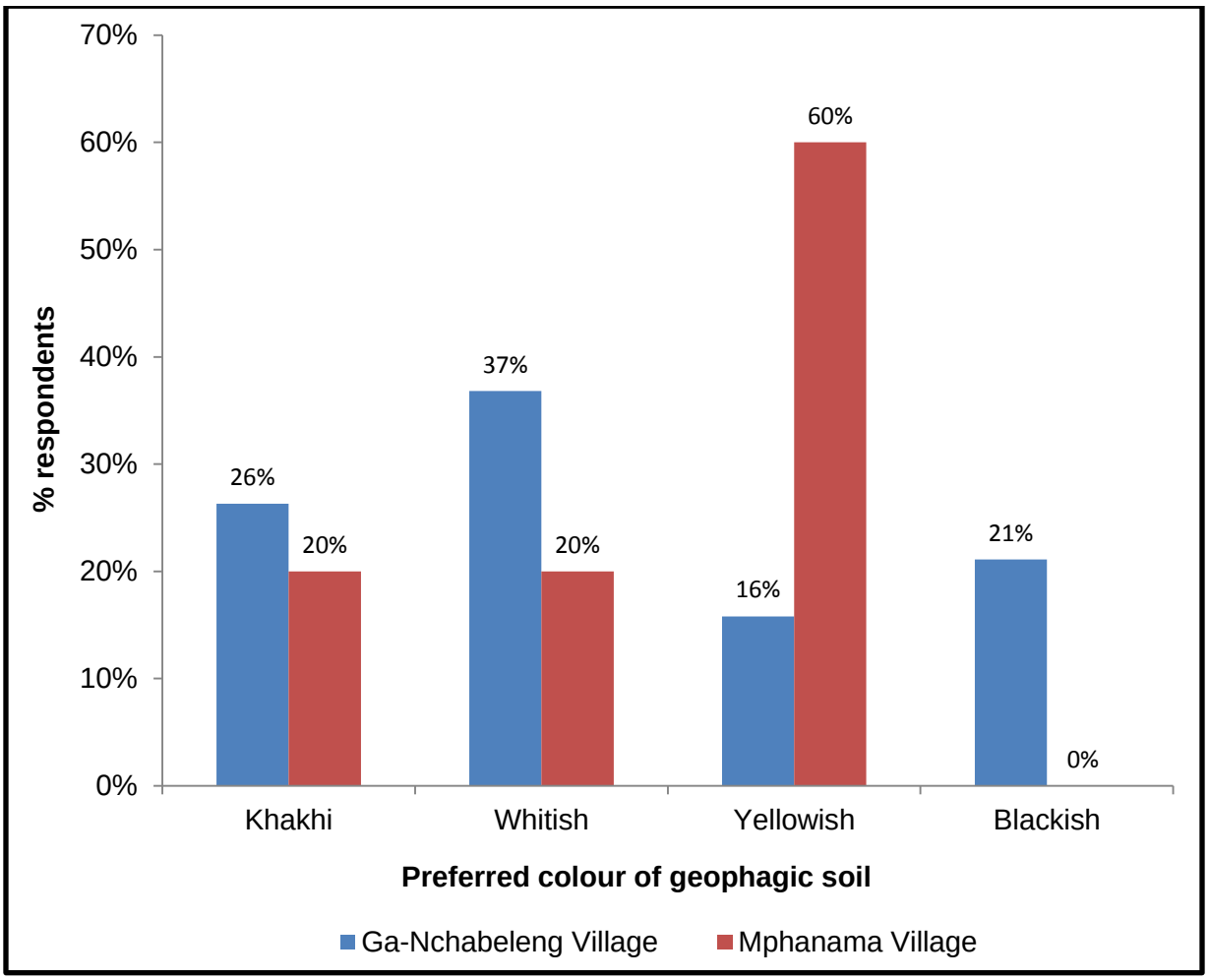

Figure 4.5: Preferred colour of geophagic soil consumed by respondents.

4.1.5 Physico-chemical, mineralogical, geological and chemical aspects.

Soil collection as indicated by respondents from Ga-Nchabeleng Village was not collected close to rocks (84.9%). About 83.3% of the respondents indicated to dig dry (95.6%) soil at 0-10 cm deep. The majority of the respondent also indicated not to process the soil (97.9%) before consumption neither does heat treatment (98.9%), (Table 4.7). However, previous studies revealed geophagic soils often undergo some degree of processing which may include pounding, grinding, slurrying, and various heat treatments (Ekosse *et al*., 2010). Typically, heat treatment of geophagic soils is believed to enhance physical properties, such as colour, taste, and also eliminates potentially pathogenic micro-organisms present in the soils (Reilly and Henry, 2001; Hunter, 2003; Young *et al*., 2007; Ekosse *et al*., 2010). The heat treatment also reduces water content, making the soil to attain that powdery, chunky nature preferred by most soil consumers and palatability (Msibi *et al*., 2014).

Table 4.7: Physico-chemical, mineralogical, geological and chemical aspects of geophagia in Ga-Nchabeleng Village (n=135).

Are substances obtained close to rocks	Yes	No		
% (n=93)	15.1	84.9		
If yes, what is the type of rock	Hard	Soft	Very hard	
% (n=14)	57.1	28.6	14.3	
If digging how deep	0-10 cm	10-20 cm	>30 cm	others
% (n=90)	83.3	14.4	2.2	1.5
When are substances collected?	Dry	Doesn't matter		
% (n=90)	95.6	4.4		
Are substances processed before consumption?	Yes	No	Sometimes	
% (n=94)	1.1	97.9	1.1	
Is there any heat treatment of substance before consumption?	Yes	No		
% (n=91)	1.1	98.9		

Unlike Ga-Nchabeleng Village, respondents from Mphanama Village collected soil close to rocks (61.4%). Respondents' also preferred dry soil (72.7%) collected from hard rocks (88.2%). No form of treatment is done before consumption (95.5%) (Table 4.8).

Table 4.8: Physico-chemical, mineralogical, geological and chemical aspects of geophagia in Mphanama Village (n=65).

Are substances obtained close to rocks	Yes	No	
% (n=44)	61.4	38.6	
If yes, what is the type of rock	Hard	Soft	Very hard
% (n=17)	88.2%	5.9	5.9
If digging how deep	0-10 cm	10-20 cm	20-30 cm
% (n=45)	62.2%	35.6%	2.2%
When are substances collected?	Dry	Doesn't matter	
% (n=44)	72.7%	27.3%	
If collected wet, how does the substance feel?	Sticky		
% (n=1)	100%		
Are substances processed before consumption?	Yes	No	
% (n=44)	2.3%	97.7%	
Is there any heat treatment of substance before consumption?	Yes	No	
% (n=44)	4.5%	95.5%	

Respondents in Ga-Nchabeleng Village collected geophagic soil primarily from riverbeds (64%) not close to rocks, whereas Mphanama respondents collected from hill/mountains (43%) which were close to rocks. Some respondents also indicated that they obtained, to

a considerable extent, their geophagic soil from valleys, termitaria etc (Figure 4.6). These results are similar to findings conducted elsewhere (Geissler *et al*., 1998; Reilly and Henry, 2000; Allport, 2006; Ekosse *et al*., 2010; Msibi *et al*., 2014).

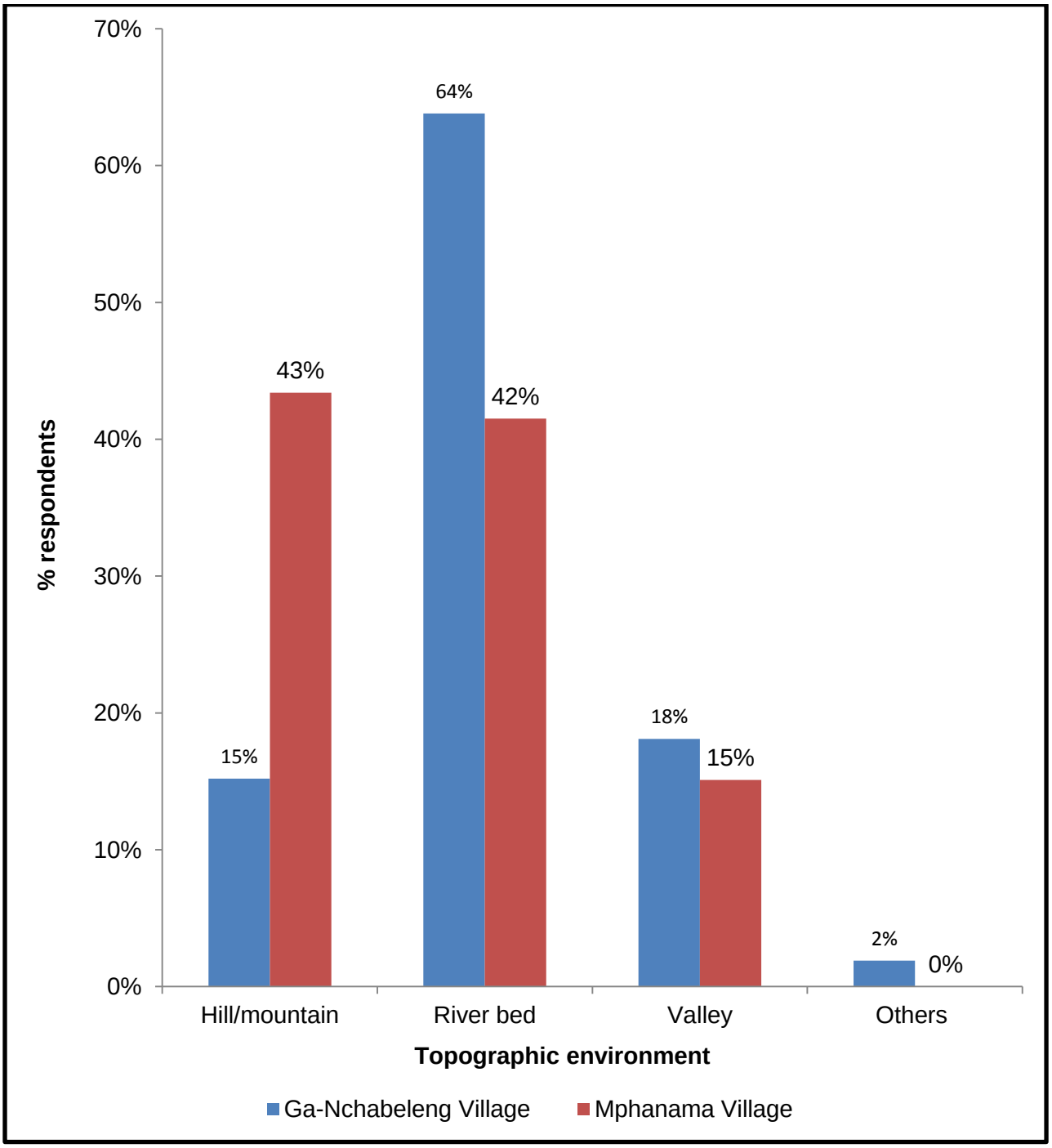

Figure 4.6: Type of geomorphological environment where geophagic soils were obtained in the study sites.

Respondents reported that geophagic soils were collected by employing one or more of the following traditional mining techniques: Digging, hand grabbing, scraping, and selective hand picking (Figure 4.7). Over 80% of respondents from both Villages indicated that geophagic soils were traditionally mined using digging technique (Figure 4.7). The digging technique in Sekhukhune studied Villages was similar to techniques used in Qwa-Qwa and Mangaung. Over 80% of the geophagic consumers used digging technique to collect soils (Ekosse *et al*., 2010; Perridge *et al*., 2010).

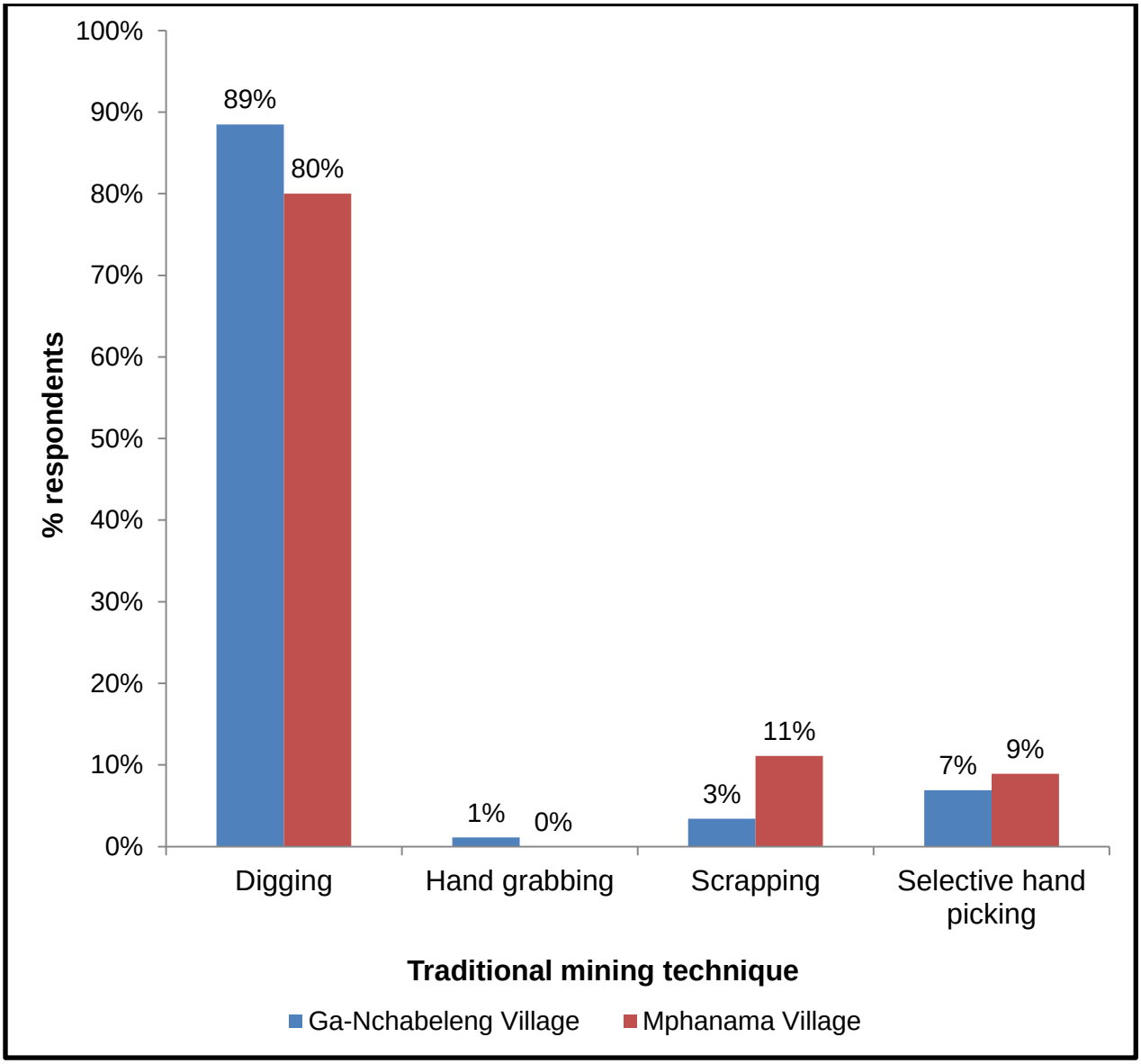

Figure 4.7: Type of traditional mining technique employed by geophagic consumers in the study sites.

According to the respondents from both Villages, the state of how the soils felt like did not matter to them. Only 42% from Ga-Nchabeleng respondents reported the soils were powdery, whereas Mphanama respondents were 33%. Some of the respondents preferred geophagic soils which were gritty and silky (Figure 4.8). The findings are similar to study conducted by Ekosse *et al*. (2010) indicating that geophagic clays from Limpopo Province were grittier and more powdery than those from Free State Province.

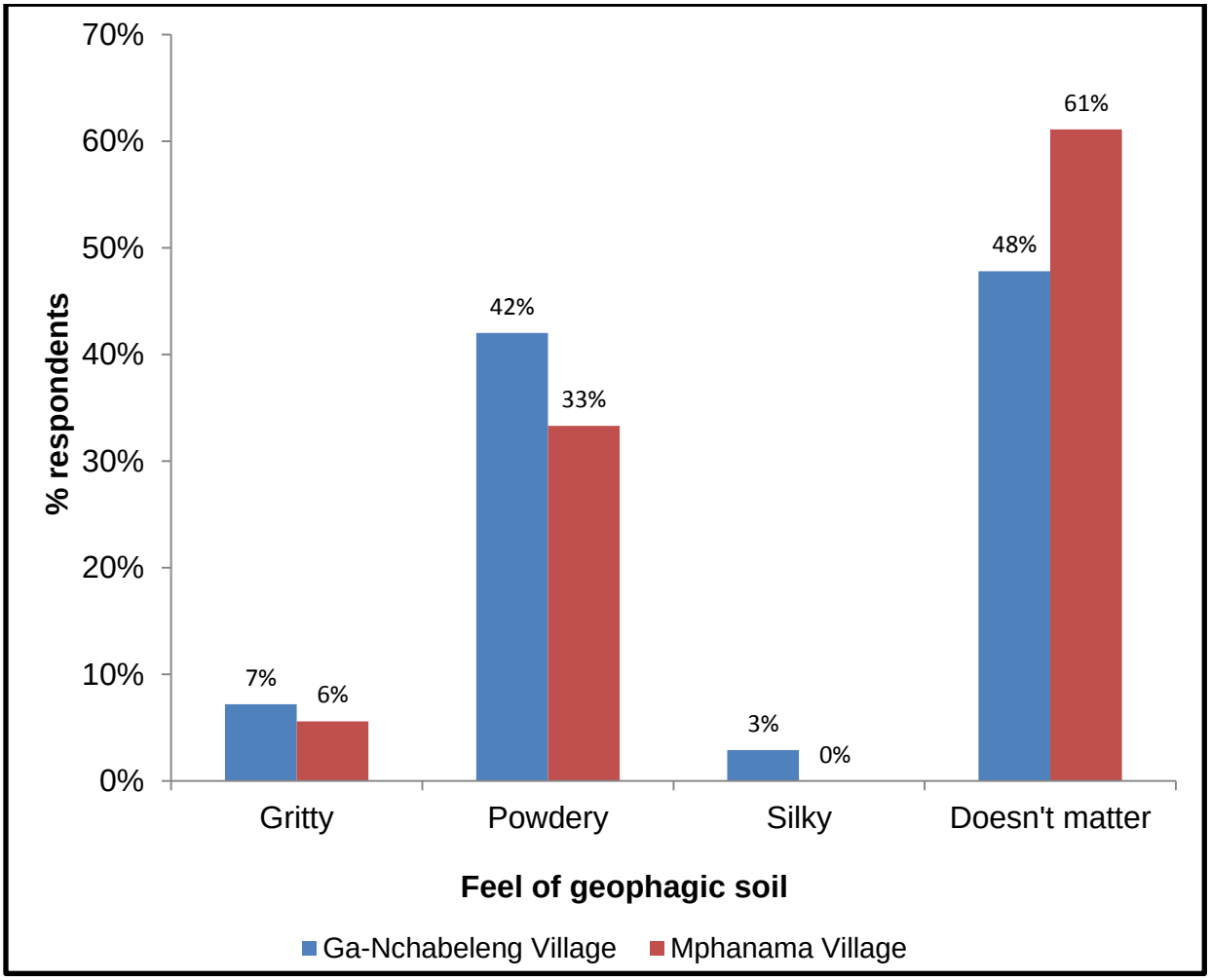

Figure 4.8: Textural feel of geophagic soils from the study sites.

4.1.6 Ecological aspects of geophagia

The respondents from Ga-Nchabeleng Village indicated to collect soil from trees (81.8%) than mound and 75% preferred to collect soil from the terrain of the valley. Respondents also indicated preferences of particular trees (75%), (Table 4.9).

Table 4.9: Ecological aspects in Ga-Nchabeleng Village (n=135).

If you consume substance from a termiteria, from which one?	Mound	Tree
% (n=11)	18.2	81.8
Do you prefer to consume the substance when?	Old	Does not matter
% (n=11)	45.5	54.5
What type of terrain do you normally find these mounds?	Undulating	Valley
%(n=4)	25.0	75.0
If substance is collected from a tree, do you prefer it from a particular tree?	Yes	No
% (n=8)	75.0	25.0

The ecological aspects of geophagic respondents from Mphanama Village were similar to Ga-Nchabeleng Village. Respondents preferred soil from tree (94.4%), from the valley (66.7%) type of terrain and also had specific type of trees from where soils were collected (Table 4.10).

Table 4.10: Ecological aspects in Mphanama Village (n=65).

If you consume substance from a termiteria, from which one?	Mound	Tree	
% (n=18)	5.6	94.4	
Do you prefer to consume the substance when?	Old	Does not matter	
% (n=16)	56.3	43.8	
What type of terrain do you normally find these mounds?	Hilly	Undulating	Valley
%(n=6)	16.7	16.7	66.7
If substance is collected from a tree, do you prefer it from a particular tree?	Yes		
% (n=14)	100.0		

The respondents showed to have particular trees where soil were collected thus it was important to establish names of particular trees preferred by the respondents from both Villages. Respondents from Mphanama Village preferred soil from the Red bushwillow (*Combretum apiculatum*) tree (38%) and Ga-Nchabeleng respondents (29%). Ga-Nchabeleng respondents preferred soil from the White thorn *Acacia* (*Vachellia tortilis*)

tree (32%) whereas Mphanama respondents preferred soil from Mountain Kirkia (*Kirkia wilmsii*) and Shepherd trees (*Boscia albitrunca*) (17%). Responds from the participants on plants have indicated that, some of these trees will contribute to the toxicity in geophagic soils (Figure 4.9).

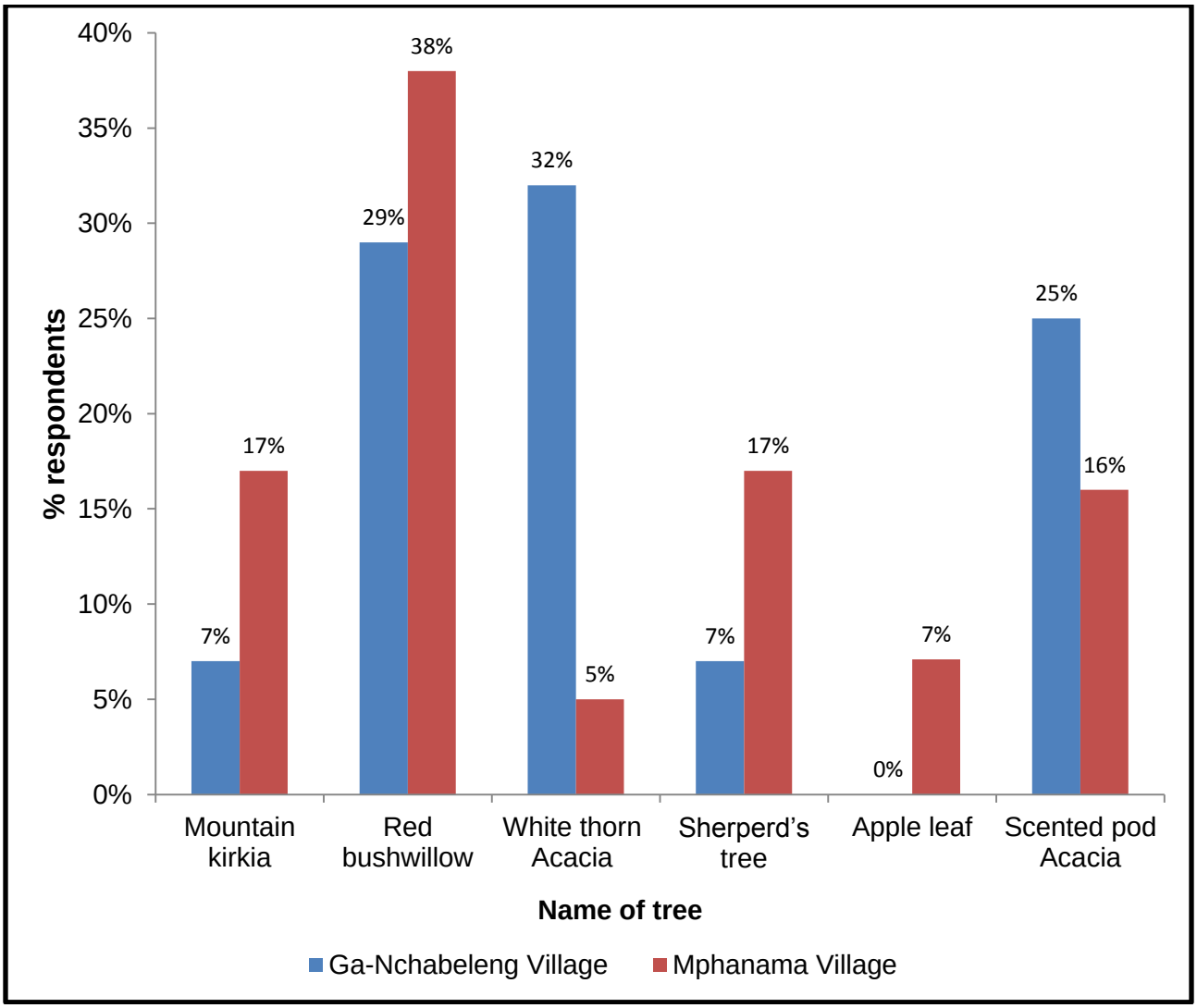

Figure 4.9: Trees preferred by geophagic consumers from the study sites.

4.1.7 Human health associated with geophagia

About sixty one percent of geophagic consumers in Ga-Nchabeleng Village did not know the soil they were consuming could be harmful to their health. They were not aware (94.4%) of the harmful substances/plant remains or parasites present in the soil. Only a

small percentage (3.2%) of the respondents did not know but suspected what was contained in the soils they ingested (Table 4.11).

Table 4.11: Human health associated with geophagia in Ga-Nchabeleng Village (n=135).

Do you know that the substance could be harmful to your health?	Yes	No		
% (n=126)	38.9	61.1		
Any operation done in the stomach?	No	Yes		
% (n=125)	100	0		
Are you aware of the harmful substances/ parasites that may be present in the substance?	Yes	No		
% (n=126)	5.6	94.4		
Do you know the content of the substance?	Yes	No		
% (n=125)	3.2	96.8		
If yes name these contents	Iron (Fe)			
%(n=4)	100			
Reason for consumption	For additional nutritional value	Don't know		
% (n=126)	2.4	97.6		
Do you often get infected with cold, flu?	Once a month	Once every 3 months	Twice yearly	Yearly
% (n=122)	1.6	1.6	7.4	89.4
Do you ingest when infected?	Yes	No	Sometimes	
% (n=126)	8.7	82.6	8.7	

Results from the respondents in Mphanama Village with regards to human health were similar to respondents from Ga-Nchabeleng Village. About 75.5% of geophagic

respondents in Mphanama Village did not know that the soil they were consuming could be harmful (Table 4.15). They were not even aware (100%) of the harmful substances/plant remains or parasites present in the soil. Only a small percentage (1.9%) of the respondents knew what was contained in the soils they ingested (Table 4.12).

Table 4.12: Human health associated with geophagia in Mphanama Village (n=65).

Do you know that the substance could be harmful to your health	Yes	No	
% (n=53)	24.5	75.5	
Any operation done in the stomach?	No	Yes	
% (n=53)	100.0	0	
Are you aware of the harmful substances/parasites present in the substance?	Yes	No	
% (n=53)	0	100.00	
Do you know the content of the substance	Yes	No	
% (n=53)	1.9	98.1	
If yes name these contents	Iron (Fe)		
%(n=11)	100.0		
Why do you consume substance?	For additional nutritional value	Don't know	
% (n=53)	1.9	98.1	
Do you often get infected with cold, flu	Once every 3 months	Twice yearly	Yearly
% (n=43)	4.7	9.3	86.0
Do you ingest when infected	Yes	No	Sometimes
% (n=50)	2.0	86.0	12.0

Reasons advanced by geophagic consumers to justify their belief that the soil they were ingesting could be harmful vary from one individual to another. Majority of the respondents admitted that eating soil was not helpful because it resulted in many health

problems. Respondents from both sites Ga-Nchabeleng (58%) and Mphanama (77%) Villages indicated that constipation could be the reason why soil is believed to be harmful (Figure 4.10).

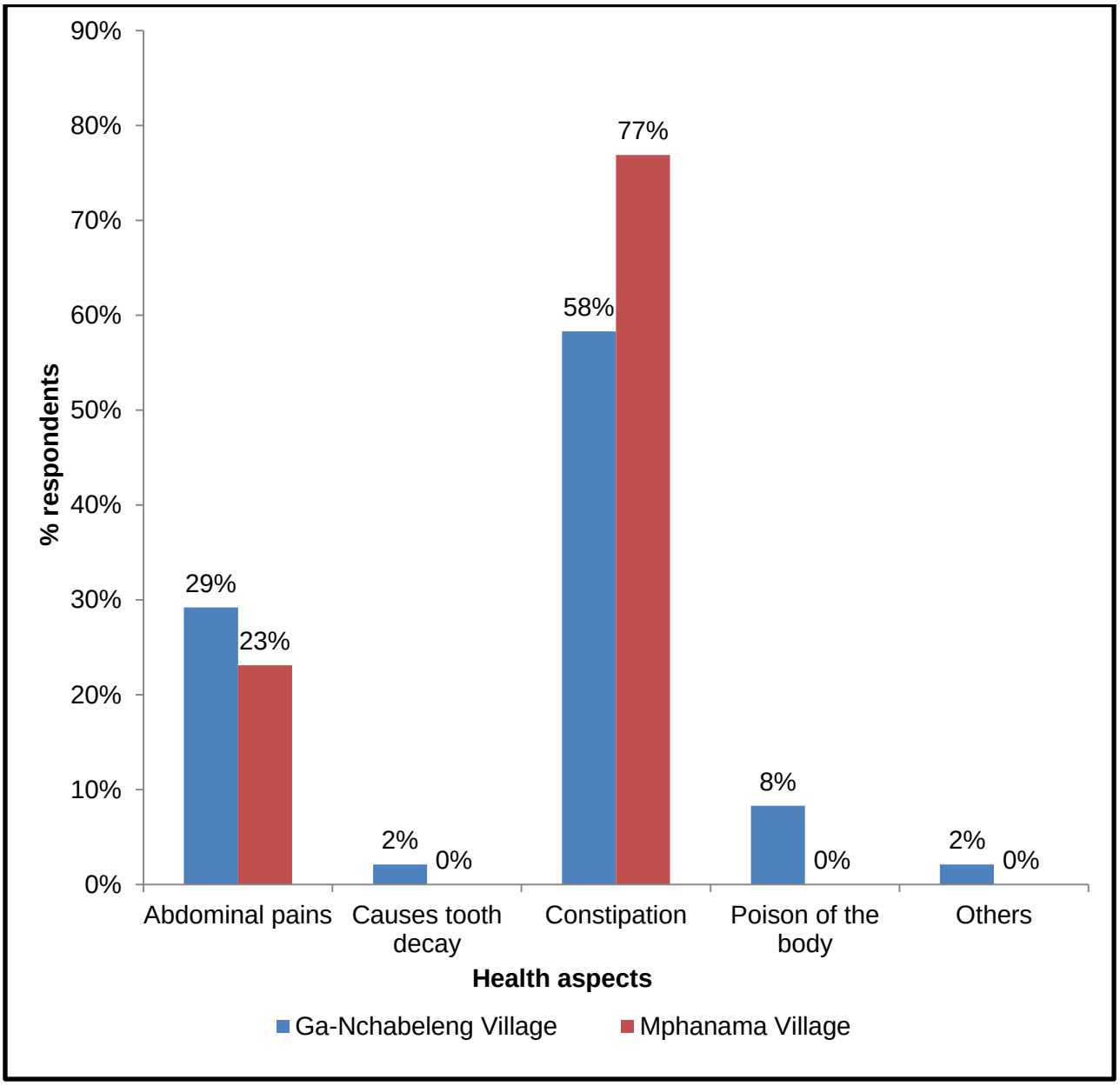

Figure 4.10: Health effects of soil ingestion from studied areas.

4.1.8 Control group analyses.

The control group was drawn from 28 non-geophagic consumers compared with geophagic consumers from the area studied (Table 4.13).

Table 4.13: Demographic, socio-economic and cultural aspects of geophagic group and control group.

Gender	Male	Female			
% Geophagic group (n=172)	24.40%	75.60%			
% Control group (n=28)	3.6%	96.4%			
Age	≤20	21-30	31-40	41-50	≥ 50
% Geophagic group (n=172)	16.30%	22.10%	32.60%	20.90%	8.10%
% Control group (n=28)	17.9%	42.9%	32.1%	7.1%	0.0%
Marital status	Married	Divorced	Single	Living together	
% Geophagic group (n=172)	22.70%	0.00%	70.30%	7.00%	
% Control group (n=28)	14.3%	3.6%	78.6%	3.6%	
Income source	Wage employment	None wage employment	Other		
% Geophagic group (n=172)	9.90%	89.50%	0.60%		
% Control group (n=28)	21.4%	78.6%	0.0%		
Educational level	No schooling	Primary	Secondary	Tertiary	
% Geophagic group (n=172)	3.50%	12.80%	80.20%	1.00%	
% Control group (n=28)	1.6%	3.6%	92.3%	2.50%	
Do geophagic consumers consume other none food substances?	Yes	No			
% Geophagic group (n=40)	97.50%	2.50%			
Control group (n=28)	84.7%	15.3%			

Crosstabulation with Chi-square ($\chi 2$) test was used to associate age, gender, economic and cultural status/aspects with geophagia. Chi-square ($\chi 2$) analyses revealed a significant association of gender with geophagic habits ($p<0.05$), while there was no association of age, educational level, income source and marital status ($p>0.05$) with geophagic habits. Findings of the survey revealed that more females (75.60%) practice geophagia compared to males (24.40%), (Figure 4.14).

Table 4.14: Factors associated with geophagia satisfaction.

Characteristic	Number of individuals	Ever practised N (%)	P-value
Age group:			0.064
≤20	33	28 (16.3%)	
21-30	50	38 (22.15)	
31-40	65	56 (32.6%)	
41-50	38	36 (20.9%)	
≥ 50	14	14 (8.1%)	
Gender:			0.013*
Male	43	42 (24.40%)	
Female	157	130 (75.60%)	
Marital status:			0.055
Married	43	39 (22.70%)	
Divorced	1	0 (0%)	
Single	143	121 (70.30%)	
Living together	13	12 (7.00%)	
Income source:			0.194
Wage employment	23	17 (9.90%)	
None wage employment	176	154 (89.59%)	
Other	1	1 (0.69%)	
Occupation:			0.606
cleaner	23	17 (10.40%)	
unemployed	145	122 (74.80%)	
student	32	24 (14.70%)	
Income:			0.84
≤ R2000	22	16 (9.80%)	
> R2000	1	1 (0.60%)	
none	177	146 (89.60%)	
Educational level:			0.853
No schooling	7	6 (3.70%)	
Primary	23	22 (13.50%)	
Secondary	164	129 (79.10%)	
Tertiary	6	6 (3.70%)	

*df= degree of freedom, †statistically significant

Chi-square ($\chi 2$) analyses also revealed that there was no association of geophagic habits and consumption of other non-food substances ($p>0.05$). There was also no association between occupation, income and educational level with buying of consumed earth materials ($p>0.05$) (Table 4.15). No association was found to all questions related to health aspects with geophagia (Table 4.16).

Table 4.15: Factors associated with geophagia and other non-food substances.

	Chi-square	df*	*p*-value
Do geophagic consumers consume other non-food substances?	5.374[a]	2	0.068
Occupation of geophagic consumers who buy soil	1.002[a]	2	0.606
Income of geophagic consumers who buy soil	.349[a]	2	0.84
Education level of geophagic consumers who buy soil	.787[a]	3	0.853

Table 4.16: Human health associated with geophagia & statistical comparison of the two groups (n=200).

					p-value
Are you aware of the harmful substances/ parasites that may be present in the substance?	Yes	No			
Geophagic group (%, n=151)	4.6%	95.4%			
Control group (%, n=28)	0%	100.00%			**0.245**
Do you know the content of the substance?	Yes	No			
Geophagic group (%, n=151)	2.6%	97.4%			
Control group (%, n=27)	3.7%	96.3%			**0.760**
Do you often get infected (common cold, flu etc)?	Once a month	Once every 3 months	Twice yearly	Yearly	
Geophagic group (%, n=138)	0.7%	2.2%	9.4%	87.7%	
Control group (%, n=27)	3.7%	3.7%	0.00%	92.6%	**0.214**
Do you experience chronic illnesses?	Yes	No			
Geophagic group (%, n=139)	9.4%	90.6%			
Control group (%, n=26)	0.00%	100.00%			**0.104**

4.2 Soil colour classification of geophagic sites

Colour is the first diagnostic parameter and most important criterion used by geophagic consumers to determine suitability of soil for consumption (Ekosse and Anyangwe, 2012). It is used to infer on palatability of the geophagic soil. Geophagic consumers also have different sites where they collect their soils. The study group was drawn from sites where geophagia was extensively used and very common, whereas the control site was drawn from sites where geophagia was not practiced in the study area.

Six geophagic sites were from Ga-Nchabeleng Village and another six sites from Mphanama Village, whereas the five control sites were collected from areas not consumed. The soil samples from studied sites were classified using the Munsell Soil Color Chart (2000). Most of the samples taken from geophagic sites were fine loose dry material with decomposed vegetation and dead plant remains. The colours of the geophagic soils comprised of three main colour groupings, namely brownish, yellowish and reddish, with brownish the most prevalent. Colour of sampled soils varied with geographical regions of Sekhukhune area.

Soil colour from Ga-Nchabeleng Village (Table 4.17) geophagic sites ranged from dark reddish brown at (*Sekubeng Ga-Solly and leotswaneng* 2) location sites, followed by brown at *Mmashaku* and *Magotwaneng* to very dark brown at (*Sekubeng-leotswaneng* 1) location sites, then to very dark grayish brown at *Phororong* location sites. These colours were related to the colours described by von Well. (2013), who stated that soils from Ga-Nchabeleng Village are brownish black to reddish. The table below shows variety of soil colours as classified by the Munsell soil colour chart (Table 4.17) from Ga-Nchabeleng Village geophagic studied sites.

Table 4.17: Summary of information on six well known geophagic sites in Ga-Nchabeleng Village.

Sample site no	Location name	Geographic coordinates	Hue, value and chroma	Colour of sample
1	Sekubeng Ga-Solly	24°26´36´´S 29°50´34´´E	5 YR 3/3	Dark reddish brown
2	*Sekubeng-leotswaneng* 1	24°26´52´´S 29°50´00´´E	7.5 YR 2.5/3	Very dark brown
3	*Sekubeng-leotswaneng 2*	24°26´52´´S 29°49´59´´E	2.5 YR 3/3	Dark reddish brown
4	*Mmatadi*	24°26´35´´S 29°50´05´´E	7.5 YR 4/3	Brown
5	*Phororong*	24°26´15´´S 29°50´17´´E	10 YR 3/2	Very dark brown
6	*Magotwaneng*	24°26´19´´S 29°50´25´´E	7.5 YR 4/3	Brown

Type of soil consumed according to percentage of consumers

The type of soil consumption was important because it gave an indication of the type of soil colour preferred. Figure 4.11 gives the colour of soil consumed according to the consumers. Consumers in Ga-Nchabeleng Village consumed variety of colours evenly which were dark reddish brown (33.3%), very dark brown (33.3%) and brown (33.3%).

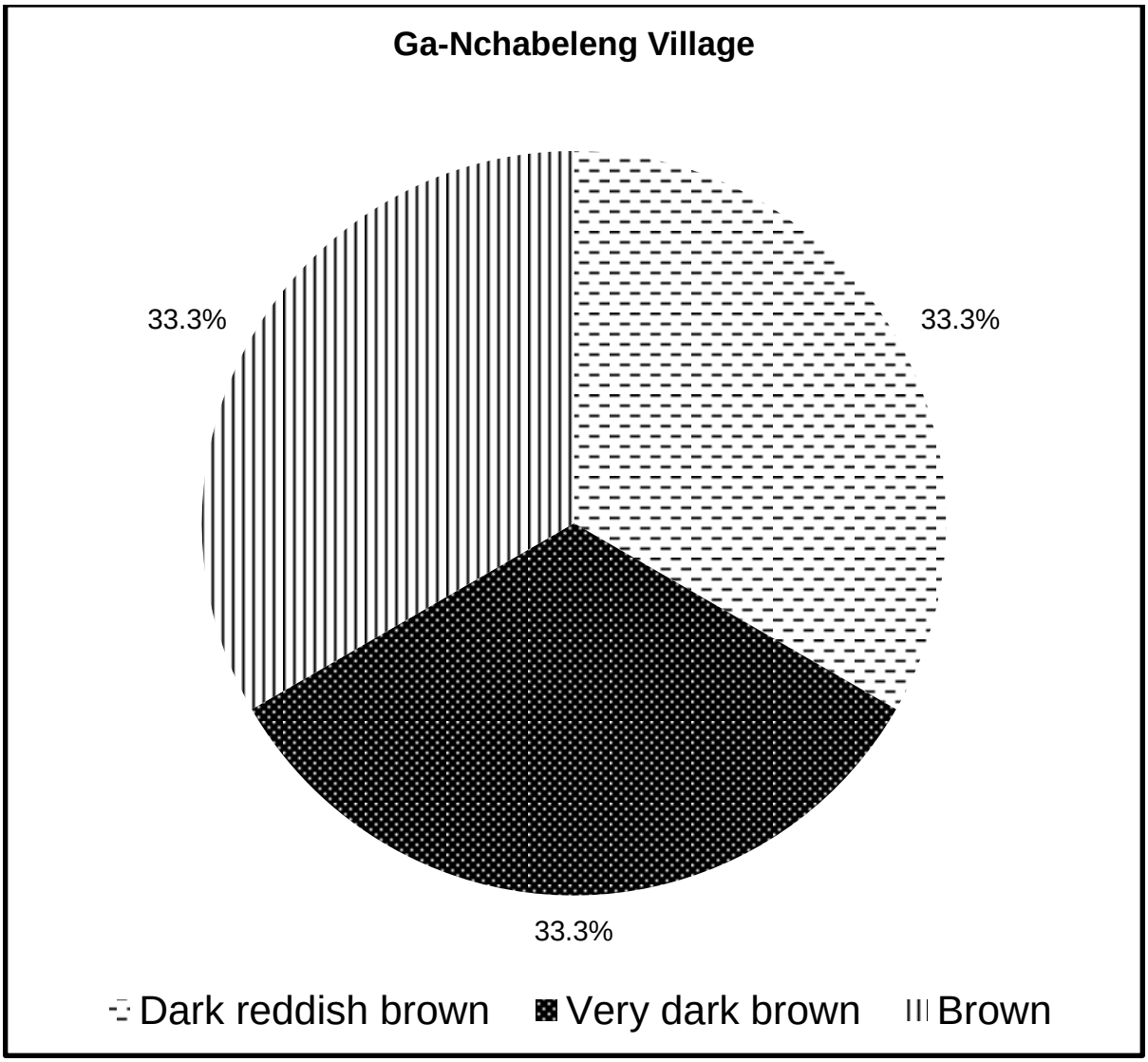

Figure 4.11: Percentage distribution of different soil colours consumed from Ga-Nchabeleng Village.

However the colours from Ga-Nchabeleng Village were different from those in Mphanama Village (Table 4.18). According to soil colour classification, soils from Mphanama Village ranged from yellowish red at *Moshate* location site to dark yellowish brown at *Bodula* location site. *Ga-Photo*, *Malaeneng-leotswaneng* and *Malaeneng-mmako* location sites had red colour. The colour from *Morakong* location site was bright reddish brown. The colours classified from Mphanama Village were also related to those described by von Well, (2013), who stated that Mphanama Village has red-yellow soils of which the red-coloured are weakly structured clay soils.

Table 4.18: Summary of information on 6 well-known geophagic sites in Mphanama Village.

Site no	Location name	Geographic coordinates	Hue, value and chroma	Colour of sample
7	*Morakong*	24°35´39´´S 29°48´43´´E	2.5 YR 5/8	Bright reddish brown
8	*Ga-Photho*	24°34´42´´S 29°48´43´´E	2.5 YR 4/8	Red
9	*Malaeneng-leotswaneng*	24°34´43´´S 29°48´42´´E	2.5 YR 4/8	Red
10	*Moshate*	24°35´38.9´´S 29°48´43.3´´E	5 YR 4/6	Yellowish red
11	*Malaeneng-mmako*	24°35´39´´S 29°48´43´´E	2.5 YR 4/6	Red
12	*Bodula*	24°35´42´´S 29°48´45´´E	10 YR 4/6	Dark yellowish brown

Half of the consumers in Mphanama Village (50%) consumed red soil, followed by bright reddish brown, yellowish red and dark yellowish brown at 16.7% each (Figure 4.12). One reason for human geophagic practice is mineral nutrient supplementation (Momoh *et al*., 2011). It suggests that colour; particularly the common reddish *hue* is an indicative of a useful presence of iron found in majority of soils may act as a primary or secondary stimulus in this respect (Halsted, 1968; Wilson, 2003; Young *et al*., 2010). This is in agreement with the study conducted in Limpopo and Free State by Songca et al. (2010) where most people preferred red soils. The *hue* value is consistent with the work of Ngole et al. (2010) which infer the occurrence of goethite and hematite in the samples of geophagic materials from South Africa. Other studies have reported that reddish and yellowish coloured clay are used as sun screens in the Eastern Cape Province (Hoang-Minh *et al*., 2010).

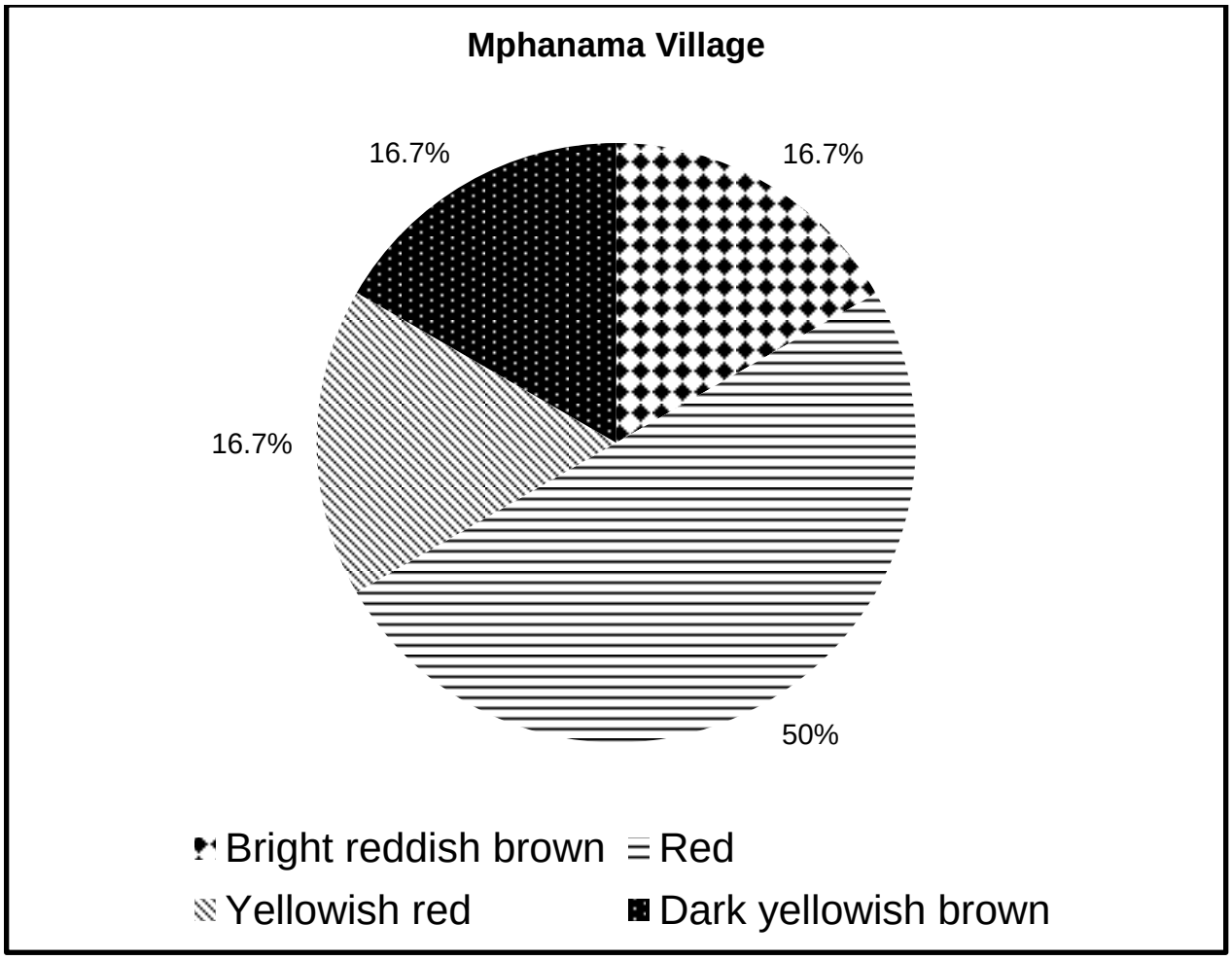

Figure 4.12: Percentage distribution of different soil colours consumed from Mphanama Village.

Soil samples from control sites were also classified according to Munsell Soil Colour Chart as well (Table 4.19). Unlike the colours obtained from geophagic sites in Ga-Nchabeleng and Mphanama Villages which were brownish red and reddish yellow respectively, control sites were different shades of brown. However, it was also observed that soils from some control sites had similar colour classification such as *Makgaleng* and *Lekoribeng* locations, the colours were all brown similar to *Magotwaneng* location. *Phororong* control site which was few metres away from *Phororong* location site had similar colour of very dark brown. *Sekubeng* control site also had similar colour of dark reddish brown to *Sekubeng* location site which was extensive. Lastly *leribeng* control site had yellowish brown (Table 4.19).

Table 4.19: Summary of information on five control sites

Control Site no	Location name	Geographic coordinates	Hue, value and chroma	Colour of sample
1	*Makgaleng*	24°26´24´´S 29°50´00,6´´E	7.5 YR 4/4	Brown
2	*Phororong*	24°26´14.2"S 29°50´15.2´´E	10 YR 3/2	Very dark brown
3	*Leribeng*	24°26´19.7´´S 29°50´25.4´´E	10 YR 5/4	Yellowish brown
4	*Sekubeng*	24°26´53.4´´S 29°49´58.9´´E	5 YR 3/3	Dark reddish brown
5	*lekoribeng*	24°26´54´´S 29°48´00.7´´E	7.5 YR 4/4	Brown

The control sites had similar soil colours as compared to Ga-Nchabeleng and Mphanama Villages. Forty percent was brown soil, followed by dark reddish brown (20%) and very dark brown (20%) followed by yellowish brown (20%), which were not consumed. However, the higher percentage of brown from control could indicate geophagic consumers find brown non-attractive and unpalatable to their taste as compared to red which contains iron supplement, even though previous studies have indicated use of brown for cosmetic purposes (Figure 4.13).

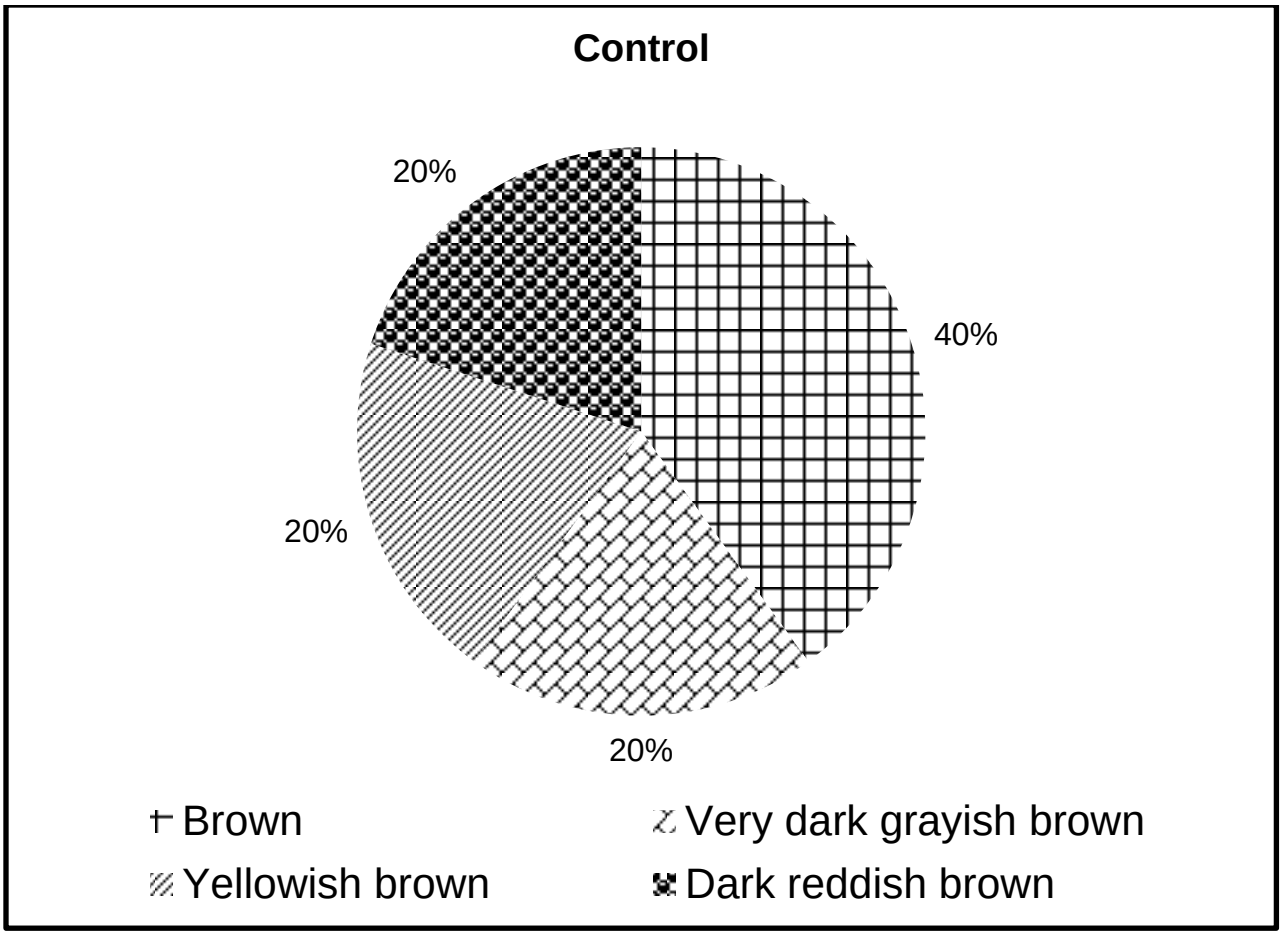

Figure 4.13: Percentage distribution of different soil colours from the control sites.

4.3 Plant remains

4.3.1 Identification of plants

Geophagic consumers in Ga-Nchabeleng Village identified four plant species of plant remains in soil that were being consumed in the area. The dead plant species were found to be mostly grasses and *Acacia* species. The plants were generally called by their vernacular names; *Jwang*-Couch grass, *Mookana*-White thorn *Acacia, Sehlwa*-Tassel three-awn *and Sehlwa sa noka*-Broad-leaved curly leaf. During collection of the plant remains samples it was discovered that roots (50%) were the most parts of plant remains found in geophagic soils consumed, followed by stems (33.3%) and leaves (16.7%), (Table 4.20).

Table 4.20: Plant species consumed as plant remains in geophagic soils from Ga-Nchabeleng Village.

Geophagic site no	Scientific name	Common name	Vernacular name	Parts of dead plant parts
1	*Cynodon dactylon*	Couch grass	*Jwang*	Leaves, roots
2	*Vachellia tortilis*	White thorn *Acacia*	*Mookana*	Stems, roots
3	*Vachellia tortilis*	White thorn *Acacia*	*Mookana*	Stems, roots
4	*Vachellia tortilis*	White thorn *Acacia*	*Mookana*	Stems, leaves and roots
5	*Aristida congesta subsp. Congesta,*	Tassel three-awn	*Sehlwa-*	Roots and stems
6	*Eragrostis rigidior*	Broad-leaved curly leaf	*Sehlwa sa noka*	Roots

Geophagic consumers in Mphanama Village identified five plant species of plant remains consumed in geophagic soils. The plant remains in the area were different as compared to Ga-Nchabeleng Village plants. They were of prostrate perennial herbs and big woody trees. The perennial herbs consumed were called *Tšhašo*-Rhodesian weeds, *Tshehlo*-Khaki weed and woody plants were *Mohwelere*-Red bushwillow tree, *Mohlopi*-Shepherd tree and *Modumela*-Mountain Kirkia tree. Geophagic consumers in Mphanama Village consumed soils with stem parts (54.5%), followed by roots (36.4%) and leaves (9.1%), (Table 4.21). Control plant species were discovered on sites where geophagia was not practiced. The plant remains were found to be of Acacia species. The local names of two Acacia plants were: Mogohlo-Scented pod Acacia and Mošu-Black wattle. The control site of plant remains had more leaves (62.5%), followed by stems (25%) and roots

(12.5%). This is an indication that geophagic consumers from both Villages preferred soils with roots than leaves (Table 4.21).

Table 4.21: Plant species consumed as plant remains in geophagic soils from Mphanama Village.

Geophagic site no	Scientific name	Common name	Vernacular name	Parts of dead plant parts
7	*Alternanthera pungens* kunth	Rhodesian weeds	*Tšhašo*	Stems, roots
8	*Alternanthera lorentzii*	Khaki weed	*Tshehlo*	Stems, leaves
9	*Alternanthera pungens* kunth	Rhodesian weeds	*Tšhašo*	Stems, roots
10	*Combretum apiculutum*	Red bushwillow	*Mohwelere*	Stems
	Kirkia wilmsii	Mountain kirkia	*Modumela*	
	Boscia albitrunca	Shepherd's tree	*Mohlopi*	
11	*Boscia albitrunca*	Shepherd's tree	*Mohlopi*	Roots and stems
12	*Kirkia wilmsii*	Mountain Kirkia	*Modumela*	Roots and stems
Control plant remains species				
13	*Vachellia nilotica*	Scented pod *Acacia*	*Mogohlo*	Leaves, roots
14	*Acacia mearnsii*	Black wattle	*Mošu*	Leaves
15	*Acacia mearnsii*	Black wattle	*Mošu*	Leaves
16	*Vachellia tortilis*	White thorn *Acacia*	*Mookana*	Leaves, stems
17	*Vachelia tortilis*	White thorn *Acacia*	*Mookana*	Leaves, stems

The parts of plant remains mostly consumed were roots (50%) in Ga-Nchabeleng, whereas Mphanama Village consumed more stems (54.5%) and the control site had leaves at 62.5% (Figure 4.14). The results obtained in this study compares favorably with

findings of Steenkamp (2003). He reported the widespread use of roots by most South African women for certain ailments. Geophagic consumers in Sekhukhune area consumed more roots in the soils and that might contribute to particular ailments, however only speculations. Even similar findings reported by Hedge *et al*. (2008) noted an extensive use of roots for treating reproductive ailments in India.

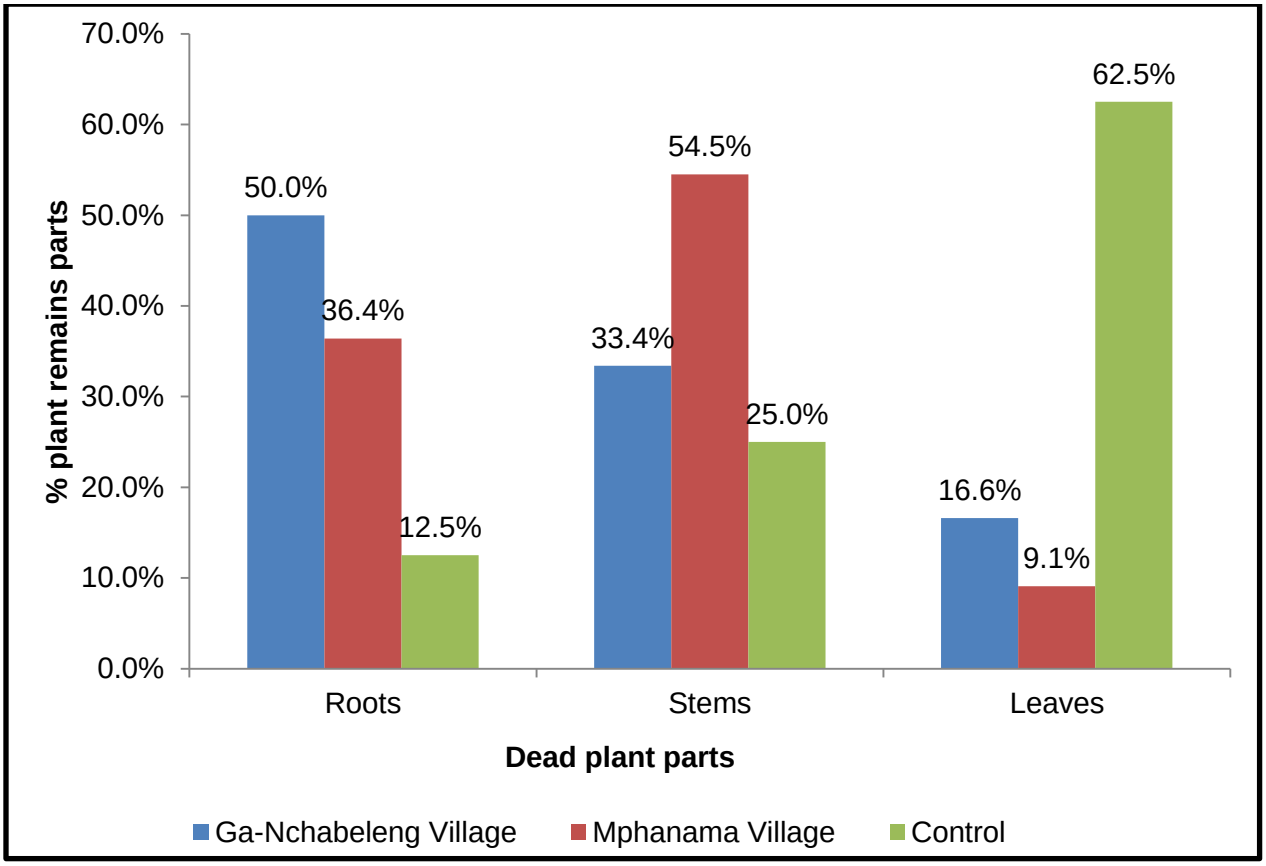

Figure 4.14: Plant remains parts consumed in geophagic soils.

4.4 Cytotoxicity of plant remains extracts.

A major component of the study was to characterise the plant remains content in geophagic soils consumed by humans in Sekhukhune area and infer their possible health effects using cytotoxic effect of methanolic extracts of plant remains on HEK-293T cells. Consumers in Sekhukhune area consumed geophagic soils containing a variety of plant remains and did not know the content of the plant remains if were toxic or non-toxic. The plant remains were grouped as a composite study focusing on their similar characteristics/features.

To evaluate this, screening for cytotoxicity effects of the plant remains extracts were tested on HEK-293T cells. Most *in vivo* and *in vitro* studies have reported results based on the screening of chemical constituents isolated from different plants on HEK-293T cells and other disorders (Bylund *et al*., 2004; Patel and Tikoo, 2006; Nanyonga *et al*., 2013; Lin *et al*., 2014; Ahmadi *et al.*, 2016). However, studies conducted on plant remains in geophagic soils used as extracts on HEK-293T cells for cytotoxicity evaluation have not been adequately documented so far. Below is a figure showing a 96 well plate of plant remains extracts with cells in a two-fold dilution in triplicate from the concentration (500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 µg/ml). Lane 1 to 3 (plant remains extracts one), followed by lane 4 to 6 (plant remains extracts two), lane 7 to 9 (plant remains extracts three) then lane 10 to 12 were for (plant remains four) (Figure 4.15).

1 2 3 4 5 6 7 8 9 10 11 12

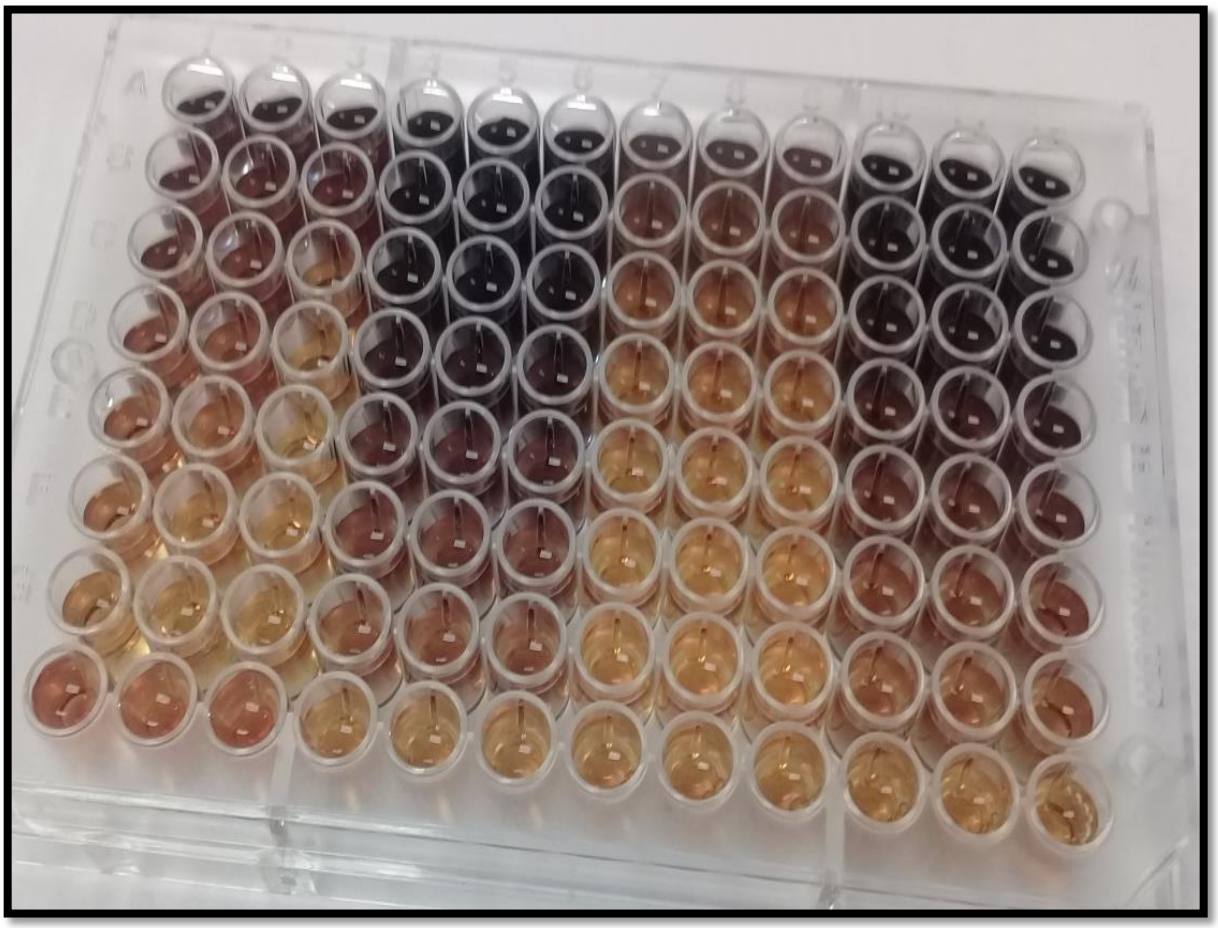

Figure 4.15: 96 well plate layouts for cytotoxicity assay.

4.4.1 Cytotoxicity effect of plant remains extracts on HEK-293T cells by MTT

To evaluate the cytotoxic effect of plant remains extracts on HEK-293T cells and also account for 50% viable cells, MTT assay was conducted. MTT assay measured the cell viability based on the reduction of yellow tetrazolium MTT to a purple formazan dye mitochondrial dehydrogenase enzyme. So, the amount of formazan produced reflected the number of metabolically active viable cells. MTT results showed the extracts possessed cytotoxic effect on HEK-293T cells at different concentrations (Figure 4. 16).

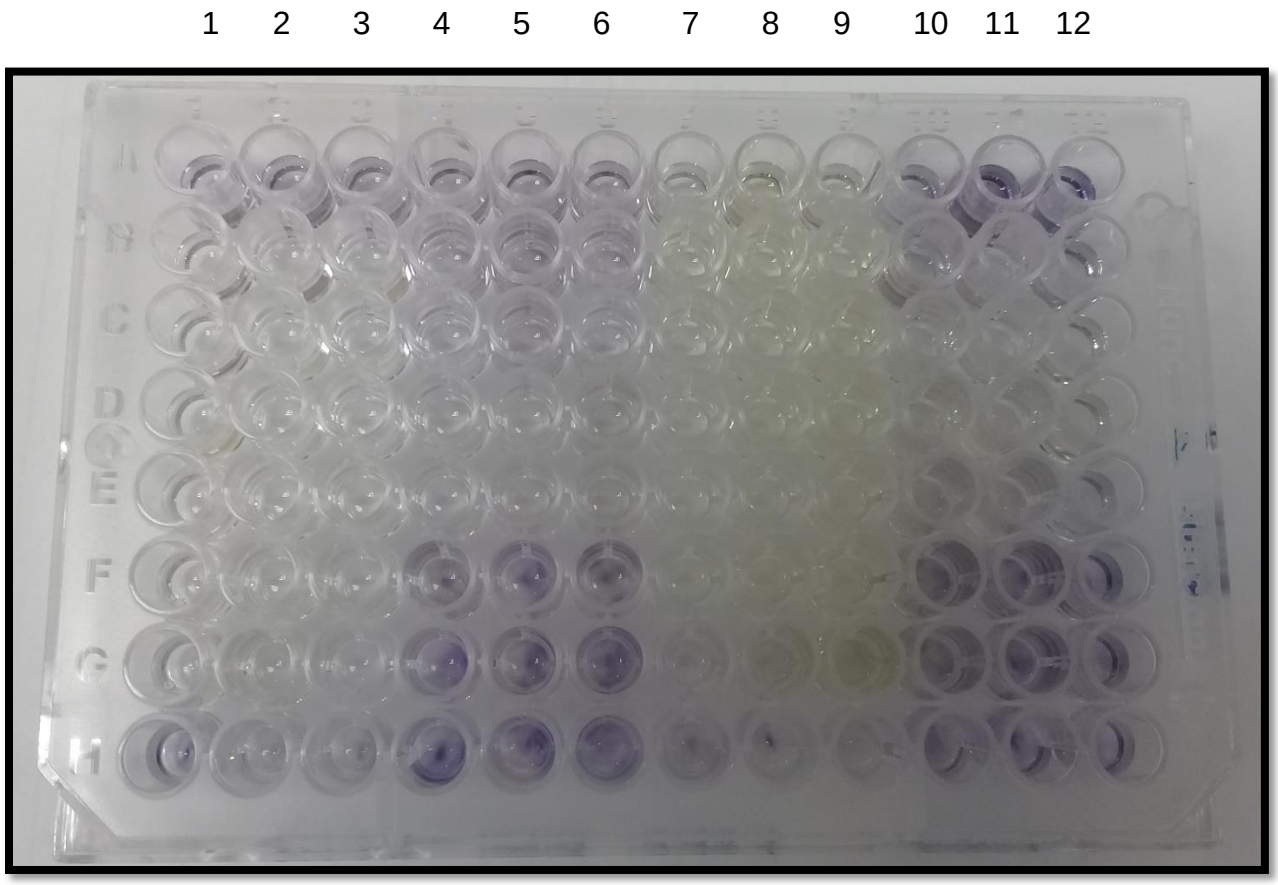

Figure 4.16: 96 well plate layouts of cytotoxicity assay results showing the purple formazan.

Table 4.22 below shows the results obtained from cytotoxicity profile on HEK-293T cell line by MTT. The concentration used was (500-7.8125 µg/ml). Methanol solvent showed the mean value of the plant remains extracts and the percentages of cell viability as well as the control (Table 4.22).

Table 4.22: **Cytotoxicity of plant remains extracts against 293T cell line by MTT assay.**

Solvent		**Methanol**						
Concentrations µg/ml		**500**	**250**	**125**	**62.5**	**31.25**	**15.625**	**7.8126**
Optical Density (OD) sample	Plant remains extracts 1	0.064	0.054	0.054	0.048	0.054	0.075	0.170
	% of Cell viability	27.9	23.5	23.9	20.9	23.6	32.8	74.2
	Plant remains extracts 2	0.080	0.083	0.084	0.108	0.118	0.226	0.230
	% of Cell viability	34.9	36.2	36.7	46.9	82.1	98.8	100.0
	Plant remains extracts 3	0.050	0.056	0.048	0.045	0.049	0.073	0.133
	% of Cell viability	21.8	24.5	20.9	19.7	21.4	31.9	58.1
	Plant remains extracts 4	0.096	0.065	0.061	0.079	0.128	0.137	0.226
	% of Cell viability	41.9	28.4	26.6	34.5	55.9	59.8	98.5
	Plant remains extracts 5 (control)	0.053	0.084	0.163	0.161	0.179	0.162	0.164
	% of Cell viability	23.1	36.8	71.2	70.3	78.5	70.7	71.6
OD Control (Cells only)		0.229						

% Cell Viability=OD Sample/OD Control x 100

Line graph below represented the percentage cell viability of the different methanol plant remains concentrations (500-7.8125 µg/ml) on HEK-293T cell line. The graph showed the cytotoxicity of different plant remains extracts on HEK-293T cell line. Cell viability increased when the concentration decreased. As the concentration of the plant remains extracts decreases, cell viability increases. The results of the study revealed plant remains extracts ranged in the order from most toxic to less toxic (13.75>16.68>58.95>92.75>251.4 µg/ml) on HEK-293T cells (Figure 4.17).

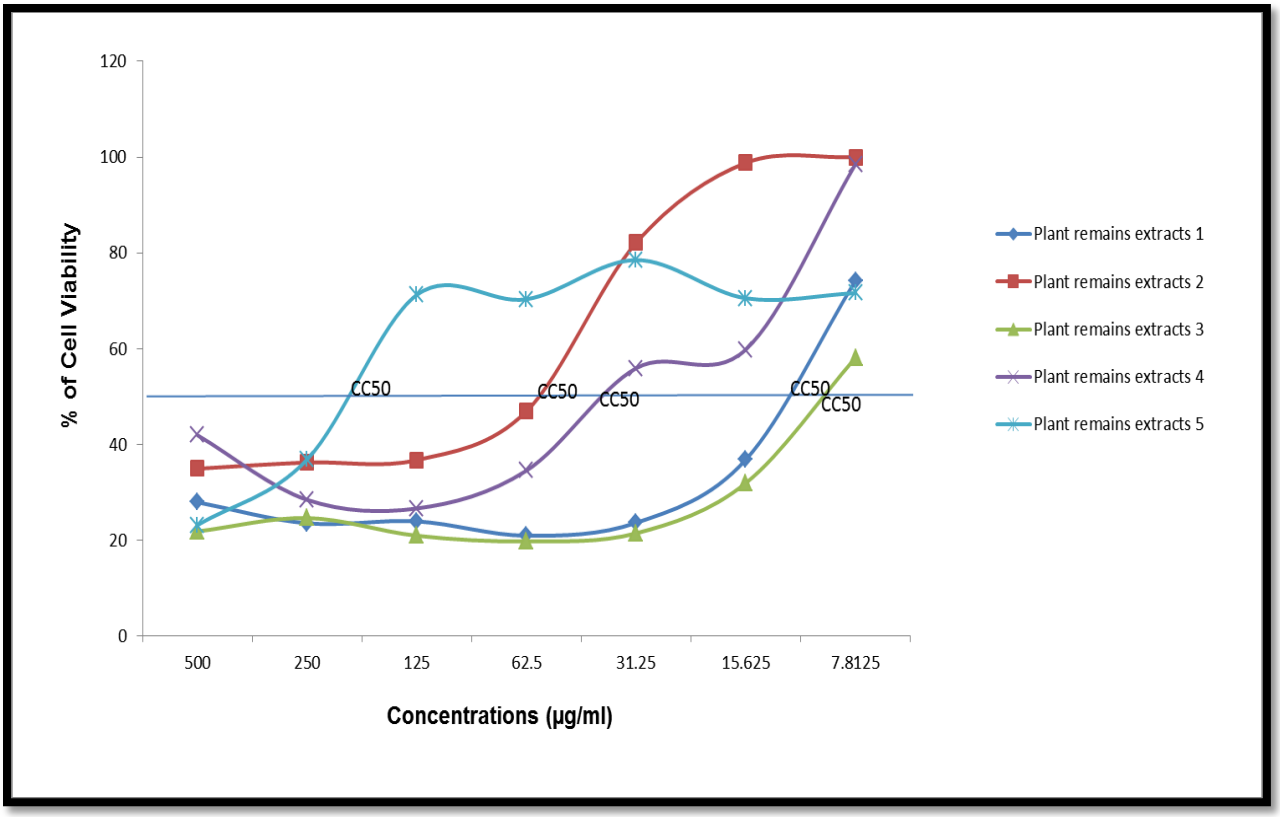

Figure 4.17: Line graph representation of percentage cell viability of 293T cell line and plant remains extracts concentrations.

Couch grass (*Cynodon dactylon),* Tassel three-awn *(Aristida congesta subsp. Congesta)* and Broad-leaved curly leaf *(Eragrostis rigidior)* are all perennial grasses that belong to the family Poaceae. The grasses are commonly known as *Jwang, Sehlwa* and *Sehlwa sa noka* by Bapedi speaking people in Sekhukhune area. Grasses contain several nutrients and possess medicinal properties (Dhanalakshmi *et al*., 2016). Grasses are related to soils and dead plants which promote the formation of different soil types (Nabradi, 2007). The use of grasses, herbs and plants gathered from meadows and the wild is still traditional and characteristic in many countries and their use is in health care as medicinal raw materials and wellness (Nabradi, 2007). In the present chapter of the study we aimed to evaluate the cytotoxicity of plant remains of grasses consumed in soils by geophagic consumers in Sekhukhune area.

The results of plant remains extracts 1 from Ga-Nchabeleng Village which was composed of the above combined grasses showed a Cytotoxic concentration of 50%

(CC_{50}) at 16.68 µg/ml. Geophagic site one had 12.6% of geophagic consumers who consumed soil with *Cynodon dactylon* grass (Table 4.23). Although the concentration value of the combined grasses was shown to be 16.68 µg/ml and killing 50% of viable cells in the study, previous studies have indicated that *Cynodon dactylon* grass is not toxic. Pringproa *et al*. (2014) conducted a study on *in vitro* virustatic and virucidal tests of the crude extract of *Cynodon dactylon* against infection with porcine reproductive and respiratory syndrome virus (PRRSV). Crude extract of *Cynodon dactylon* was prepared for cytotoxicity on tissue-culture cells that were used to measure virustatic and virucidal activities against PRRSV. The crude extract at 0.78 mg/ml showed no cytotoxicity on the cell line and at that concentration significantly inhibited replication of PRRSV as early as 24 hours post infection (Pringproa *et al*., 2014). Another study by Kidder *et al*. (1961) and Ndyanabo (1974) recorded that 1:10% total of oxalic is present in the dry matter but no toxicity. Majority of the *Cynodon dactylon* grasses are non-toxic but an occasional of Prussic acid may occur (Kidder *et al*., 1961; Skerman and Riveros 1990). About 17.1% of geophagic consumers indicated to consume soils with *Eragrostis rigidior* grass from geophagic site five and studies conducted by Skerman and Riveros (1990) showed the grass is not toxic. The remaining grass *Aristida congesta subsp congesta* consumed by 23.4% geophagic consumers from geophagic site six is the possible toxic grass in plant remains extracts 1.

Vachellia tortilis commonly known as *Acacia tortilis* or *Acacia Karroo* is a member in the family Fabaceae (Taylor and Barker, 2012; Idamokoro *et al*., 2017; Maroyi, 2017). It is known as *Mookana* by people in Sekhukhune area. The plant is the most widespread *Acacia* in southern Africa (Taylor and Barker, 2012) and has been used by the indigenous people of southern Africa for centuries. The roots and leaves of the plant are used for treatment of diarrhoea, dysentery (Idamokoro *et al*., 2017; Maroyi, 2017). Scientific studies indicate it has a wide range of pharmacological activities which include cytotoxicity activities (Maroyi, 2017).

Plant remains extract 2 which were of the combined *Vachellia tortilis* plant extracts from three different sites were consumed by geophagic consumers from Ga-Nchabeleng village. Geophagic site two had 20.7% of geophagic consumers who consumed the plant

remains of this plant in soils; geophagic site three had 15.3% and geophagic site four had 10.8% of consumers. The CC_{50} of plant remains extracts 2 was at 92.75 µg/ml indicating the extracts not toxic as compared to plant remains extracts 1 that killed viable cells at a lower concentration (Table 4.23). A study by Kigondu *et al*. (2009) showed the plant extracts of *Acacia tortilis* now *Vachellia tortilis* to have very low cytotoxicity (CC_{50}>500 µg/ml) on human embryonic lung fibroblast cells. The study by Yadav *et al*. (2013) indicated that *Vachellia tortilis* have no known high toxicity effects, so it may prove important alternative therapy to those who consume it for treatment of various diseases; however the results are not conclusive.

Table 4.23: Plant remains composite analysis according to characteristics: Ga-Nchabeleng Village

Plant remains	Site no	Number of consumers per site (%)	Characteristics/ features	References	Plant Species	CC_{50} (µg/ml)
1	1 5 6	14 (12.6%) 26 (23.4%) 19 (17.1%)	Perennial grasses, very variable with long rapid-growing, creeping runner or stolons, rooting at nodes, forming a dense tuft on the surface of the soils	Watson and Dallwitz, 1992; AL-Snafi, 2016; Dhanalakshmi *et al*., 2016;	*Cynodon dactylon* *Aristida congesta subsp. Congesta* *Eragrostis rigidior*	16.68 µg/ml
2	2 3 4	23 (20.7%) 17 (15.3%) 12 (10.8%) Total= 111(100%)	It tolerates high alkanity. Drought resistant and dominates regions of arid and semi-arid ecosystems. Grow on stony soils, strongly sloped rooting surfaces.	Carr, 1976; EL-demerdash *et al*., 1995; Siebert *et al*., 2002; Abderlrahman and Krzywinski, 2008.	*Vachellia tortilis*	92.75 µg/ml

Alternanthera pungens kunth and *Alternanthera lorentzii* commonly known as Rhodesian and Khaki weeds belong to the family Amaranthaceae. They are commonly known as *Tšhašo* and *Tshehlo* by people in Sekhukhune area. They are prostrate, creeping, perennial herbs with stems up to 60cm long (Parsons, 1973). Weeds are the important and unused components (Njoroge *et al*., 2004). The role of weeds, commonly found in disturbed areas, in traditional medicine has been overlooked (Stepp and Moerma, 2001). However, weeds are useful to human beings as food, erosion control, medicines, supply of organic matter and mineral nutrients to the soil (Jain *et al*., 2016). Consumption of weeds is a world-wide phenomenon as some of the plants are characterised by high nutritional value and medicinal properties (Maroyi, 2017). Young shoots and leaves of weeds are consumed as a vegetable in Southeast Asia (Jain *et al*., 2016). The leaf is very rich in iron, vitamin A and dietary fiber (Singh *et al*., 2009; Jain *et al*., 2016). Although weeds are known to be consumed as vegetables, it was significant to undertake and evaluate if there is any possible cytotoxicity for the above specific weeds which were consumed as plant remains (dead weeds) in geophagic soils.

Plant remains extracts 3 were collected from Mphanama Village and composed of two weeds combined. Geophagic site seven and nine had 8.2 and 19.7% of geophagic consumers who consumed soils with *Alternanthera pungens* kunth plant. Geophagic site eight had 4.9% of geophagic consumers who consumed soils with *Alternanthera lorentzii* plant. The CC_{50} of the combined plant remains extracts 3 was at 13.75 µg/ml and showed high cytotoxicity at the lowest concentrations as compared to the rest of plant remains extracts (Table 4.24).

A number of studies have reported cytotoxic activity of *Alternanthera* plants commonly present in the world. However, the study conducted by Jain *et al*. (2016) indicated the cytotoxicity results obtained showed a direct relation with the concentration of the *Alternanthera* extracts. The results obtained in the study suggests the presence of certain cytotoxic compounds in these extracts, but studies by Simmonds *et al*. (2000) indicated that *Alternanthera pungens* kunth has no known risk of toxicity.

Red bushwillow (*Combretum apiculutum*), Mountain Kirkia (*Kirkia wilmsii*) and Shepherd tree (*Boscia albitrunca*) are woody climbers' trees that belong to the families:

Combretaceae, Kirkiaceae and Capparaceae. The trees are commonly known as *Mohwelere, Modumela* and *Mohlopi* by people in Sekhukhune area. The trees are known for their medicinal value. In southern Africa they are used to treat abdominal disorders, constipation and diarrhoea (Eloff *et al*., 2008; de Morais Lima *et al*., 2012; Sharma and Lall, 2014). The *Kirkia wilmsii* tuber is used by the Bapedi people in Limpopo Province for treatment of various ailments (Chigayo *et al*., 2016). The local people believe that chewing it regularly helps maintain general good health (Chigayo *et al*., 2016). The roots of the *Boscia albitrunca* have been a provider of valuable food source for humans (Coates Palgrave, 1983).

Geophagic consumers in Sekhukhune area consume soil with mixed plant remains of these plants. Geophagic soils which were from Mphanama Village had plant remains mentioned by geophagic consumers. Geophagic site 10 had 9.8% of geophagic consumers who consumed soils with *Combretum apiculutum* plant remains, geophagic site 11 had 23% who consumed *Kirkia wilmsii* and geophagic site 12 had 34.4% who consumed *Boscia albitrunca*. The CC_{50} of the combined plant remains extract 4 was at 58.95 µg/ml, (Table 4.24). The value was obtained from the combined plant remains extracts.

From the above combined value of plant remains extracts 4, previous studies conducted on cytotoxicity evaluation of *Combretum apiculutum* on Propionibacterium acnes showed 50% radical scavenging activity of viable cells (EC_{50}) at concentrations ranging from 1.6 µg/mL to 3.5 µg/mL (Sharma and Lall, 2014). Another study conducted by Nopsiri et al. (2014) on methanolic extracts of leaves and root of *Combretum apiculatum* inhibited MCF-7 breast cancer cells with IC_{50} value at 25.00 µg/ml from ranged concentration of 1000-7.8 µg/ml indicating high inhibitory of this plant. *Kirkia wilmsii* extracts showed comparably low toxicity when compared with the reference agent berberine (cytotoxic agent) with hemagluttination assay titre value of 0.80 and agglutination value of 1.25 mg/mL (Suleiman *et al*., 2010). Toxicological evaluations carried out so far on Kirkia plants are preliminary considering their widespread usage as herbal medicines in southern Africa. It is important to ascertain any toxicological effects that can occur as a result of chronic of sub-chronic usage of Kirkia plants (Maroyi, 2017). The *Boscia*

albitrunca plant is regarded as not toxic as it has been used as a source of food during droughts and its roots are eaten raw (van der Walt and le Riche, 1999). The dried, unroasted root has also been important for consumption (Coates Palgrave, 1983). The plant extracts and isolated compounds of *Boscia albitrunca* were evaluated for antimicrobial activities using micro dilution technique and the values for the extracts ranged from 390.0 to 6250 µg/ml (Pendota *et al*., 2015).

Table 4.24: Plant remains composite analysis according to characteristics: Mphanama Village

Plant remains	Site no	Number of consumers per site (%)	Characteristics/ features	References	Plant species	CC_{50} (µg/ml)
3	7	5 (8.2%)	Annual perennial broadleaf weed herbs. The leaves are mostly alternate, flat or terete, sometimes opposite. The roots often develop at the nodes of spreading stems. The species forms dense mats of stems and leaves.	Parsons and Cuthbertso, 2001; Kopec *et al*., 2004; Hephner *et al*., 2013.	*Alternanthera pungens* kunth	13.75 µg/ml
	8	3 (4.9%)			*Alternanthera lorentzii*	
	9	12 (19.7%)			*Alternanthera pungens* kunth	
4	10	6 (9.8%)	All of them are prominent woody plants that grow well on open woodland, arid and shallower soils. These plants grow also on granitic and dolomitic soils in dry bushveld preferably rocky places	Coates Palgrave, 2002; Wilson, 2006; Stronkhorst *et al*., 2009	*Combretum apiculutum*	58.95 µg/ml
	11	14 (23%)			*Kirkia wilmsii*	
	12	21 (34.4%)			*Boscia albitrunca*	
		Total=61 (100%)				

Acacia is an important plant genus that is commonly used in a variety of infections. It is widely distributed and has been demonstrated in the treatment of various ailments (Idamokoro *et al*., 2017). *Vachellia nilotica* commonly called *Acacia nilotica* or Scented pod *Acacia* (Rasool *et al*., 2013) and *Acacia meansii* known as Black wattle belongs to the family *Fabaceae (*Kalaivani *et al*., 2011; Olajuyigbe *et al*., 2012*)*. The plants are commonly called *Mogohlo* and *Mošu* by people in Sekhukhune area. The third plant found on the control site was the *Vachellia tortilis* and was described in plant remain extract 2. These *Acacia* plants have yellow mimosa-like flowers or cream coloured pale yellow and long grey pods constricted between seeds. The bark and branches are dark with fissures. The branches bear spikes. The leaflets are small, densely together and covered in fine hairs (Carr, 1976; Barnes *et al*., 1996; Smit, 1999; Coates Palgrave, 2002; Dharani, 2006; Banso, 2009).

Plant remains extract 5 consisted of soils with three *Acacia* plants not consumed by geophagic consumers in the study. In geophagic site one the soil had *Vachellia nilotica*, geophagic sites two and three had *Acacia mearnsii* and geophagic sites four and five had *Vachellia tortilis* plant remains. The CC_{50} value for the control plant remains was at 251.4 µg/ml indicating low cytotoxicity of these plants (Table 4.25). From the above results on the control plant remains extract the value was from the combined *Acacia* plants.

Previous studies have shown the stem, leaves and roots of *Acacia nilotica* to be useful in treating diarrhoea (Wickens, 1995). Riaz *et al*. (2011) conducted a study on cytotoxic activities of *Acacia nilotica* methanolic extracts on extended spectrum beta-lactamase producing *Escherichia coli* and *klebsiella* species. The results showed minimal cytotoxic effects on human brain microvascular endothelial cells ranging 8.1-29% (Riaz *et al*., 2011). Another study by Kalaivani *et al*. (2011) on Free radical scavenging and cytotoxic activities of leaves of *A*cacia *nilotica* on Vero and HeLa cell lines revealed that ethanol extract was the most effective and IC_{50} value was found to be 53.6 µg/mL for Vero and 28.9 µg/mL for HeLa cell lines in cytotoxicity assays. It was revealed that none of the tested extracts possessed any haemolytic activity against the rat and human erythrocytes revealing their cytotoxic mechanism and non-toxicity (Kalaivani *et al*., 2011). Furthermore, a study by Kabbashi *et al*. (2015) carried out to evaluate cytotoxicity of

ethanol extracts of *Acacia nilotica* leaves on Vero cell line. The ethanol extracts of *Acacia nilotica* was screened for their cytotoxicity using MTT. The results obtained on *Acacia nilotica* leaves ethanol extracts exhibited 100% cell death (mortality) within 96 hours at concentration of 500 µg/ml which was the highest concentration, indicating the safety of the examined extract (Kabbashi *et al*., 2015). A study conducted by Olajuyigbe and Afolayan, (2012) on pharmacological assessment of the potential of *Acacia mearnsii* antimicrobial and toxicity activities revealed the cytotoxic activity of the extract was observed between 31.25 µg/mL and 500 µg/mL and the LC_{50} value (112.36 µg/mL) indicates that the extract was non-toxic in the brine shrimp lethality assay (LC_{50}>100 µg/mL) (Olajuyigbe and Afolayan, 2012).

Table 4.25: Control plant remains composite.

Plant remain Control	Site no	Characteristic/ features	Plant Species	References	CC_{50} (µg/ml) evaluation
5	1	Finely divided green leaflets that give the stalk a fernlike appearance. The small fragrant flowers are pea-shaped, yellow in colour though some species produce white blooms	*Vachellia nilotica*	Carr, 1976; Barnes *et al*., 1996; Smit, 1999; Coates, 2002;	251.1µg/ml
	2		*Acacia mearnsii*		
	3		*Acacia mearnsii*		
	4		*Vachellia tortilis*		
	5		*Vachelia tortilis*		

Observing the results it showed that some geophagic sites where miners collect their soils consumed with plant remains were harmful to their health and wellbeing. From the results obtained, certain plant remains in the areas studied in Sekhukhune area were most toxic to least toxic for human consumption.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The aim of this study was to characterise plant remains content in geophagic soils consumed by humans and geophagic practices in Sekhukhune District and to infer their possible health effects. The study findings indicated that there was high prevalence of geophagia in Sekhukhune area, Limpopo Province, South Africa. It appeared that geophagia in the area studied had been a daily practice within the communities. Geophagic consumers who volunteered to participate in the study questionnaire reported that other family members in some households consumed soil as well.

Previous studies have revealed that men and children indulge in the habit; however it is most common among women particularly of child-bearing age between the ages of 21-40 years with low income in Africa (Brand *et al*., 2009; Ekosse *et al*., 2010). However, for this study it was revealed that both males and females consumed soil. It was interesting to find there were males who were extensively geophagic in Mphanama Village, whereas Ga-Nchabeleng Village had more female geophagic consumers who participated. Furthermore evaluating the overall of the study females were more geophagic than males. It was also discovered that in both Villages, geophagic consumers ingested soil whether pregnant or not pregnant. The motivational factor for soil consumption mostly mentioned was craving and those who indicated to buy revealed it was because of the taste in purchased soils as compared to soil from the wild. The consumers were not even aware and did not know the constituents of the soils consumed. The colour of most consumed soil was brownish in Ga-Nchabeleng Village and red in Mphanama Village. The soil in the area is traditionally called *mobu*, *letsopa* or *leraga* and the digging method was mostly preferred.

The study findings also revealed beliefs and perceptions, both negative and positive, associated with soil consumption. Some positive beliefs were that soil consumption acts as a remedy for stomach pains and diarrhoea, similar to perceptions of other soil eaters as documented in the literature. However, the participants indicated constipation,

abdominal pains, tooth decay and poison to the body could be as a result of soil consumption. Majority of respondents admitted that eating soil was not helpful as it contributed to a number of health problems as indicated above. Yet, some of the respondents proudly mentioned that they were addicted to soil consumption and would stop only if there was assistance.

Soil samples were collected from various popular geophagic sites in the area studied. It was obvious that most of these mines were well known to geophagic practicing communities and were frequented for collection of geophagic soil, by school children and adults. Consumers in Ga-Nchabeleng Village preferred soil from riverbeds whereas Mphanama consumers preferred soil from mountains/hills.

Soil samples collected from both Villages indicated a wide range of colours and textures, providing for different preferences to various geophagic consumers. Geophagic sites varied from relatively neat to very dirty mines with pieces of glasses, plastics, and other rubbish tools. It was also ascertained that collection of soils was often done with hands and uncleaned utensils. Most of the sites were found to be located close to footpaths, houses, dumping sites and even roads, which raised a concern about the health aspects of these soils. Other consumers even preferred the soils of dead woods of trees.

It was found that the soils eaten contained plant remains which were toxic and other sites least toxic to the consumers. However the concentration of cytotoxicity does not give conclusive results due to the plant remains extracts being combined. All geophagic soils had plant remains, of which quantities depended on the site where soils were collected. Geophagic soil in Ga-Nchabeleng Village had more roots of plant remains whereas Mphanama Village had stems. Although other geophagic sites with soil collected with these plant remains may not be that harmful, there are serious risks in consuming them. It might therefore become necessary to educate geophagic consumers in the communities' with regard to possible risks in consuming soils with toxic plant remains.

In the current study, there was also no correlation between the preferred choices of colour with plant remains, except few males had specific trees which had to decayed prior to consumption. Geophagic consumers in Sekhukhune area were probably at risk

whilst others were not at risk of acquiring health threats by plant remains through consumption of these soils. The levels of toxicity in plant remains in the soils consumed may present further information about the relationship between geophagia and the toxins in geophagic soils.

5.2 Recommendations

a) Mphanana Village had more males who participated in the study than females. Further studies need to be conducted specifically on male geophagic consumers from that area. The male geophagic consumers also stated specific sites where they obtain their soils. Further investigations need to be conducted on preferred soils from specific sites with plants/trees where males collect their soils.

b) Ga-Nchabeleng and Mphanama Villages had a variety of geophagic mining sites of which only 12 study sites were investigated. Further studies from this Villages need to be investigated looking at other geophagic sites. Geophagic practices is often associated with pregnancy as stated by previous studies, however Ga-Nchabeleng and Mphanama consumers were shown to practice geophagia more even when not pregnant, further studies must be conducted on non-pregnant geophagic consumers.

c) The results obtained from plant remains experiment were collected and analysed as a composite study. Further investigations must be done from an individual plant and not as collective plants. Further studies need to be conducted on a single plant to have conclusive results. Not all plant remains in Sekhukhune area were toxic, other studies should be conducted to identify the actual plant species especially those that are harmful to human health. When the type of species is/are known, it will be much easier to predict the type of infection that might affect the consumers.

d) Further cytotoxicity evaluation need to be undertaken with the use of other assay kits and evaluate on which specific site of plant remains is consumed. The different plant parts should be tested against a wide range of cell lines such as (HeLa, MCF-7, U87, A549, SKOV-3, Vero) as well as using other *in vitro* toxicological assays (LDH and ATP).

e) Considerable processing may be required to remove fibrous skins and compounds that may be unpalatable or toxic to individuals that practice geophagia
f) Implementation of Environmental health education programmes together with the assistance of health professionals in the area is required to raise the awareness about disadvantages of consuming soils (such as constipation, abdominal pains, tooth decay and poison to the body).
g) Geophagic consumers in the communities need to be educated moderately with regard to healthy consumption practices and to improve the quality of soil they consume by processing such as baking, heat treatment etc, prior consumption.

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APPENDICES

APPENDIX 1: QUESTIONNAIRE (HUMAN GEOPHAGIA)

Individual Questionnaire Related To Human Geophagia

Introduction

The University of Venda, South Africa is carrying out a study to characterize habits related to human and enzootic geophagia in South Africa. It is also designed to physicochemically, microbiologically, mineralogically and ecologically characterize the soils that are preferred by geophagic individuals in the country. This exercise is mainly for academic purposes. However, information provided may be generally used to improve methods of harvesting geophagic soils that will guarantee the health of geophagic individuals. Strict confidentiality of the information will be guaranteed at all times. Respondents are therefore requested to cooperate with the interviewees in order to facilitate this study.

Date of interview……...
Name of interviewee (optional)……..
Area (Address)……..

1. A DEMOGRAPHIC INFORMATION

Geographic Information

1. Location	1. Mark the correct option		2. Specific town or area
2. Location	Rural	Suburban	Urban
3. Specify town or area	Rural	Suburban	Urban

Personal and Demographic Information

4. Sex	Male		Female		
5. Age					
6. Number of children					
7. Ages of children					
8. Sex of children					
9. Marital status	Married	Divorced	Single	Living together	Cohabiting
10. Income source	Wage employment		Non-wage employment		Others, please specify
11. Occupation					
12. Income					
13. Educational level	Uneducated	Primary	Secondary	Vocational	Technical
	Teacher training	Higher education	Post graduate	Professional	Other (specify)

1. B SOCIO-ECONOMIC AND CULTURAL ASPECTS

Habits

14. Are you presently consuming soil?	Yes		No	
15. If yes how often do you consume the soil?	Once a month	Once a week	Once a day	More than once a day
16. If yes, for how long have you been consuming soil?				
17. What is the reason for consuming soil?	Standard practice (cultural, traditional, spiritual)	Craving	Medical value	Complement diet
	Ritualistic	When hungry	When pregnant	Don't know / Others (specify)
18. Do you crave for it?	Regularly once a month	Regular weekly	Regular daily	Only when pregnant
19. When do you crave it?	Pregnant	Lactating	Both pregnant and lactating	Experiencing sleeplessness
	Nauseated but not pregnant	Constipated	Feeling weak	Others (specify)
20. When you are pregnant how often do you consume the soil?	Once a month	Weekly	Daily	Others (specify)
21. Do you consume other non-food substances?	Yes		No	
22. If yes name the substance				
23. How often do you consume this substance?	Daily	More than a week	Weekly	Monthly
24. How much of the substance (handfuls) do you consume?	Daily	More than once a day	Weekly	Monthly
25. Do other people know that you consume clay?	Yes	No	Don't know	
26. If yes who is aware?	Family members	Extended family members	Friends	Others (specify)
27. How do people perceive the habit of eating non-food substances?	Positive	Negative	Indifferent	Don't know
28. Is this practice common among certain members of the community?	Yes	No	Don't know	
29. If yes, specify				

1. C INDIGENOUS KNOWLEGDE

30. What substances are consumed?	Soil	Clay	Soil from termite mounds	Others (specify)
31. How are substances consumed?	Wet	Dry	With other food	Others (specify)
32. What are the traditional names of the substances consumed?				
33. Where do you obtain the substance?	From the wild	Bought	Given	Others, specify
34. If you buy them give the brand name				
35. If you buy indicate the price per handful				
36. What is the colour of the substance consumed?	Reddish	Whitish	Blackish	Yellowish
	Khaki	Others (specify)		
37. Why do you prefer to consume the specific colour type?	Taste	Tradition/belief	It is easily accessible	Other (specify)
38. Method of a storage of substance				
39. Length of storage				

1. D PHYSICO-CHEMICAL, MINERALOGICAL, GEOLOGICAL AND CHEMICAL ASPECTS

40. Where does the substance you consume come from?	Hill/mountain	River bed	Termitaria	valley	Others (specify)
41. If from a mound, where specifically on the mound is substance collected?	Surface	Inside mound above the surface of the soil	Inside mound below the surface of the soil	Does not matter	Not sure
42. Are the substances obtained close to a rock	Yes	No	Not sure		
43. If yes, what is the type of rock?	Very hard	Hard	Soft	Very soft	
44. How is the substance collected	Digging	Hand grabbing	Scrapping	Selective hand picking	Other (specify)
45. If dug how deep do u dig?	0-10 cm	10-20 cm	20-30 cm	>30 cm	Others (specify)
46. How does the substance feel when collected?	Gritty	Silky	Powdery	Does not matter	Don't know
47. When are the substances collected?	Wet	Dry	Does not matter	Others (specify)	
48. If collected wet, how does the substance feel?	Very sticky	Sticky	Very soapy	Soapy	Neither
49. Are substances processed before consumption?	Yes	No	Sometimes yes/no		
50. If yes, how are they processed?	Grinding	Pounding	Sieving	Slurrying	Other (specify)
51. Is there any heat treatment of the substances before consumption?	Yes	No	Sometimes yes/no		
52. If yes specify type of heat treatment	Baking	Boiling	Burning	Combinations (specify)	Others (specify)

1. E ECOLOGICAL ASPECTS

53. If you consume substances from a termiteria, which one is it?	Mound		Tree	
54. If the substance is collected from a termite mound (section C), describe the height of the mound preferred.	<0.5 m	0.5-1 m	1-2 m	>2 m
55. What is the shape of the mound?	Conical	Flat topped	Dome shaped	Others (specify)
56. Do you prefer to consume the substance when	Newly constructed	Old	Does not matter	Not sure
57. On what type of terrain do you normally find these mounds?	Flat	Hilly	Undulating	Valley	Others (specify)
58. Do you collect the substance from	Mound	Base of the mound	Some distance from the mound	
59. If substance is collected from a tree, do you prefer a particular tree?	Yes	No	Not sure	Does not matter
60. If yes, name the preferred tree type.				

1. F HUMAN HEALTH ASSOCIATED WITH GEOPHAGIA

61. Height					
62. Weight					
63. Do you know that the substance could be harmful to your health?	Yes		No		
64. If yes, in what sense?	Constipation	Abdominal pains	Poison the body	Causes tooth decay	Others (specify)
65. Were you ever operated on for stomach problems?	Yes		No		
66. If yes, how many times and for what reason?					
67. Are you aware of the harmful substances/ parasites that may be present in the substance?	Yes		No		
68. Do you know the content of the substance?	Yes		No		
69. If yes name these contents.	Vitamins	Calcium	Iron	Salt	Others (specify)
70. Why do you consume the substance?	To clean your body	For additional nutritional value	To protect against infections	Do not know	Others (specify)
71. Do you often get infected (common cold, flu etc)?	More than once a month	Once a month	Once every three months	Twice yearly	Yearly
72. Do you ingest these substances when infected?	Yes	No	Sometimes		
73. Do you experience chronic illnesses?	Yes		No		
74. If yes, which of these?	Headaches	Dizziness	Blood in stool	Fatique	Chest pains
	Coughs	Muscle pains	Tremors	Blood in urine	Nose bleeding
75. Number of still born					
76. Number of children born with abnormalities					
77. Name the abnormalities					
78. Did these children reach the expected	Yes	No	Others (specify)		

developmental and growth stages					
79. Did the children experience any pains in the muscle or joints?	Yes		No		Others (specify)
80. Children under age of three that experienced parasite infections					
81. Medical condition diagnosed/ experienced	Iron deficiency	High blood pressure	Constipation	Constant headaches	Other

APPENDIX 2: ETHICAL CLEARANCE CERTIFICATE

RESEARCH AND INNOVATION
OFFICE OF THE DIRECTOR

NAME OF RESEARCHER/INVESTIGATOR:
Ms MV Phakoago
Student No: 14015177

PROJECT TITLE: **Characterisation of plant remains in the geophagic soils consumed by human in Sekhukhune area, Limpopo Province, South Africa.**

PROJECT NO: **SES/15/ERM/04/2511**

SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS

NAME	INSTITUTION & DEPARTMENT	ROLE
Prof GE Ekosse	University of Venda	Supervisor
Prof JO Odiyo	University of Venda	Co-Supervisor
Ms MV Phakoago	University of Venda	Investigator - Student

ISSUED BY:
UNIVERSITY OF VENDA, RESEARCH ETHICS COMMITTEE

Date Considered: November 2015
Decision by Ethical Clearance Committee Granted
Signature of Chairperson of the Committee: .....
Name of the Chairperson of the Committee: Prof. J.E. Crafford

University of Venda
PRIVATE BAG X5050, THOHOYANDOU, 0950, LIMPOPO PROVINCE, SOUTH AFRICA
TELEPHONE (015) 962 8504/8313 FAX (015) 962 9060
"A quality driven financially sustainable, rural-based Comprehensive University"

APPENDIX 3: STATISTICAL DATA

3. A DEMOGRAPHIC INFORMATION

Q_4. Gender

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Male	43	21.5	21.5	21.5
	female	157	78.5	78.5	100.0
	Total	200	100.0	100.0	

Q_5. Age

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	≤20	33	16.5	16.5	16.5
	21-30	50	25.0	25.0	41.5
	31-40	65	32.5	32.5	74.0
	41-50	38	19.0	19.0	93.0
	≥ 50	14	7.0	7.0	100.0
	Total	200	100.0	100.0	

Q_9. Marital status

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Married	43	21.5	21.5	21.5
	Divorced	1	.5	.5	22.0
	Single	143	71.5	71.5	93.5
	living together	13	6.5	6.5	100.0
	Total	200	100.0	100.0	

Q_10. Income source

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	wage employment	23	11.5	11.5	11.5
	none wage employment	176	88.0	88.0	99.5
	Other	1	.5	.5	100.0
	Total	200	100.0	100.0	

Q_11. Occupation

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Cleaner	23	11.5	11.5	11.5
	Unemployed	145	72.5	72.5	84.0
	Student	32	16.0	16.0	100.0
	Total	200	100.0	100.0	

Q_12. Income

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	≤ R2000	22	11.0	11.0	11.0
	> R2000	1	.5	.5	11.5
	None	177	88.5	88.5	100.0
	Total	200	100.0	100.0	

Q_13. Educational level

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	no schooling	7	3.5	3.5	3.5
	Primary	23	11.5	11.5	15.0
	Secondary	164	82.0	82.0	97.0
	Tertiary	6	3.0	3.0	100.0
	Total	200	100.0	100.0	

3. B SOCIO-ECONOMIC AND CULTURAL ASPECTS

Q_14. Are you presently consuming soil?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Yes	172	86.0	86.0	86.0
	No	28	14.0	14.0	100.0
	Total	200	100.0	100.0	

Q_15. If yes how often do you consume the soil?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	once a month	4	2.0	2.3	2.3
	once a week	4	2.0	2.3	4.7
	once a day	35	17.5	20.3	25.0
	more than once a day	129	64.5	75.0	100.0
	Total	172	86.0	100.0	
Missing	System	28	14.0		
Total		200	100.0		

16. If yes, for how long have you been consuming soil?

Row Labels	Count of Q_16	Count of Q_16
>5yrs	121	71%
≤ 2yrs	17	10%
3-5yrs	33	19%

Descriptive Statistics

Q_16. If yes, for how long have you been consuming soil?

	N	Minimum	Maximum	Mean	Std. Deviation
Q_16	171	1.00	46.00	11.6608	9.70819
Valid N (listwise)	171				

Q.17. What is the reason for consuming soil?

Row Labels	Count of Q_17a
Craving	47%
when pregnant	23%
complement diet	10%
Standard practice	9%
Ritualistic	5%
don't know	3%
when hungry	2%
Medical value	1%

Q_18. Do you crave to eat soil?

	Frequency		Percent	Valid Percent	Cumulative Percent
Valid	regularly once a month	4	2.0	2.1	2.1
	regularly weekly	4	2.0	2.1	4.3
	regular daily	154	77.0	82.4	86.6
	only when pregnant	25	12.5	13.4	100.0
	Total	187	93.5	100.0	
Missing	System	13	6.5		
Total		200	100.0		

Q_19. When do you crave for the soil?

Row Labels	Count of Q_19a	Count of Q_19a
Pregnant	72	87%
both pregnant & lactating	8	10%
experiencing sleeplessness	2	2%
Lactating	1	1%
Total	83	100%

Q_20. When pregnant how often do you consume the soil?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	once a month	3	1.5	3.4	3.4
	Weekly	4	2.0	4.5	7.9
	Daily	82	41.0	92.1	100.0
	Total	89	44.5	100.0	
Missing	System	111	55.5		
Total		200	100.0		

Q_21. Do you consume other none food substances?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	yes	40	20.0	20.6	20.6
	No	150	75.0	77.3	97.9
	other	4	2.0	2.1	100.0
	Total	194	97.0	100.0	
Missing	System	6	3.0		
Total		200	100.0		

Q_22. If yes name the substance

Row Labels	Count of Q_22	Count of Q_22
Ashes	5	12%
Chalk	26	60%
cow dung	4	9%
Ice	4	9%
pencil lead	3	7%
Serokolo	1	2%
Grand Total	43	100%

Q_23. How often do you consume this substance?

Row Labels	Count of Q_23	Count of Q_23
Daily	10	28%
Monthly	7	19%
Weekly	19	53%
Grand Total	36	100%

Q_24. How much of the substance (handfuls) do you consume

Row Labels	Count of Q_24	Count of Q_24
Daily	9	25%
Monthly	8	22%
Weekly	19	53%
(blank)		0%
Grand Total	36	100%

Q_25. Do other people know that you consume clay?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Yes	177	88.5	91.7	91.7
	No	13	6.5	6.7	98.4
	don't know	3	1.5	1.6	100.0
	Total	193	96.5	100.0	
Missing	System	7	3.5		
Total		200	100.0		

Q_26. If yes who are aware?

Row Labels	Count of Q_26	Count of Q_26
family members	113	66%
Friends	58	34%
Others	1	1%
(blank)		0%
Grand Total	172	100%

Q_27. How do people perceive this habit of eating non-food substances

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Positive	3	1.5	1.5	1.5
	Negative	157	78.5	79.3	80.8
	indifferent	36	18.0	18.2	99.0
	don't know	2	1.0	1.0	100.0
	Total	198	99.0	100.0	
Missing	System	2	1.0		
Total		200	100.0		

Q_28. Is this practice more common among certain members of the community?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Yes	196	98.0	99.5	99.5
	don't know	1	.5	.5	100.0
	Total	197	98.5	100.0	
Missing	System	3	1.5		
Total		200	100.0		

Q_29. If yes, specify

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Classmates	10	5.0	9.9	9.9
	Friends	10	5.0	9.9	19.8
	Neighbours	81	40.5	80.2	100.0
	Total	101	50.5	100.0	
Missing	System	99	49.5		
Total		200	100.0		

3. C INDIGENOUS KNOWLEDGE

Q_30. What substances are consumed?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Soil	185	92.5	92.5	92.5
	Clay	13	6.5	6.5	99.0
	soil from termite mounds	2	1.0	1.0	100.0
	Total	200	100.0	100.0	

Q_31. How are substances consumed?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Wet	2	1.0	1.0	1.0
	Dry	197	98.5	98.5	99.5
	with other food	1	.5	.5	100.0
	Total	200	100.0	100.0	

Q_32. What are the traditional names of substances consumed

Row Labels	Count of Q_32	Count of Q_32
Mobu	187	90%
Letsopa	19	9%
Leraga	1	0%
Total	207	100%

Q_33. Where do you obtain the substance

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	from the wild	187	93.5	96.4	96.4
	Bought	3	1.5	1.5	97.9
	Given	4	2.0	2.1	100.0
	Total	194	97.0	100.0	
Missing	System	6	3.0		
Total		200	100.0		

Q_35. If you buy indicate the price per handful

Row Labels	Count of Q_35	Count of Q_35
50C	11	30%
R1.00	18	49%
R2.00	7	19%
R3.00	1	3%
Grand Total	37	100%

Q_36. What is the color of substance consumed

Row Labels	Count of Q_36	Count of Q_36
blackish	4	17%
khakhi	6	25%
Whitish	8	33%
yellowish	6	25%
(blank)		0%
Grand Total	24	100%

Q_37. Why do you prefer to consume the specific color type

Row Labels	Count of Q_37	Count of Q_37
easily accessible	2	8%
Taste	21	88%
tradition/belief	1	4%
(blank)		0%
Grand Total	24	100%

Q_39. Length of storage

Row Labels	Count of Q_39	Count of Q_39
1 day	1	20%
2 days	2	40%
3 days	1	20%
one week	1	20%
(blank)		0%
Grand Total	5	100%

3. D PHYSICO-CHEMICAL, MINERALOGICAL, GEOLOGICAL AND CHEMICAL ASPECTS

Q_40. Where does the substance you consume come from

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	hill/mountain	39	19.5	24.7	24.7
	river bed	89	44.5	56.3	81.0
	Valley	27	13.5	17.1	98.1
	Others	3	1.5	1.9	100.0
	Total	158	79.0	100.0	
Missing	System	42	21.0		
Total		200	100.0		

Q_41. If from a mound, where specifically on the mound is substance collected

Row Labels	Count of Q_41	Count of Q_41
does not matter	1	33%
inside mound above the surface of the soil	1	33%
Surface	1	33%
(blank)		0%
Grand Total	3	100%

Q_42. Are substances obtained close to rocks

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Yes	31	15.5	22.6	22.6
	No	106	53.0	77.4	100.0
	Total	137	68.5	100.0	
Missing	System	63	31.5		
Total		200	100.0		

Q_43. If yes, what is the type of rock

Row Labels	Count of Q_43	Count of Q_43
hard	23	74%
soft	5	16%
very hard	3	10%
(blank)		0%
Grand Total	31	100%

Q_44. How is the substance collected

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Digging	113	56.5	85.6	85.6
	hand grabbing	1	.5	.8	86.4
	scrapping	8	4.0	6.1	92.4
	selective hand picking	10	5.0	7.6	100.0
	Total	132	66.0	100.0	
Missing	System	68	34.0		
Total		200	100.0		

Q_45. If digging how deep

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	0-10cm	103	51.5	76.3	76.3
	10-20cm	29	14.5	21.5	97.8
	20-30cm	1	.5	.7	98.5
	Othere	2	1.0	1.5	100.0
	Total	135	67.5	100.0	
Missing	System	65	32.5		
Total		200	100.0		

Q_46. How does the substance feel when collected?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Gritty	7	3.5	6.7	6.7
	Silky	2	1.0	1.9	8.6
	Powdery	41	20.5	39.0	47.6
	does not matter	55	27.5	52.4	100.0
	Total	105	52.5	100.0	
Missing	System	95	47.5		
Total		200	100.0		

Q_47. When are substances collected?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Dry	118	59.0	88.1	88.1
	doesn't matter	16	8.0	11.9	100.0
	Total	134	67.0	100.0	
Missing	System	66	33.0		
Total		200	100.0		

Q_48. If collected wet, how does the substance feel?

Row Labels	Count of Q_48	Count of Q_48
Sticky	1	50%
very sticky	1	50%
(blank)		0%
Grand Total	**2**	100%

Q_49. Are substances processed before consumption?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	yes	2	1.0	1.4	1.4
	no	135	67.5	97.8	99.3
	sometimes	1	.5	.7	100.0
	Total	138	69.0	100.0	
Missing	System	62	31.0		
Total		200	100.0		

Q_51. Is there any heat treatment of substance before consumption?

Row Labels	Count of Q_51	Count of Q_51
No	132	98%
Yes	3	2%
(blank)		0%
Grand Total	135	100%

3. E ECOLOGICAL ASPECTS

Q_53. If you consume substance from a termiteria, from which one

Row Labels	Count of Q_53	Count of Q_53
Mound	3	10%
Tree	26	90%
(blank)		0%
Grand Total	29	100%

Q_54. If substance is collected from termite mound (section C), describe the height of the mound preferred

Row Labels	Count of Q_54	Count of Q_54
<0.5 m	7	24%
0.5-1 m	22	76%
(blank)		0%
Grand Total	29	100%

Q_56. Do you prefer to consume the substance when

Row Labels	Count of Q_56	Count of Q_56
does not matter	13	48%
Old	14	52%
(blank)		0%
Grand Total	27	100%

Q_57. What type of terrain do you normally find these mounds?

Row Labels	Count of Q_57	Count of Q_57
Hilly	1	10%
Undulating	2	20%
Valley	7	70%
(blank)		0%
Grand Total	10	100%

Q_58. Do you collect the substance from

Row Labels	Count of Q_58	Count of Q_58
base of the mound	4	50%
Mound	3	38%
some distance from the mound	1	13%
(blank)		0%
Grand Total	8	100%

Q_59. If substance is collected from a tree, do you prefer it from a particular tree

Row Labels	Count of Q_59	Count of Q_59
no	2	9%
yes	20	91%
(blank)		0%
Grand Total	**22**	100%

Q_60. If yes, name the preferred tree type

Row Labels	Count of Q_60	Count of Q_60
megaba	1	5%
mohlwa	1	5%
mohwa	7	35%
mohwelere	1	5%
mojakwane	9	45%
moshwana	1	5%
(blank)		0%
Grand Total	20	100%

3. F HUMAN HEALTH ASSOCIATED WITH GEOPHAGIA

Q_63. Do you know that the substance could be harmful to your health

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	yes	62	31.0	34.6	34.6
	no	117	58.5	65.4	100.0
	Total	179	89.5	100.0	
Missing	System	21	10.5		
Total		200	100.0		

Q_64. If yes, in what sense

Row Labels	Count of Q_64	Count of Q_64
abdominal pains	17	28%
causes tooth decay	1	2%
constipation	38	62%
others	1	2%
poison of the body	4	7%
(blank)		0%
Grand Total	61	100%

Q_65. Were you ever operated upon for stomach problems?

Row Labels	Count of Q_65	Count of Q_65
no	178	100.00%
(blank)		0.00%
Grand Total	178	100.00%

Q_67. Are you aware of the harmful substances/ parasites that may be present in the substance?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	yes	7	3.5	3.9	3.9
	no	172	86.0	96.1	100.0
	Total	179	89.5	100.0	
Missing	System	21	10.5		
Total		200	100.0		

Q_68. Do you know the content of the substance

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	yes	5	2.5	2.8	2.8
	no	173	86.5	97.2	100.0
	Total	178	89.0	100.0	
Missing	System	22	11.0		
Total		200	100.0		

Q_70. Why do you consume substance?

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	for additional nutritional value	4	2.0	2.2	2.2
	do not know	175	87.5	97.8	100.0
	Total	179	89.5	100.0	
Missing	System	21	10.5		
Total		200	100.0		

Q_71. Do you often get infected (common cold, flu etc)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	once a month	2	1.0	1.2	1.2
	once every 3 months	4	2.0	2.4	3.6
	twice yearly	13	6.5	7.9	11.5
	Yearly	146	73.0	88.5	100.0
	Total	165	82.5	100.0	
Missing	System	35	17.5		
Total		200	100.0		

Q_72. Do you ingest these substances when infected

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Yes	12	6.0	6.8	6.8
	No	147	73.5	83.5	90.3
	sometimes	17	8.5	9.7	100.0
	Total	176	88.0	100.0	
Missing	System	24	12.0		
Total		200	100.0		

Q_73. Do you experience chronic illnesses

Row Labels	Count of Q_73	Count of Q_73
No	152	92%
Yes	13	8%
(blank)		0%
Grand Total	165	100%