Nutritional and Medicinal value of the edible stinkbug, *Encosternum delegorguei* Spinola consumed in the Limpopo Province of South Africa and its host plant *Dodonaea viscosa* Jacq. var. *angustifolia*

By

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PREFACE

I, Leah Snow Teffo, declare that the thesis hereby submitted to the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria for the degree of Philosophiac Doctor has not been submitted by me for a degree at this or any other University. The results obtained from this study are my own work in design and execution and except where specifically acknowledged.

Signed: .....……

Dated: ……..

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Conference Proceedings and Publications

Conferences:

Paper Presentations

1. Teffo LS, Toms RB and Eloff JN. *Medicinal and nutritional value of edible stink-bug Encosternum delegorguei consumed in Limpopo Province of South Africa*. Faculty Day, University of Pretoria, September 2004.

2. Teffo LS, Toms RB and Eloff JN. *Nutritional value of edible stink-bug Encosternum delegorguei consumed in Limpopo Province of South Africa.* IPUF Conference, Clanwilliams Town, Cape Town; June 2004.

3. Teffo LS, Toms RB and Eloff JN. *Medicinal value of edible stink-bug Encosternum delegorguei consumed in Limpopo Province of South Africa and its host plant Dodonaea viscosa*. IPUF Conference, Rhodes University, Grahamstown; July 2005.

4. Teffo LS, Aderogba MA, Toms RB and Eloff JN. *Antibacterial and antioxidant activity of isolated flavanoids from Dodonaea viscosa.* IPUF Conference, Botswana University, Gaborone; July 2006.

5. Toms RB and Teffo LS. *The role of edible insects in culture and medicine in South Africa and other countries*. Indigenous Knowledge System, Dept. of Science and Technology, Indaba Hotel, Fourways, Johannesburg; July 2006

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1. Teffo LS, and Eloff JN. *Antibacterial and antioxidant activities of Dodonaea viscosa Jacq. var. angustifolia leaf extracts.* To be submitted to South African Journal of Botany.

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Summary

In most rural areas, reliance on traditional medicine and food is high and this is attributed to both economic and cultural factors. The edible stink-bug (*Encosternum delegorguei* Spinola), local name "thongolifha" is consumed by the Venda tribe in Limpopo Province of South Africa. Thongolifha is important to the Venda tribe in terms of culture, nutrition and economical value. They eat it raw or cooked with porridge or as a snack. The edible stink-bug is sold at the Thoyandou open market and provides valuable income for the harvesters. Many insects have been reported to contain good source of proteins minerals and vitamins. In terms of medicinal value, insects have also been used in folk medicine in the past by various cultures to treat many ailments such as stroke, asthma, cold, etc. Some insects are also known to sequester compounds from their host plant and use them for its defensive mechanism. The host plant for thongolifha was identified as *Dodonaea viscosa* Jacq. var. *angustifolia* which has been reported to have antibacterial activity.

The aim of this study was to investigate the possible interaction between the thongolifha and its host plant *Dodonaea viscosa*. Since thongolifha is so important to the Venda tribe and its nutritional value was never analysed before it was also relevant to determine its nutritional composition. Nutritional components of thongolifha such as the proteins, fats, amino acids and carbohydrates contents were detected by using standardized methods. Thongolifha contained 35.2% protein, 50.6% fat and 7.63 g/100 g carbohydrate with an energy content of 2599 kJ/100 g. These results compare well to nutritional values of other edible insects such as termites (41.8% protein and 44.3% fat) and wasps (31% protein and 62% fat).The mopane worm has higher protein content (63.5%) and a lower fat content of (18%). Mineral content of thongolifha (1.2 g/100 g) was found to be at a comparable level to other bugs such as the *Acantocepphala declivis* (1.0 g/100g). Analysis of essential amino acids varied from 0.82 mg/100 g (threonine) and 1.32 mg/100 g (valine). The nutritional composition of thongolifha is acceptable and thongolifha is not just a traditional delicacy but also contributes as a diet supplement of the Venda tribe. Between 680 and 3400 of thongolifha will however have to be consumed to supply the daily nutritional needs for the essential amino acids phenylalanine and methionine.

Analysis for antibacterial activity of thongolifha was carried out after extracting with solvents of varying polarities, separation by thin layer chromatography (TLC) and bioautography against four nosocomial bacteria; Gram-negative; *Escherichia coli* and *Pseudomonas aeruginosa;* Gram positive; *Staphylococcus aureus* and *Enterococcus faecalis.* Bioautography results of the thongolifha extracts revealed some activity against *S. aureus* and *E. faecalis.* Evaluation of the minimal inhibitory concentration (MIC) by using a serial dilution microplate method indicated low antibacterial activity (MIC > 2.5 mg/ml). Thongolifha therefore does not contain antibacterial compounds but there may be present in a low concentration or the serial dilution microplate method does not work well with insects extracts. Some insects contain peptides as antibacterial compounds. Spraying thongolifha extracts chromatograms with several spraying reagents indicated that these antibacterial compounds were not peptides

Methanol crude leaf extracts of *D. viscosa* was reported in the literature to have antibacterial activity against *S. aureus* however the results were obtained by using the agar diffusion method which does not provide convincing quantitative results. In our study we extracted components from *D. viscosa* using solvents of varied polarities to evaluate its antibacterial and antioxidant activities. Dichloromethane and acetone extracted more compounds with good antibacterial activity against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa*. The average MIC values varied from 0.04 to 0.28 mg/ml, indicating excellent to good antibacterial activity. The antioxidant activity using the DPPH Spectrophotometric assay gave the EC_{50} of the extracts of 212 ± 4 to 469 ± 9 μg/ml. As expected, the more polar crude extracts, ethyl acetate and acetone demonstrated higher activity compared to other extracts. This was supported by the TLC qualitative assay showing more yellow bands in these extract on TLC chromatograms after spraying with 0.2% DPPH in methanol.

To determine whether thongolifha has sequestered compounds from the host plant, *D. viscosa*, a serial exhaustive extraction was performed in order to extract components from the crude extracts of both insect and the host plant by using various solvents and separating the extracts on TLC plates by using various eluant systems. After the TLC chromatograms were sprayed with vanillin-sulphuric acid to reveal separated components, there were few similarities of components from both insect and host plant. This could mean that either a sequestered plant compounds are present in low quantities in the insect extract, that insect may have metabolized the sequestered compounds or alternatively that the insect might not have sequestered the compounds from the host plant at all. It should be kept in mind that the stink-bugs feed by sucking sap from the host plant.

By using bioassay-guided fractionation based mainly on silica gel chromatography, four compounds (5-hydroxy-3,74'-trimethoxyflavone (1); 5,7-dihydroxy-3,6,4'-trimethoxyflavone (2); 5,7,4'-trihydroxy-3,6-dimethoxyflavone (3); and 4'-O-methylkaempferol (4) where isolated from dichloromethane fraction of a crude *D. viscosa* leaf extract, acetone crude leaf extract yielded only one compound, Kaempferol (5). Compounds 1, 2, 3 and 5 where previously isolated from *D. viscosa* whereas compound 4 was isolated for the first time from *D. viscosa*. Compounds 4 is not a novel compound because it was previously isolated from other plants species such as the *Lens culinaris.* Compounds 3, 4 and 5 have good antibacterial activity against *E. coli* and *E. faecalis* at MIC values of 15.63 μg/ml and 31.25 μg/ml respectively. In addition, the DPPH quantitative assay has demonstrated that Compounds 4 and 5 have strong antioxidant activities (EC_{50} = 35.1 ± 0.85 and 75.5 ± 1.76 μM respectively) but lower than L-ascorbic acid (EC_{50} = 13.5 ± 0.28 μM) used as a standard antioxidant agent. The antibacterial and antioxidant activity of most of the isolated compounds were not known previously. Investigation of structure antibacterial-activity relationship in the isolated compounds did not yield a clear correlation.

When investigating the possible interaction between the insect and the host plant, bioautography assay has shown compounds with antibacterial activity against *S. aureus* (Rf 0.79) *E. faecalis* (Rf 0.66) when separated with CEF

eluent system. Some of the isolated compounds from *D. viscosa* leaf extracts had an Rf of 0.79 (5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone) and Rf 0.61 (5, 7, 4'-trimethoxxyflavone) when separated with CEF eluent system. These findings indicate that thongolifha may have sequestered the two compounds from *D. viscosa* leaves, but this possibility has to be confirmed.

This study has indicated that thongolifha is good source of protein, vitamins, minerals and amino acids; however we have not found evidence of significant antibacterial activity of thongolifha. If fresh material of thongolifha can be analysed in future work the probability of finding medicinal properties may be greater. On the other hand, *D. viscosa* crude leaf extracts have demonstrated strong antibacterial and antioxidant activities; although isolated compounds have shown moderate level of activities. Because practically all antibacterial compounds based on bioautography have been isolated, it is clear that in the crude dichloromethane extract some synergism must have taken place because the antibacterial activity on a mass basis was nearly as good as the isolated compound without removing >90% of non active compounds.

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GLOSSARY

List of Abbreviations

BEA- benzene/ethanol/ammonia (18:2:0.2)

CE- chloroform/ethyl acetate (8:2)

CEF- chloroform/ethyl acetate/formic acid (10:8:2)

^{13}C NMR- Carbon 13 Nuclear Magnetic Resonance

CSIR- Centre for Scientific and Industrial Research

DCM- Dichloromethane

EMW- ethyl acetate/methanol/water (10:1.35:1)

EtOAc- Ethyl Acetate

EtOH- Ethanol

DPPH- 2, 2 Diphenyl-1-picrylhydrazyl

Hex- Hexane

H NMR- Proton Nuclear Magnetic Resonance

INT- *p*-iodonitrotetrazolium violet

IR- Infra Red

LD_{50}- the concentration that would kill 50% of the organism

M^+- Molecular ion

MEDUNSA- Medical University of South Africa

MIC- Minimum Inhibitory Concentartion

MH- Mueller-Hinton broth

MS- Mass Spectrometry

M/Z- Mass to charge ratio

NMR- Nuclear Magnetic Resonance

RAU- Rand Afrikaans Universiteit

SANAS- South African National Accreditation System

TA- Total Activity value

TEAC- Trolox Equevalent Antioxidant Capacity

TLC- Thin Layer Chromatography

UV- Ultra violet

VLC- Vacuum Liquid Chromatography

Codes for Isolated Compounds

T1055= 5-hydroxy -3, 7, 4'-trimethoxyflavone (Compound 1)

C= 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone (Compound 2)

E= 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone (Compound 3)

Cpd2= 4'-O-methylkaempferol (Compound 4)

AB= kaempferol (Compound 5)

D. viscosa is used to indicate *Dodonaea viscosa* Jacq. var. *angustifolia*

CHAPTER 1

INTRODUCTION

1. The Insects

Insects are arthropods, i.e. a type of invertebrate animals that lack backbones. At present, the estimated figure for total living species on earth is 10 million, of which a mere 3% are vertebrates and over 60% are insects. Insects are by far the most diverse group of organisms on earth. They have existed for around 500 million years in comparison to mammals which appeared around 200 million years ago, with human ancestors *Homo sapiens sapiens* only arriving ~ 120.000 years ago. Insects have colonized most parts of the globe and as such, have confronted microorganisms and predators during their existence. To survive in this wide variety of environmental conditions and to combat enemies, insects have evolved powerful defense systems. These rely mainly on the synthesis of peptides and organic small molecules with predefined biological activities (Dimarcq and Hunneyball, 2003). The larva of the pine sawfly (*Neodiprion sp*., Hymenoptera), when attacked by a predator such as an ant, it raises its prolegs and emits from its mouth a viscous droplet composed of terpenes such as α- and β-pinene and resin acids (Edwards and Wratten, 1980).

1.1 Insects as human food

Insects have played an important role in the history of human nutrition, and it is probable that the first hominids in Africa were eating insects. They are good source of protein, with high fat content (and thus energy) and many important minerals and vitamins (DeFoliart, 1992). One major problem with consumption of insects in southern Africa is that many people are more into Western lifestyle and that makes them ashamed or ignorant of consuming insects. The more educated population, are more reluctant to admit that entomophagy still exists. This can affect populations that are economically marginal, as they cannot afford meat or fish in order to provide protein. It is well documented that iron deficiency is a major problem in women's diets in developing countries, especially in poorer continents such as Africa (Orr, 1986). Vegetarians also are at risk of zinc deficiency because zinc content in vegetables is very low; as such eating of insects should be promoted as insects also contain high levels of iron and zinc (DeFoliart, 1992).

Consumption of insects is widespread especially in Africa and Asia. In the Bikita district of Zimbabwe, *Encosternum delegorguei* which commonly known as "*harurwa"* is much sought after and can be bartered for grain (Wilson, 1990; O'Flaherty, 2003). Harurwa is such an important insect that it is also distributed as gifts to the local chiefs, district administrator and the local police. The management of the forest in which harurwa is found is regulated by a team made up of a representative of 24 villages in Bikita district, with members rotating every year. Harurwa is a highly

priced insect and is therefore an important source of income in Bikita, Central Zimbabwe (O'Flaherty, 2003). Other species that are consumed in Zimbabwe includes the *Pentascelis remipes (local name "magodo"*) which feeds on *Combretum molle* and *C. imberbe as we as*. *P. wahlbergi* (*local name "nharara"*) that feeds on *Gardenia resiniflua (mutara*) and occur in clusters. The insects are a delicacy among the Manyika and Ndau tribes. *Gonimbrasia belina*, the"mopane-worm", is a particularly a major food item and is collected, transported and sold on an industrial basis at price of Z$0.60 per 100 g dry (during mid-1986), its price is similar to that of fresh beef (Wilson, 1990).

Caterpillars, termites, locusts, honeybees and ants are among the favorites and mostly consumed insects. Harvesting of insects is seasonal, but they can be collected, processes and stored for longer periods. In southern Africa, the most widely consumed insects are mopane worms, locusts, bugs, termites, honeybees and crickets. Chavunduka, (1975) has highlighted the significant role of insects in curing kwashiorkor. According to the above author, winged termites and giant crickets (*Brachytrupes membranaceus*) are frequently consumed in Zimbabwe. They are collected during rainy season. These insects are processed by grilling or frying without additional fat or they can be eaten raw. They are storable for later use. In South Africa, mopane worm (*Imbrasia belina*) is the most frequently consumed insect. Quin, (1959) reported that the Pedi tribe preferred mopane worm to beef and the availability of mopane worm could seriously affect the sale of beef. This worm is also widely consumed in other parts of southern Africa, such as Botswana, Zambia and Zimbabwe. Consumption of mopane worms can to a substantial degree supplement the predominantly cereal diet with many of the protective nutrients (Dreyer and Wehmeyer, 1982).

In Japan the most popular and widely eaten insect is the rice-field grasshoppers (mainly *Oxyya yezoensis* or *Oxyjaponica sp*.). They are fried and slightly seasoned with soy sauce to prepare a luxury dish called "inago", mostly sold as a luxury item in Japan (Mitsuhashi, 1997). Another widely consumed insect in Japan is "hachinoko", a bee or a wasp larva that is eaten raw, boiled down in soy sauce or served over boiled rice. Bee and wasp brood are canned and sold at a high price. The wasp-rice was reported to be the late Emperor Hirohito's favorite dish. Following surgery in 1987, the Emperor Hirohito "would finish the wasp-rice dish even when he had no appetite and left most of the other dishes" (Mitsuhashi, 1988).

1.2 Chemical composition of insects

A series of studies over the last 25 years have established the basic nutritional requirements of most major taxonomic groups of insects, the nutritional ecology of immature forms, and also the behavioral and physiological mechanisms by which individuals respond to variation in diet quality (Dadd, 1985; Simpson and Simpson, 1990, Slansky and Scriber, 1985). More recent work has focused on multidimensional aspects of insect nutrition. A fundamental problem faced by feeding insects is how to adjust physiology and behaviour to obtain simultaneously all necessary nutrients at reasonable rates (Raubenheimer, 1992; Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993).

It has been proven that insects can be an important source of protein (DeFoliart, 1975; Ramos-Elorduy, 1974, 1982a, 1982b), with caterpillars and termites being the most widely eaten and marketed insects in Africa. They have been shown to contain high concentrations of good quality proteins and high digestibility (Ramos-Elorduy *et al*., 1993a; Defoliart, 1989; Dreyer and Wehmeyer, 1982). According to Dreyer, (1968) the amino acids composition of dried mopane worms is relatively complete, with high proportions of lysine and tryptophan (which are limiting in maize protein) and of methionine (limiting in legume seed proteins). Dreyer and Wehmeyer, (1982), have concluded that the consumption of mopane worms can sustainably supplement the predominantly cereal diet with many of the required nutrients. The nutritional content of the mopane worm has been found to comprise of 60.7% crude protein, 16.7% crude fat and 10.72% minerals, on a dry matter basis and it is therefore a highly nutritious supplement to the diet of people consuming them (Dreyer and Wehmeyer, 1982).

Some caterpillars that are eaten in Africa Countries like Angola, Zaire and South Africa have higher protein content than those of America that have a deficiency in isoleucine (Kodonki *et al*., 1987), phenylalanine, and tyrosine and/or in tryptophan (Santos-Oliveira, 1976). The insects' species with the largest deficiency in essential amino acids were some members of the Family Saturnidae in America, reported by Landry (1986), with up to six species that do not reach the requirements of the WHO/FAO/UNU. The least deficient were the caterpillars of Zaire (Kodonki *et al*., 1987).

Ramos-Elorduy *et al*., (1997), analyzed nutrient composition of seventy-eight species of edible insects representing twenty-three families from the state of Oaxaca in Mexico. They include the orders of Anoplura, Diptera, Orthoptera, Hemiptera, Homoptera, Lepidoptera, Coleoptera and Hymeaoptera. The dry basis protein content ranged from 15-81%. The highest was found in a wasp of the genus *Ploybia*. Fat content ranged from 4.2% (several grasshopper species, *Boopedon flaviventris*; *Sphenarium* species, *Melanoplus mexicanus*) to 77.2% in the larvae of a butterfly *Phasus triangularis*. The insect richest in carbohydrates was found to be the ant *Myrmecosis tusmelliger* with 77.7%. Protein digestibility varied between 76% and 98%. The calorie contribution varied from 293-762 kcal/100 g, the highest value also being for the butterfly larvae of *Phasus triangularis* constituting a significant component of the diet of some rural communities in Oaxaca. Consumption parameters vary depending on the species, season, habitat, climate and biotope. The fact that seventy-eight species were registered and obtained for analysis in only a few places and over a short period indicates that, eating insects occurs generally as a habit both in the poor (Mixteca) as well as the more developed regions (La Costa).

Insects are not just emergency foods; they constitute an important product of the daily diet of a large proportion of some communities. In several cases, they are more of luxury product, and/or culturally important delicacy, a specialty eaten by custom due to their taste or special properties. It is also interesting that some families complement their income harvesting insects and selling them at the markets.

1.3 Why care for insects?

Mbata, (1995), has demonstrated the multiple importances of honeybees of the species *Apis mellifera adansoni* and *Apismellifera capensis* in Zambia. Honey if obtained from these species can be used as a source of sugar for rural people, and it is also used to brew local beer. The wax is used to make candles and to condition animal skins on the traditional drums. The brood (larvae and pupae) may be eaten with honey, or they may be extracted, fried and consumed as relish with the main meal. Silk from the cocoons of silkworm moths, *Bombyx mori* and related species has been used for fabric for centuries. Insects are essential in maintaining the ecosystem in aspects such as: plant propagation which includes seed dispersal and pollination, nutrient recycling via leaf-cutter and wood degradation, dispersal of fungi, disposal of carrion and dung and soil turnover (Gullan and Cranston, 1986).

1.4 Economical importance of insects

Insects are regarded as important food source and as such they contribute significantly to local economies. They are not only sold widely in the village markets but many of the favorites have made their way to urban markets and restaurants of the developing world. In Mexico white maguey worm (larva of the Hesperriid, *Aegiale hesperiaris*) as well as the “ahuahutle” (egg of several species of aquatic Hemiptera) are being exported to the United States and Europe. Thailand exports giant water bug (*Lethocerus indicus*) and canned silkworm pupae (*Bombyx mori*) to the Asian community shops in the United States (Ramos-Elorduy, 1997).

In sourthern Africa, mopane worm is widely sold and exported between African countries such as Botswana, Zimbabwe, Angola, Namibia and South Africa. Harvesters mainly sell mopane worm at roadsides vendors, open markets, tuck shops, supermarkets and bus termini and in some instances the marketing chain is quite long. Mopane worms are also being exchanged for goods and for gifts (Stack et *al*., 2003). In South African about 16 000 tonnes were traded during 1996 on the commercial market during the year 1982 (Dreyer and Wehmeyer, 1982). Styles, (1994) estimated that an annual population of 9,500 m mopane worms in South Africa’s 20,000km^2 of mopane veld worth £57m, of which approximately 40% goes to producers who are primarily the poor rural women. There are industries in South Africa, Botswana and Zimbabwe that are involved in canning and processing of mopane worm. The sale price range from $2.50-$4.00 per kg (Marais, 1996) which compares favorably with that of beef retailing at approximately -$4.00 per kg. Mopane worm is therefore a cheaper source of protein for low-income earners.

1.5 Insects as animal feed

Insects have also been incorporated into animal feeds to enrich their nutritional contents. House cricket (*Acheta domesticas*) when fed to weaning rats, was found to be superior to soy protein as a source of amino acids at all levels of intake and mormon cricket, (*Anabrus simplex*) was found to be equivalent to soy protein (DeFoliart, 1999). In a similar study conducted with a variety of insects in feeding trials in poultry, (Phelp *et al*, 1975; Dreyer and

Wehmeyer, 1982) obtained the same convincing results. The concept of feeding insects to domesticated animals such as poultry, pigs and farm-grown mink is popular in China, whereby more feeding trials have shown that insects-derived diets can be cost-effective to more conventional fish meal diets. The insects that are utilized are primarily the pupae of silworms, *Bombyx mori*, the larvae and pupae of house flies, *Musca domestica*, and the larvae of mealworms, *Tenebrio molitor*. In India they feed chickens with the meal that remains after the oil has been extracted from the pupae (Gullan and Cranston, 1986).

1.6 Insects as source of drugs

Insects are also an important source of novel drugs. The first antibacterial peptide, cecropin was isolated from an insect (the pupae of the moth *Hyalophoria cecropia)*, (Steiner *et al*. 1981) and since then more than 170 antimicrobial peptides have been found in insects. Furthermore, a cysteine-rich peptide drosomycin was the first inducible antifungal peptide to be isolated from insects and to date, has only been reported in the fruit-fly *Drosophila melanogaster* (Fehlbaum *et al*., 1994). Drosomycin is active against both human and plant fungal pathogens at concentrations often below 5 μM, delaying fungal hyphae growth at low concentrations and inhibiting spore germination at high concentrations (Dimarcq *et al.*, 1988).

Insect defensins also have activity against a wide range of Gram-positive bacteria, exerting an almost immediate lytic effect (Dimarcq *et al*., 1988). Studies conducted on a recombinant version of *Phormia terranovae* defensin demonstrated that the peptide instantly disrupts the permeability of the bacterial cytoplasmic membrane in *Micrococcus luteus*, resulting in loss of cytoplasmic potassium and ultimately, inhibition of respiration (Cociancich *et al*., 1993). The first broad-spectrum insect peptide to be reported was thanatin with activity against both Gram-negative and Gram-positive bacteria as well as filamentous fungi. It exerts bactericidal and fungicidal effects at minimal concentrations, often below 2.5 μM and is highly specific (with no side effects on red blood cells for example). Although the mode of action for thanatin is not yet understood, data suggest that it differs depending on the microorganism being targeted (Fehlbaum *et al.,* 1996).

Current antibiotics act by inhibiting protein synthesis or nucleic acid synthesis or by disrupting the cell wall. To achieve this, they only target the specific receptors, enzymes or a protein which makes the antibiotics vulnerable to the development of resistant strains. Insect peptides are being explored because insect antimicrobial peptides exhibit a high level of structural diversity, which could prove to be the good candidates to resolve the increasing problem of microbial drug resistance This is because insect-derived peptides use modes of action that should restrict the development of resistant strain because they have much broader target i.e., the microbe cell membrane. One promising insect derived compound being taken for clinical development is the ETD151, an antifungal 44 amino acid peptide analogue based on naturally occurring peptide from the Lepidopteran *Heliothis virescens*. This compound has been optimized by genetic manipulation of the native peptide and it is now in advanced preclinical development for treatment of life threatening hospital acquired fungal infections in immunosuppressed patients. Insect derived

molecules are not only sought solely as a source of antimicrobials, approximately 25% of the peptides involved in the immune response are antimicrobial, with the remaining 75% performing an array of different biological roles such as anti-inflammatory, anti-proliferative and anti-viral activities as well as ion channel modulators (Dimarcq and Hunneyball, 2003).

2. The plants

2.1 The use of plants extracts

Over three quarters of the world's population rely mainly on plants extract for healthcare. The study of plants that are used in traditional medicine in various cultures has yielded important drugs that are critical to modern medicine. The use of crude plant extracts poses some dangers. Many plants extract display variation of activity due to environmental conditions and time of collection. Due to these factors the amount of particular active constituents in plant extract may vary. This makes a difference when a particular dose of the extract is applied. If the amount is higher than normal, toxic effects may occur and if lower than normal the desired effect may not be achieved. For these reasons, it is necessary and desirable to identify the active constituents from plant extract to be able to have a reliable degree of efficacy and safety.

Since the development of organic chemistry at the beginning of this century, extraction and fractionation techniques have improved significantly. It is now possible to isolate and identify many of the active chemicals from plants (Srivastava *et al*., 2000). For example, quinine derived from the bark of the cinchona tree was used for treating fevers as early as the 17th century, although not until 1820 was the active ingredient of the bark isolated by French Scientists, Caventou and Pellentier and was used to treat malaria (Wright, 2005). Sertürner in 1806 identified morphine alkaloids from opium, which is the dried latex material that exudes from cut seed capsules of the opium poppy *Papaver somniferum*. Morphine is the major alkaloid component of opium, making up approximately 42% of total alkaloid content and is used mainly as analgesics ('pain relief drugs') (Huang and Kutchar, 2000).

2.2 The problem of drug resistance

Worldwide, a large burden of diseases is due to bacterial infections. Recently, treatment of bacterial infections in modern medicine has become a major problem due to emergence of bacterial strains, which are resistant to antibiotics (Rice, 2006; Tenover, 2006). The use of traditional herbs is more wide-spread than the use of modern medicine. This is one of the reasons why so many people especially in Africa and Asia are still relying on traditional herbs to treat diseases because of it costs less and easily accessible to the people in rural communities. Plants contain reservoirs of secondary metabolites that present potential for developing new drugs as they are now widely screened for medicinal purposes and their biological properties are determined. Thus far, ethnopharmacology of plants that are used in traditional medicine in various cultures has yielded important drugs that are critical to modern medicine (Farnsworth, 1984).

2.3 Importance of antioxidants

Antioxidants are natural compounds occurring widely in plants or synthetic compounds and are added to food in order to delay free radicals accumulation and hence strengthen its stability against oxidation and are thus very useful in human health (Halliwel and Gutteridge, 1995). Oxidative stress causes cell damage that later induces various kinds of diseases/disorders such as neurodegenerative disorders, inflammation, cancer and ulcers in humans. The balance between reduction and oxidation is believed to be a critical concept maintaining a healthy biological system (Aruoma, 2003).

Many medicinal plants have potential antioxidant components. Medicinal plants are recognized as sources of antioxidants components mainly because they contain flavonoids, phenolic acids, coumarins and antioxidant micronutrients. These antioxidants components contained in medicinal plants are of high interest because of their natural origin and the ability to act as efficient free radical scavengers (Langley-Evans, 2000). Potential sources of antioxidant compounds have been found in many types of plant materials such as fruits, leaves, seeds etc. Teas like mate, green and black tea, rooibos and honeybush are reported to have multiple biological effects, including antioxidant activity. Rooibos tea (*Aspalathus linearis*) and honeybush tea (*Cyclopia intermedia)* have been marketed for their high antioxidant potential largely due to their high polyphenol content. Green tea has been reported to have high antioxidant activity because it contains tannin with most of its antioxidant activity attributed to catechins (Nanjo *et al*., 1996).

2.3.1 Flavonoids

Flavonoids are important phytonutrient components that are present mainly in vegetables, fruits, nuts and beverages (Kim *et al*., 2006) and they are also formed in plants from the aromatic amino acids phenylalanine, tyrosine and malonate (Harbone, 1986). The basic structure of flavonoid is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C_6-C_3-C_6) which are labeled A, B and C. Various classes of flavonoids have been identified and they include: flavones, flavonones, iosflavones, flavonols, flavononols, flavan-3-ols, coumarins and anthocyanidins (Pietta, 2000).

The flavonols such as myricetin, qurcetin, kaempferol and galangin are effective scavenger of free radicals generated by both enzymatic and nonenzymatic systems (Kim *et al*., 2006). The B-ring -OH moiety is the most significant determinant factor in the scavenging of reactive oxygen species (Sekher *et.al.*, 2001; Burda and Oleszek, 2001). The flavonols, myricetin, qurcetin, kaempferol and galangin contain three, two, one and none –OH moieties on the B-ring respectively. Myricetin has been reported to have antiviral activity by inhibiting the reverse

transcriptase, antiaggregatory effects on blood platelets, and antiatherosclerotic effect via inhibition of oxidative modification of low density lipoprotein (de Whalley *et al*., 1990). Quercetin has been reported to prevent atherosclerosis chronic inflammation (Havsteen, 1983). Biological activities for kaempferol includes mainly the inhibitin of lipoxygenase and cyclooxygenase, it also posses antiaggregatory, antibacterial, and anticancer activities (Moriyama *et al*., 2003). Galangin is present in high concentrations in honey (propolis) and it is widely used in Asia countries for the treatment of respiratory infections, subcutanueo-mucosal and viral infections (Park *et al*., 1995). Galangin has antioxidative, radical scavenging, antimutagenic, anti-inflammatory activities as well as an inhibitory effect on cytochrome P450 hydroxylase in human liver microsomes (Cholbi *et al*., 1991; So *et al*., 1997; Kang *et al*., 2003; Buening *et al*., 1981; Ciolino and Yeh, 1999).

The presence of more –OH moieties on the B-ring plays a vital role in electron resonance and in the donation of electrons to the oxidizing agent. Oxidation pattern is however, affected by the number and pattern of –OH substitutions on the B-ring and by the presence of structural groups required for extended conjugated between the B- and C-rings. A study by Kim *et al.*, (2006) has demonstrated that among this group of flavonols, myricitin and quercetin which contains three and two-OH moieties on the B-ring, respectively, have the highest antioxidant capacity and intracellular antioxidant activity whereas, kaempferol and galangin with one and no-OH moiety on the B-ring respectively, have the lowest antioxidant capacity and intracellular antioxidant activity.

3. The host plants

Insects are known to sequester toxic materials from food plants. *Encosternum delegorguei*, local name (thongolifha); the edible stink bug that was investigated in this study was found mainly on *Dodonaea viscosa* although it was also found scarcely on other host plant such as *Diospyros mespiliformis.* Moreover, Getie *et al*., (2003) have reported that the two host plants posses medicinal properties and so far, *D. mespiliformis* is been widely studied. In this study we decided to focus on analyzing some of the medicinal properties of *D. viscosa* because the plant is widely spread at Modjadji, a village where thongolifha is harvested.

3.1 *D. viscosa*

3.1.1 Taxonomy and description of *D. viscosa*

In the latest taxonomic treatment of the plants of southern Africa (Germizhuisen and Meyer, 2003) two taxa are acknowledged.

Dodonaea viscosa var *angustifolia* (L.f.) Benth. includes *D. angustifolia* (L.f.) and *D. viscosa* Jacq. subsp. *angustifolia* (L.f.).

D. viscosa Jacq. var *viscosa* includes *D. viscosa* Jacq. subsp. *viscosa. This species occurs mainly in KwaZulu-Natal.*

Dodonaea viscosa Jacq. var. *angustifolia* (L.f.) Benth. (Family Sapindaceae) is the species investigated in this study. For ease of reference it will be referred to as *D. viscosa* in this thesis. It is a shrub of 3-4.6 m tall and prefers full sun. The shrub has green leaves all year with yellow flowers and it prefers moist soil (Fig 1.1). The soil conditions can either be acidic, neutral or alkaline (Palgrave, 2002).

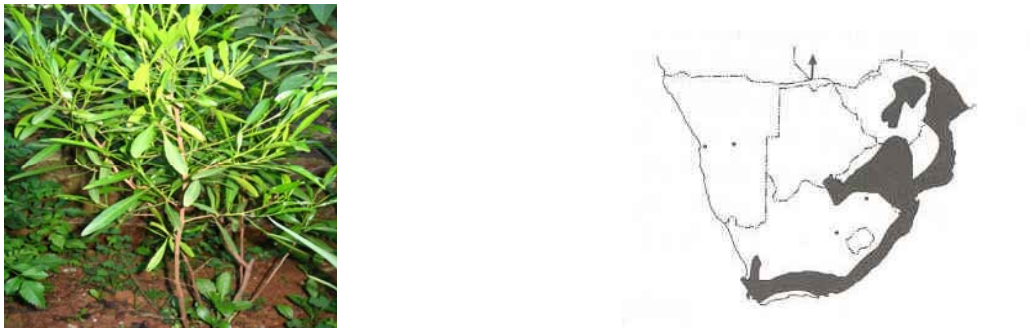


Figure 1.1: *D. viscosa* (Picture by: Cathy Dzerefos) and its distribution map (Palgrave, 2002).

3.1.2 Biological activity of *D. viscosa*

The leaves of *D. viscosa* have traditionally been applied internally to treat fevers, toothache and sore throat or externally to treat skin rashes, wounds and stings. The bark is employed in astringent baths and poultices (Bown, 1995). The plant can also be pruned to make a neat hedge that is popular in rural areas whereas the leafy branches are used to make brooms that are used to sweep around the yard. Getie *et al*., (2003) reported that methanol leaf extract from Ethiopia demonstrated antibacterial activity against *Staphylococcus aureus, Streptococcus pyogenes, Cornybacterium diphtheriae* and exhibited antiviral activity against Coxsackie virus B3 and Influenza A virus. However, their quantitative data was performed by using the agar diffusion technique which can only measure the width of a zone of growth inhibition to indicate the activity of the plant extract. This method is however not convincing enough to quantify activity of plant extract. It was therefore necessary in our study to quantify the activity of *D. viscosa* with solvents of various polarities by applying the microdilution assay as described by Eloff, (1998) and to further determine the total activity of the *D. viscosa* extracts.

Apart from these, the leaves of *D. viscosa* are reported to be toxic, as dairy cattle have died of poisoning after consuming the leaves; postmortem results have shown liver damage with massive hepatocellular necrosis (Colodel *et al.*, 2003). This has never been found in southern Africa based on the latest highly regarded publication in this field (Kellerman *et al*., 2005).

3.1.3 Major chemical constituents of *D. viscosa*

Chemical constituents previously isolated from *D. viscosa* include the following:

diterpernoid acids (hautriwaic acid, dodonic acid, oleic acid, linolenic acid);

flavonoids (5-hydroxy-3,6,7,4'-tetramethoxyflavone, santin, penduletin, quercetin, kaempferol, acacetin-7-methyl ether and aliarin and a new flavonoid with an isoprenoid side chain 5,7-dihydroxy-3'-(3-hydroxymethylbutyl)-3,6,4'-trimethoxyflavone; isorhamnetin; pinocembrin)

and quinones, tannins, saponins, triterpene steroids as well as a new clerodane diterpenoid identified as 15, 16-epoxy-5,9-diepicleroda-3,13(16),14-trien-20,19-olide (Dominguez, 1980; Ibid, 1983, Sachdev and Kulsreshtha, 1983, 1984; Rojas *et al*., 1992; Getie *et al*., 2000; Abdel-Mogib, 2001). The chemical composition is also discussed later in the thesis.

Problem statement

It is not widely appreciated in the western world that insects are a good and cheap source of protein. Although it is well evident that all societies have consumed insects during the past, including biblical times with reference to John the Baptist who reputedly depended almost entirely for his diet on locust and honey (New Testament, Mark 1:6). However, in Africa entomophagy, is widely appreciated, although it is more prevalent in rural than urbanized areas. More educated persons are sometimes reluctant to admit that indigenous customs, including eating of insects still exist. Some people secretly eat insects and are ashamed of their culture. However, eating insects can significantly reduce protein deficiency and this should be promoted through education especially in schools. Malnutrition remains a major problem, for which one reason is the lack of a mixed, balanced diet. Information on multi-use of insects' products should be known. Sustainable harvesting must be achieved in order to optimize the harvest and protect the environment and limit the loss of host plants. Sustainable harvesting of host plant is necessary because the insects can not survive without their host plants.

E. delegorguei feeds mainly on *D. viscosa*, a plant with known antibacterial and antiviral activities. It may be possible that some compounds that are present in *D.viscosa* are also present in *E. delegorguei* since insects are known to sequester compounds from host plants. The chemistry of *D. viscosa* has been investigated thoroughly and it is unlikely that any new compounds will be identified. Compounds with antibacterial activity extracted from *D. viscosa* will be isolated and characterized. The possibility that these compounds also occur within *E. delegorguei* will be investigated.

Aim of the Study

To investigate the nutritional value of the edible stink-bug *E. delegorguei* and the possible interaction between the insect and its host plant, *D. viscosa* (In this thesis *D. viscosa* is used as an abbreviation to indicate *Dodonaea viscosa* Jacq. var. *angustifolia*).

Objectives of the study

- ✓ To determine the nutritional components of thongolifha that will benefit the Venda community both economically and ecologically.
- ✓ To evaluate the antibacterial activity of insect extracts.
- ✓ To extract components from *D. viscosa* and determine its medicinal properties.
- ✓ To isolate antibacterial compounds from *D. viscosa* and elucidate their structures.
- ✓ To evaluate whether bioactive compounds present in *D. viscosa* possibly occur in *E. delegorguei.*

C H A P T E R 2

2. METHODOLOGY

2.1 Insect Experimental Procedures

2.1.1 Collection of Insects

Thongolifha is a traditional delicacy for the Venda tribe of Limpopo Province. The scientific name for thongolifha is *Encosternum delegorguei* and the identity of this insect was confirmed by expert Entomologist Dr RB Toms of Transvaal Museum, Pretoria South Africa; as *Encosternum delegorguei* Spinola. The Insects are mainly collected at the mountainous area of Modjadji village in the Limpopo Province. During our field trips we observed that the insects prefer to settle and feed on evergreen plants such as the *Dodonaea viscosa* and *Diospyros mespiliformis*. The insects were harvested during winter season (May-August) at dawn or during sunset. The harvesters collect them in a plastic bag with bare hands and the defensive secretion leaves an orange stain on their hands.

2.1.2 Traditional Knowledge of Insects Preparation

The processes described here are used by expert pickers from Venda. Different or shorter procedures are often used. The first step of preparation is to separate all dead bugs, leaves and debris from the live bugs. The dead bugs are removed, as they are unable to release the defensive secretions and may be prepared in a different way or used for different purposes. The live bugs are placed in a bucket with a small amount of warm water and stirred with a wooden spoon. During the stirring procedure the bugs release a defensive secretion with a strong odour. This is painful if it reaches human eyes and the collectors close their eyes to protect them. The bugs are rinsed with warm water and the process is repeated about three times. The body is full of fat and some fatty compounds can be seen floating on the water like oil during the preparation process. Dead bugs that were missed during the first sorting are easily identifiable during subsequent quality control. Their surface is blackened by the stink glands and they are therefore, rejected for human consumption. An alternative procedure is used for dead bugs whereby the heads of the dead bugs are removed and the thorax and abdomen are squeezed between the thumb and index finger. The translucent pale green gland is exuded through the neck of the dead bug and wiped off on a stone. The bugs are then sun-dried. The dried bugs are ready for consumption either raw or cooked.

Step 1

Step 2

Step 3

Step 4

Step 5

Step 6

Figure 2.1 Steps that are taken during the traditional preparation of thongolifha from the period it is harvested until it is taken to the Venda market to be sold. Step 1- Freshly harvested thongolifha and the dead ones are sorted out and discarded; step 2- Live ones are placed in a bowl; step 3- heads of dead thongolifha are removed and the thorax squeezed on top of the stone to exude translucent pale green gland; Step 4- live bugs are placed in warm water and stirred to get rid of the defensive secretion and after this the bugs are blanched; Step 5- processed bugs are dried; Step 6- bugs are sold at Venda market. (Pictures by: Dr Rob Toms).

2.2.1 Nutritional Analysis of Insects

Because of lack of facilities from both Phytomedicine Programme laboratories as well as the Transvaal Museum, samples were send to a subcontracted laboratory and an analysis of the chemical composition of the stink-bug was performed. Traditionally prepared specimens of *E. delegorguei* were purchased at the Thohoyandou market. Some of these were ground and 100 g material was analyzed using standard procedures for the measurement of nutritional qualities of food at the Food and Feed SANAS accredited laboratory of the Agricultural Research Council-Irene Analytical Services. The following tests were done and procedures briefly explained and method followed as indicated:

2.2.1.1 Determination of Macronutrients and Amino acids

The method of Harris (1970) was used to determine ash content of stinkbug. The inorganic matter of a sample is the total ash. The organic matter of a sample was removed by heating at 550 ^{o}C overnight and the remaining residue is the ash**.** For determination of fat content, Allihan Condenser Soxhlet extraction apparatus was used with ether as extractant. Fat is made up of fatty acids and glycerol. Most fats are soluble in ether; therefore the stinkbug sample was dissolved in ether followed by boiling at boiling temperature. The ether was evaporated and the fat was left inside the beaker. The weight gained was used to calculate the fat content. For the composition of proteins and peptides, the method used for the analysis of amino acids which involves acid hydrolysis extraction, pre-column derivitisation, separation by High Performance Liquid Chromatography (HPLC) and detection using a fluorescence detector was applied (Einarsson *et al*., 1983). Protein in the stinkbug material was extracted and further precipitated with tungstate and the concentration of the different carbohydrates in the filtrate was determined by HPLC with refractive index detection (Smit and Nel 1987). For the conversion of nitrogen content to protein the factor 6.25 was used.

2.2.1.2 Determination of Minerals

Edible stink bug (1 g) was digested with 7 ml concentrated nitric acid and 3 ml perchloric acid at temperatures up to 200 ^{0}C and brought to volume in a 100 ml volumetric flask (Zasoski and Burau, 1977). For potassium and sodium an aliquot of the digested solution was subjected to flame emission spectroscopy in a LPG-air flame using lithium as an internal standard. For determination of iron and zinc minerals the digest solution was subjected to atomic absorption spectrometry using an Air-Acetylene flame with wavelength of 248.3 and 213.9 nm respectively. For calcium, an aliquot of the solution was subjected to atomic absorption spectrometry in a Nitrous Oxide-Acetylene Flame, using wavelength of 422.7 nm (Antanasopoulos, 1982).

2.2.1.3 Determination of Vitamins

Thiamine (vitamin B_1) and riboflavin (vitamin B_2) were determined by HPLC (Wimalasiri and Wills, 1985). Vitamin C was extracted using acetic acid and meta-phosporic acid, followed by determination with HPLC and fluorescence detection (Dodson *et al*., 1992). For vitamin A and vitamin E detection, alkaline saponification of the test material was done, which involves elimination of fats, liberation of natural retinol in the cells. Unsaponifiable material was extracted with ether and vitamin A was determined by using HPLC and detection done by UV and fluorescence respectively (Manz and Philip, 1981)

2.2 Plant Experimental Procedures

2.2.1 Collection and Identification of Host Plant

The leaves of *D. viscosa* were collected from Modjadji kraal in the Limpopo Province during August 2004. The identification and voucher specimen no. of the plant was sorted out and deposited at the National Botanical

Institution, Pretoria as voucher specimen no. 1, Gen spec no. 48310004. The plant was identified as *Dodonaea viscosa* Jacq. var. *angustifolia*.

2.2.2 Plant Preparation

The leaf material was allowed to dry at room temperature and ground into a powder, labeled and stored in a tightly closed dark bottle container in a dark cupboard at room temperature until they are needed for analysis.

2.3 General Materials and Methods

2.3.1 Extraction Procedure

The dried insect material as well as the leaf material of the host plant, *D.viscosa* were analyzed by using standard procedures at the Programme for Phytomedicine, University of Pretoria. The extraction was carried out as described by the method of Eloff, (1998a). Briefly, 1.00 g of the powdered material was serially extracted with 10 ml of the following solvents: hexane, DCM, EtOAc, acetone and EtOH. This system is preferred because it separates components over a wide range of polarities. The extracts were centrifuged at 3000x g for 10 min. The supernatant was decanted and the extract centrifuged again and the process was repeated twice. The extracts were filtered using Whatman No.1 filter paper and the solvents evaporated under a cold airflow. The yield was determined and extracts were freshly prepared with acetone at concentrations of 10 mg/ml prior to use for the bioassays.

2.3.2 Thin Layer Chromatography analysis

To determine the composition of the extracts, 10 µl of 10 mg/ml of components of either *E. delegorguei* or *D. viscosa* were loaded onto TLC plates (Silica gel 60 F_{254}, Merck) and developed using the following eluent systems: the intermediate eluent system CEF (Chloroform/Ethyl acetate/Formic acid) at ratio of 10:8:2; the polar eluent system EMW (Ethyl acetate/Methanol/Water) at ratio of 10:1.35:1 and non-polar eluent system BEA (Benzene/Ethanol/Ammonia) at the ratio of 18:2:0.2. To reveal the constituents in the crude extracts, the developed TLC plates were air dried and visualized under ultraviolet light (245-360nm, Camac Universal UV lamp TL-600) and subsequently sprayed with ninhydrin (0.2 g ninhydrin dissolved in 100 ml ethanol with addition of 0.2% acetic acid) for insect material and vanillin (0.1 g vanillin dissolved in 28 ml methanol with careful addition of 1 ml sulphuric acid) for plant material and allow to develop under oven at 110 ^{0}C (Stahl, 1969). Ninhydrin and vanillin were both purchased from SIGMA, Aldrich, Germany.

2.3.3 Bioassays

Test Microorganisms - Gram-positive microorganism *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212); Gram-negative microorganism *Pseudomonas aeruginosa,* (ATCC 25922) and *Escherichia*

coli (ATCC 27853) were used. These strains which are responsible for nosocomial infections are recommended by the National Committee for Clinical Laboratory Standards (NCCLS) 1990, Villanova, Pennyslavania, USA for antibacterial testing

2.3.1.1 Bioautography assay

Bioautography assay locates the activity of individual components on developed TLC plates. To achieve this, the TLC plates (10 x 10 cm) were developed in EMW, CEF and BEA and allowed to dry overnight. Four test bacterial cultures (*S aureus, P. aeruginosa, E faecalis and E coli*) were prepared as follows: actively growing test bacterial cultures were centrifuged at 3000 x g for 10 min. The supernatant was discarded and the pellets were then redissolved in 10ml fresh Mueller-Hinton (MH) broth (Fluka BioChemika). The suspension of concentrated bacterial suspension was sprayed onto developed TLC plates, placed into a tank and incubated overnight at 37 ^{0}C in 100% relative humidity. After incubation the TLC plates were sprayed with 2 mg/ml solution of INT and incubated for a further 30 min or until activity is observed. Activity of individual constituents was determined by observing any inhibition of bacterial growth, which is indicated by a clear zone against a red-violet background on the chromatograms. This method was previously described by Begue and Kline, (1972).

2.3.3.2 Microdilution assay

The minimal inhibitory concentration (MIC) value is taken as the lowest concentration at which no growth has taken place after approximately 24 hours of incubation. To determine the MIC values of the extracts, the microplate method described by Eloff, (1998b) was used. The following test microorganisms were used: Gram-positive microorganism: *S. aureus* and *E. faecalis;* Gram-negative microorganism: *P. aeruginosa and E. coli.* Briefly, 100 µl of distilled water was added to 96- well microtitre plates followed by addition of 100 µl of 10mg/ml extracts and serially diluted two fold from 2.5 to 0.02 mg/ml after which 100 µl of the above mentioned 4 actively growing test microorganisms were added to each microtitre plates in all the wells to give final volume of 200 µl. Gentamycin (SIGMA Aldrich, Germany) was used as positive control (0.1 mg/ml stock solution) whereas acetone and water were used as negative controls. The prepared microtitre plates were sealed so that they do not dry and incubated overnight at 37 ^{0}C in 100% relative humidity. An indicator of bacterial growth, 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) (SIGMA Aldrich, Germany) dissolved in water was then added to all the microtitre plate wells and incubated for a further 30 min-2 hrs. Bacterial growth was indicated by the red color of the INT reduced to formazan. The MIC values were recorded as the lowest concentration at which a decrease in red color is apparent compared to the next dilution.

2.3.3.3 Antioxidant assay

2.3.3.3.1 Qualitative assay

Qualitative screening for antioxidants from the extracts was carried out using the 2.2 Diphenyl-1-picrylhydrazyl (DPPH) (SIGMA Aldrich, Germany) method as described by Glavind and Holmer, (1967). DPPH, which is a stable purple free radical and its reactions with antioxidants results into discoloration of free radicals. To determine antioxidants activity, 10 µl of 10 mg/ml of extracts were loaded on TLC plates and developed in CEF, EMW and BEA eluent systems. The plates were air-dried followed by spraying with 0.2% DPPH in methanol to screen for any antioxidants activity. The presence of antioxidant components was confirmed by initially purple background that turns yellow on spots where antioxidant components are present.

2.3.3.3.2 Quantitative (DPPH Spectrophotometric) assay for crude extract

Sample stock solution of each of the crude extracts: hexane, dichloromethane, ethyl acetate and methanol (1000 µg/ml) were diluted to a final concentration of 500, 250, 125, 63 and 31 µg/ml in ethanol. Ten microlitres of 0.25 mM DPPH ethanol solution was added to 50.0 µL of sample stock solution of different concentrations and allowed to react at room temperature for 30 minutes in a dark chamber. The blank solutions were prepared with sample solution 50.0 µL and 20.0 µL of ethanol only while the negative control was DPPH solution, 20.0 µL plus 50.0 µL ethanol. This method was previously described by Mensor *et al.,* (2001). The absorbance values showing a change in colour from deep violet to yellow was measured at 517 nm on a microplate reader and converted to percentage antioxidant activity (AA%) using the formula:

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$$

1. Abs_{sample} is the absorbance of the sample + DPPH solution,

2. Abs_{blank} is the absorbance of the sample solution + methanol only (without DPPH) this corrects for any absorbance due to interaction of the sample and methanol

3. $Abs_{control}$ is the absorbance of DPPH + methanol only (without sample) this corrects for any absorbance due to interaction of the DPPH and methanol.

L-ascorbic acid (Sigma Aldrich) was used as a positive control (antioxidant agent)

In this study, EC_{50} is the concentration of the test sample that will bring about 50% inhibition of the DPPH free radicals. Its value was calculated from the separate linear regression of plots of concentration of the test extracts (µg /mL) against the mean percentage of the antioxidant activity obtained from the three replicate assays.

2.3.3.3.3 Quantitative-DPPH Spectrophotometric assay for isolated compounds

This was carried out as previously described by Mensor *et al*., (2001) with slight modification. Sample stock solution of each of the isolated compounds (200 µM) from *D. viscosa* leaf extracts was diluted to a final concentration of 100.0, 50.0, 125.0, 25.0 and 12.5 µM in MeOH. Ten microliter of 0.2 mM DPPH MeOH solution was added to 50.0 µL of sample stock solution of different concentration and allowed to react at room temperature for 30 minutes in a dark chamber. The blank solutions were prepared with sample solution 50.0 µL and 20.0 µL of MeOH only while the negative control was DPPH solution, 20.0 µL plus 50.0 µL MeOH. The absorbance values showing a change in colour from deep violet to yellow was measured at 515 nm on a microplate reader and converted to percentage antioxidant activity (AA%) using the formula:

$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$

(Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control). L-ascorbic acid (vitamin C) was used as a positive control (antioxidant agent).

EC_{50} is the concentration of the test sample that will bring about 50% inhibition of the DPPH free radicals. Its value was calculated from the separate linear regression of plots of the mean percentage of the antioxidant activity against concentration of the test compounds (µM) obtained from three replicate assays.

2.4 Statistical analysis

The results were expressed as mean ± SEM (Standard error of mean). The software for regression plots (SigmaPlot® 2001, SPSS Science) was used.

C H A P T E R 3

3. NUTRITIONAL VALUE *OF E. DELEGORGUEI*

3.1 Background

In Thoyandou region of Limpopo Province of South Africa; traditions, beliefs, myths, habits and customs are still deeply rooted among the Venda people. The consumption of various insects such as grasshoppers, termites, mopane worms and stinkbugs is a tradition and custom that persists. Edible insects are harvested and sold at the open markets around Thoyandou. The edible stinkbug, *Encosternum delegorguei* Spinola Tessaratomidae (Order Heteropreta) is one of the most important insects in South Africa from a cultural point of view. Thongolifha is consumed by the va-Venda tribe in Thoyandou of the Limpopo Province. The stinkbug, which is popularly known as thongolifha, it is in great demand at the Thoyandou market although it emits a very strong odour. Among the Venda tribe, young and old are fond of the thongolifha and they eat it raw or cooked either with porridge or alone (Faure, 1944). The harvesters travel to other areas such as Modjadji village that is about 200 km from Thoyandou to collect them. The insect is also imported from southern African countries such as Mozambique and Zimbabwe as the local supply can not meet the demand. During our field trips we have observed that, thongolifha feeds on several plants including *Dodonaea viscosa* and *Diospyros mespiliformis.* These two host plants have well-established antimicrobial value (Getie *et al*., 2003; Adzu *et al*., 2002). Thongolifha is probably an important diet supplement and it was therefore important in this study to analyse its chemical composition for nutritional supplement because this has not yet been done before.

3.1.1 Morphology of the thongolifha

The body is c. 25 mm in length, green-yellow or brown (Fig. 1) with lateral margins of abdomen not exposed as in similar *Natalicola* species. Nymphs are circular and green. Their habitat is subtropical and open woodland and bushveld (Picker *et al*., 2000; Faure, 1944).

Thongolifha is mainly harvested during winter season either at sundown or early in the morning or better still on misty, cloudy days. At these times the insects are cold and less active and congregate in clusters, possibly to conserve heat. They fly in swarms on hot days and settle in the evening on evergreen plants such as *D. viscosa* and *D. mespiliformis*. The bug is scarce during summer or wet seasons. It is believed that they congregate and lay their eggs during spring and the development of nymphs will then occur in summer (Toms and Thagwana, 2003).

Figure 3.1: *E. delegorguei* settling on *D. viscosa* and on a paper (Photograph- Dr R.Toms).

3.2 Results and Discussion

Available literature on nutritional values of insects is scarce; but indicates that insects have high protein, energy, mineral and vitamin content as well as low to high fat content. The results for macro nutrient composition of thongolifha (*E. delegorguei)* are represented in Table 3.1. Thongolifha in this study had a protein content of 35.5%. The fat content of the thongolifha was 50.6%. This is not surprising since during processing of the bug; fat can be seen floating on the water. Calculated carbohydrate content was 7.63 g/100g. The energy value of 2599 kJ/100g is acceptable and comparable with other edible insects (Table 3.6). High energy content based on carbohydrates and fats is very important in foodstuffs because it complements diet and helps in complete utilization of protein thereby increasing nutrition significantly. Vitamins A, B_1, B_2, and E were detected at reasonable concentrations of 0.23, 0.63, 0.86 and 2.17 mg/100 g respectively (Table 3.2) with recommended daily allowance of 1.7-8 mg/day.

Essential amino acids were also detected, the concentration of essential amino acids varied from 0.82 mg/100 g (threonine) to 1.32 mg/100g (valine). Other essential amino acids were also present in reasonable concentrations (Table 3.4). The percentage of different essential amino acid of thongolifha protein (Table 3.5) was compared to that of beef and chicken meat (Beach *et al*., 1943) The data has indicated that although the percentage of methionine and lysine for thongolifha has scored low values compared to beef and chicken, however the values for phenylalanine, tyrosine, threonine and tryptophan are closer to that of beef and chicken. This implies that thongolifha protein quality is not nearly as good as that of beef and chicken and therefore consumption of thongolifha will provide to a degree for most essential amino acids.

Important minerals diet supplements such as iron, potassium, phosphorus and selenium were also found at high concentrations (Table 3.3). Total minerals content was at 1.2 g/100g which is at comparable levels to other bugs such as *Acantocephala declivis* (1.0 g/100g). The data about the nutritional content of thongolifha has confirmed that consumption of these bugs by the vhaVenda tribe is not just a traditional delicacy but it provides them with good source of protein, various minerals and essential amino acids

The nutritional contents of the most widely consumed insects and other animal food has been determined and their values are compared in Table 3.6. The protein content varied from as low as 31% (wasp) to as high as 63.5% for mopane worm. Because of mopane worm's high protein content, it is used in health care whereby it is crushed and mixed with porridge to cure children with kwashiorkor. Termite and thongolifha have high fat content compared to other insects and they present with high energy content when consumed. From the data in Table 3.6, it is evident that insects have higher protein content than animal food. Insects are comparable with expensive fish in their protein content. Insects can be a cheaper sources of protein, so people should be encouraged to accept them as a respectable food item based on their nutritional and economic implications.

Some of the insects that are regarded as food are sometimes available in abundance and could be regarded as pests by farmers. Widespread harvesting and consumption of insect might serve as a form of biological control of these pests. Harvesting of insects for consumption will results in reduction in the use of pesticides as well as creating new economic opportunities for indigenous people. However, public education is vital if we need to preserve safe insects foods, the subject can also be incorporated into school curriculum for young people to learn and appreciate insects as food as they grow up. Harvesters must also be taught to look after the forest in order to preserve insects. Communities must be encouraged to manage the forest very wisely thereby enhancing the sustainable harvesting of plants and insects.

Not all insects are edible, some are toxic and may create allergy problems but the ones that are edible should be looked after and their host plants must be preserved. Some toxic insects may be used in medicine and thongolifha may have some toxic elements as it was mentioned earlier in chapter 1 that its host plant *D. viscosa* contain some toxic substances. Traditional preparation of insect should also be improved to avoid contamination and wastage, thereby providing for a high quality and acceptable product. As people are encouraged to eat insects, the subject of food safety should be looked into. Food contamination is now regarded as a serious public health problem worldwide. Insect are mostly collected by rural people and in rural areas, there is problem of food hazard, which is linked to poor sanitation, lack of water supply food storage, and marketing of food that includes mainly vending site. At Modjadji, an important village were thongolifha is harvested, all these health hazards exist and one cannot guarantee that the end product is always safe. In reality, many insects are far cleaner than other creatures, for example, grasshoppers and crickets eat fresh clean plants whereas crabs, lobster and pigs (that many people enjoy eating) eat any kind of decomposing material as a scavenger. The other important factor about insects is that, by

weight insects such as termites, grasshoppers, caterpillars are a better source of protein than beef, chicken, pork or lamb (Lyon, 2005).

If one evaluates the number of thongolifha that have to be consumed per day to provide in the daily required minimum for essential amino acid (Table 3.4) the number varies from 680 for phenylalanine to 3 400 for methionine.

Table 3.1: Macro Nutrient composition of *E delegorguei* (g/100 g edible weight)

Protein	**Fat**	**Energy (kJ/100 g)**	**Carbohydrate (g/100 g)**	**Moisture**	**Dry matter**	**Ash**
35.2%	50.5%	2599	7.63	4.9%	95.1%	1.7%

Table 3.2: Vitamin content of *E. delegorguei* (mg/100g)

Vit A mg/100 g	**Vit B_1 mg/100 g**	**Vit B2 mg/100 g**	**Vit C mg/100 g**	**Vit E mg/100 g**
0.23	0.63	0.86	Not detected	2.17

Table 3.3: Mineral content of E. *delegorguei* (mg/100 g)

Chloride	**Sodium**	**Copper**	**Sulphate**	**Calcium**	**Magnesium**	**Potassium**	**Phosphorus**	**Iron**	**Selenium**	**Manganese**	**Zinc**
85.4	55.3	4.4	66.7	91	109	275	575	20.2	0.2	0.8	4.6

Table 3.4: Essential amino acid profile of *E. delegorguei* and daily requirement

Essential Amino Acids	**Amino acid content of *E.delegorguei* (mg/100g)**	**Daily minimum requirement (mg)**	**Mass (g) required for daily minimum**
Isoleucine	830	450-700	54
Leucine	1050	620-1100	59
Methionine	400	550-1100	137
Phenylalanin	810	220-1100	27
Tryrosine	1260	900-1100	71
Threonine	820	310-500	37
Tryptophan	160	160-250	100
Valine	1320	650-800	49
Lysine	850	500-800	58

Table 3.5: Percentage of protein of different essential amino acid of *E. delegorguei* compared to that of beef and chicken.

Essential amino acids	**Methionine**	**Phenylalanin**	**Tyrosine**	**Threonine**	**Tryptophan**	**Lysine**
E. delegorguei	1.1	2.3	3.5	2.3	0.4	2.4
Beef (Beach *et al*., 1943)	3.1	4.9	4.3	4.5	1.4	8.1
Chicken (Beach *et al*., 1943)	3.2	3.8	4.2	4.6	1.3	8.4

Table 3.6: Nutritional components of some of the commonly consumed insects and other mammals based on 100g serving.

Species	**Protein (%)**	**Energy K.cal/100 g**	**Minerals (g/100 g)**	**Carbohydrates (g/100 g)**	**Fat (%)**	**Reference**
Thongolifha (*E. delegorguei*)	35.2	2599	1.2	7.63	50.5	Chapter 3 results
Termite (*Macrotermes falcigen)*	41.8	7611	0.75	N/A	44.3	Phelps *et al*., 1975
Beetles (*Callipogon barbatus*)	41.5	474	2.1	23.2	34.8	Ramos-Elorduy, 1997
Mopane worm (*Imbrasia belina*)	63.5	543	3.5	11.4	18	Dreyer and Wehmeyer, 1982
Wasp (*Polistes instabilis*)	31.0	655	2.1	3.0	62	Ramos-Elorduy, 1997
Larvae *(Apis mellifera)*	42.0	475	3.1	1.0	19.1	Ramos-Elorduy, 1997
Bug (*Acantocephala declivis*)	35.1	547	1.0	18	45.3	Ramos-Elorduy, 1997
Grasshoppers/locust (*Sphenarium histrio*)	77.2	363	2.1	12.4	12.0	Ramos-Elorduy, 1997
Beef	27.4	219	3.5	*N/A	*N/A	Lyon, 2005
Fish	28.5	170	1.0	*N/A	*N/A	Lyon, 2005

*N/A- results not available.

3.3 Conclusion

Thongolifha is not just a traditional food; it has high protein content. It is therefore, recommended as good source of protein and people should be encouraged to consume it. However, sustainable harvesting is recommended in order to maintain availability of stink-bugs. The harvesters from Venda are already traveling as far as Modjadji area to collect this traditional delicacy. Thongolifha is not only a traditional food but many families rely on the sale of the insect to maintain their standard of living. For these reasons sustainable harvesting and preservation is vital. Most widely consumed insects have been proven to have high protein content. This will ultimately encourage people to consume insects, especially if they know that they are acquiring high quality protein for less cost.

The large number of insects required to satisfy the daily required essential amino acids indicates that insects can only be an additional food source and would not easily provide all the protein requirements.

CHAPTER 4

4. MEDICINAL VALUES OF INSECTS

4.1 Background to use of insects

4.1.1 Use of insects in Folk Medicine

Entemotherapy, which is the use of Insects and insects' derived products in medical systems have been used by different human cultures throughout the world for centuries. Insects are used live, cooked, ground, in infusions, in plasters, and as ointments (Costa-Neto, 2002). For example the Maya were already using maggots for therapeutic purposes a thousand years ago (Zimmer, 1993). In Brazil, about 42 insects species were reported to have been used in folk medicine (Costa-Neto, 2002) and in India, the use of insects in folk medicine is also common (Hitchcock, 1962). Different species of insects are used as remedies. The mud wasps, leaf-cutting ants, cockroaches, termites and crickets are mainly used to treat asthma, bronchitis and sore throat. They are usually crushed and made into tea which is drunk three times a day and the patient is not supposed to know what they are drinking (Costa-Neto, 2002) In order to treat stroke, insects such as dung beetle, coconut borer, stingless bee, grasshoppers are burned and patient must inhale the smoke (Costa-Neto, 1994, Lima, 2000). The larvae of coconut borer are fried in their own fat in order to extract oil that is used as a treatment for dandruff (Costa-Neto, 1994).

Herbalists from Feira de Santana prescribe the sting of honey bee, *Apis melifera scutellata* for the treatment of rheumatism, backache and joint problems. In India the Pankarare people use honey bee to cure whooping cough and tuberculosis, and the Ichu people eat it for curing ulcers. The Remanso community use honey bee to cure cough and diabetes (Costa-Neto, 2002). The mud wasp is used by the Pankarare community to treat mumps, whereby the nest is melted in water and the mixture is applied on mumps, they also use honey of stingless bee as an antidote against snakebites (Costa-Neto, 1994, Lenko and Papavero, 1979). Honey is used as an eye drop to treat cataract and glaucoma by the rural people of Tanquinho and Serrinha. In the city of Itaberaba, tea of toasted ant is given to a child to make the child to stop wetting the bed (Costa-Neto, 2002).

In the city of Feira de Santana, the cockroach is cooked and its tea is drunk in order to treat heartburn, on the other hand the people from Matinha dos Pretos put the exoskeleton of a cockroach over wounds to promote their scarring. The sting bug, *Tritoma sp*. is used mainly to treat toothache and earache (Costa-Neto, 1994). The bumble bee, (*Bombus sp*), is used as love charms (Lenko and Papavero, 1979).

Furthermore, insects and other anthropods are used in Korean traditional medicine. The centipede (*Scolopendra sp.*) is used primarily to treat arthritis because centipedes have so many legs it is believed to cure leg problems. The silk moth fungus (*Beauveria bassiana*, which infects silk moth larvae) is used mostly to treat stroke (Pemberton, 1999). In Australia, the Aborigine community use insects such as bush cockroach (Blattidae) for local anaesthetic, they use the green tea ant (Formicidae) to prepare a refreshing drink, cure headaches, as a cold remedy, as an antiseptic and expectorant. The caterpillar is used for wound dressing and honey bees are used to treat sore throat (Cherry, 1993).

4.1.2 Insects as source of new drugs

Insects are known to sequester compounds from host plants and store them for their defense mechanism (Harborne, 1993). This phenomenon has developed much interest in searching for novel drugs derived from insect components. Insects have thus far proven to be promising new source of drugs in modern medicine since they possess immunological, analgesic, antibacterial, diuretic, anaesthetic and antirheumatic properties (Yamakawa, 1998). Chemical screening of 14 species of insects has confirmed the presence of proteins, terpenoids, sugars, polyols and mucilages, saponins, polyphenolic glycosides, quinones, glycosides and alkaloids (Andary *et al*., 1996).

Chitosan which is a compound derived from chitin has been used for various purposes: as an anticoagulant, for lowering serum cholesterol levels; to repair tissues and even used to fabricate contact lenses (Goodman, 1989). Chitosan is used widely for various purposes such as pesticides (Cohen, 1993; Watkins et al., 2002), and can also be used for decreasing skin irritation caused by shaving (Lazarowitz and Morris, 2004). Chitosan and chitosan derivatives were found to posses high insecticidal and fungicidal activity tested against cotton leaf worm *Spodoptera littoralis*, the grey mould *Botrytis cinerea* and rice leaf blast *Pyricularia grisea* Cavara (Rabea *et al*., 2005; Badaway *et al*., 2005). Ethanolic extracts of propolis of *Apis mellifera* collected from Brazil have shown anticancer and anti-HIV properties (Park 2000). Kunin and Lawton, (1996) have developed promising anticancer drugs, isoxanthopterin and dichostatin which were derived from the wings of Asian sulphur butterflies (*Catopsilia crocale*, Pieridae) and from the legs of Taiwanese stag beetles (*Allomyrina dichotomus*, Scarabaeidae) respectively.

Because our group has expertise in antibacterial natural products, I decided to evaluate thongolifha and *D. viscosa* for antibacterial activity against the four most important nosocomial pathogens (*S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa*).

4.2 Results and Discussion

4.2.1 Thin Layer Chromatography analysis

Serial extraction using hexane, DCM, acetone and methanol was performed as outlined in Chapter 2 section 2.3.1. Various extracts were loaded on TLC plates (10 µl of 10 mg/ml) and separated using EMW, CEF and BEA eluent systems. After spraying with vanillin-sulphuric acid, various separated components were observed. Various

components of different Rf values both from the plant (*D. viscosa*) and the insect *(E. delegorguei)* were separated (Fig. 4.1). When duplicate chromatograms were sprayed with ninhydrin- ethanol in 0.2% acetic acid only at the origin was any positive bands for amino acid detected (results not shown). The results demonstrated no similarity in components extracted from the insect and the plant. Developed TLC plates by EMW system revealed major plant components of Rf 0.49, 0.59 and 0.68 whereas the major components of the insect had Rf 0.46 and 0.61. CEF eluent system was able to separate major components of the plant at Rf 0.52, 0.59, and 0.69 and for insect separated components were at Rf 0.79 and 0.66. For non-polar eluent system, BEA, major components of the plant had Rf of 0.3, 0.46 and 0.46 whereas the insect had Rf 0.57 and 0.44. These Rf values obtained from both the plant and insect indicate that the major components separated by the three eluent system are completely different from each other. Although a completely different matrix was present in the insect extracts, it was satisfying that the methods developed for plants extracts gave reasonable results with the insect extracts. If the insect had sequestered any components from the plant, it may be present in lower quantities that were not possible to detect by our TLC method. Alternatively the insect could have metabolized the structure of the compounds resulting into new compounds.

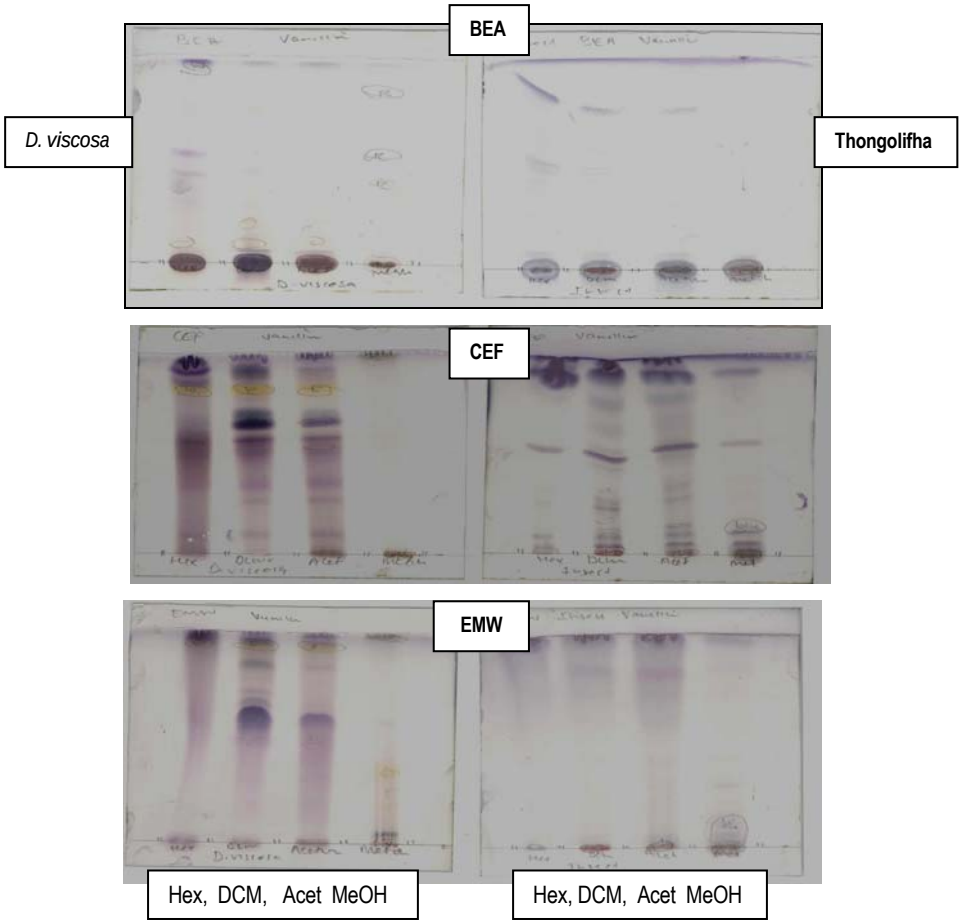


Figure 4.1: TLC chromatogram of *D. viscosa* (L) and thongolifha (R) serially extracted with hexane, DCM, acetone and MeOH followed by spraying with vanillin in sulphuric acid. The TLC plates were developed in BEA, CEF and EMW.

4.2.2 Bioautography assay

The component at Rf of 0.82 exhibited a bactericidal effect against *E. coli* (Fig. 4.2). The EMW, CEF and BEA solvent systems separated insect extracts components that are susceptible to S*. aureus* at Rf 0.79 and 0.67 (EMW); Rf 0.79 and 0.66 (CEF) and Rf 0.72 and 0.37 (BEA). It is possible that the two major components exhibiting antibacterial activity against *S. aureus* are the same in all the three eluent system. Furthermore, the most active components against *E. faecalis* were only separated by CEF solvent system at Rf 0.66, and the same component was also active against *S. aureus*. The two Gram negative bacteria, *E. coli* and *P. aeruginosa* were resistant to the insect extract.

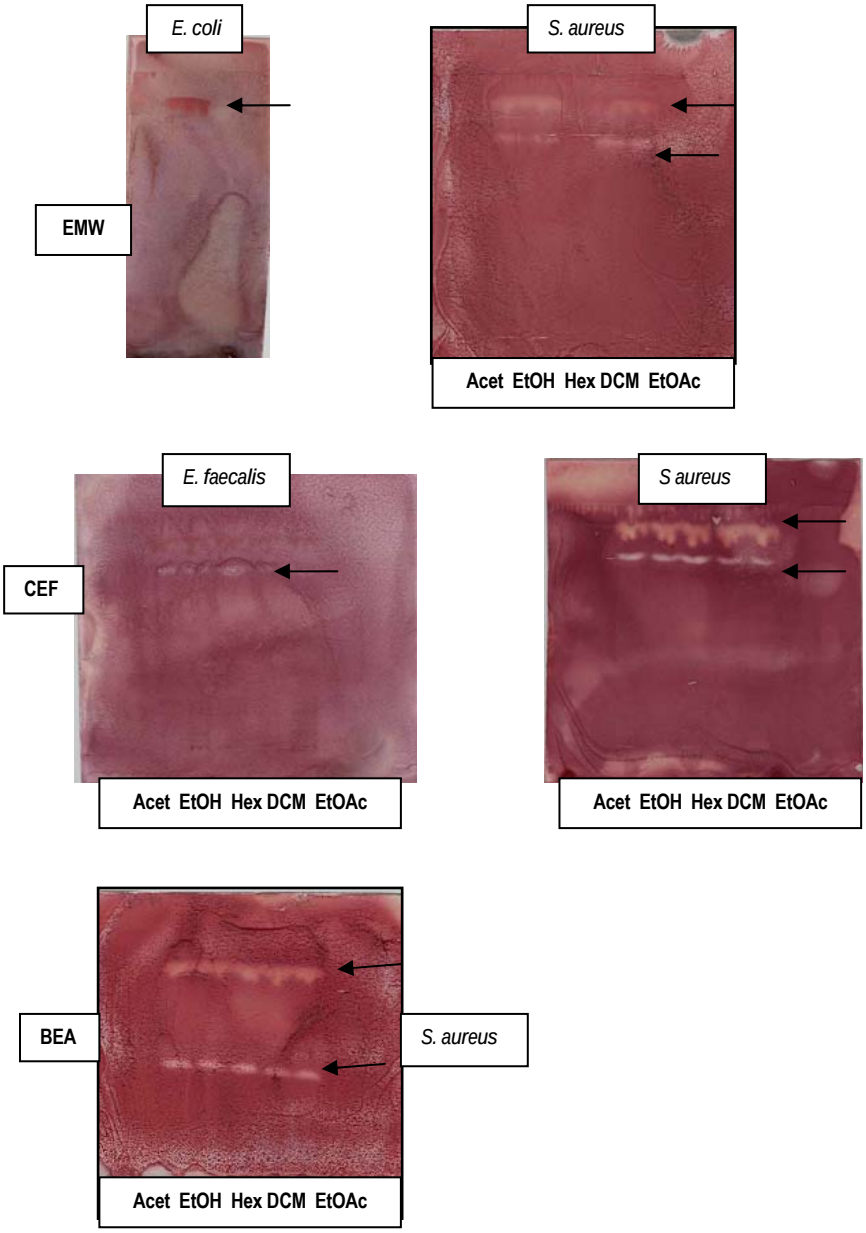


Figure 4.2: Bioautograms of *E. delegorguei* extracts against *E. coli, S. aureus* and *E faecalis*. The clear bands on the chromatograms show an area where the compound is active against the test microorganism.

4.2.3 Microdilution assay

All the extracts have showed the MIC value of >2.5 mg/ml against all four test microorganisms, *S. aureus, E. faecalis*, *E. coli* and. *P. aeruginosa,* indicating that the insects extracts have no antibacterial activity.

4.3 Conclusion

Based on the bioautography results there were at least three compounds active against one or more of the nosocomial pathogens in the thongolifha extracts (Fig.4.2). The MIC values indicated that the antibacterial activity was very low. This may be an artefact of the serial dilution microplate method used, because this method has not yet been tested using insects or animal extracts. Based on the relatively high Rf values found with the different solvent systems used, it is likely that the antibacterial compounds are not peptides, which would probably have been retained much stronger on silica gel due to its polar characteristics. This conclusion was confirmed when only compounds at the origin reacted positively with the amino acid/peptide ninhydrin-ethanol spray reagent.

It appears that the antibacterial compounds separated by CEF (Fig. 4.2) reacted positively with vanillin-sulphuric acid spray reagent (Fig. 4.1) again indicating that the antibacterial compound is possibly a flavonoid or a terpenoid (Fig. 4.1) and not a peptide.

CHAPTER 5

5. MEDICINAL VALUE OF HOST PLANT

5.1 Introduction

Based on the results obtained in Chapter 4, it now makes sense to investigate the host plant rather than thongolifha. The main reasons been that, the plant material were easy to obtain, the methods to analyse those plant material have been already developed and the facilities to perform the analysis in our laboratory are in place. At the end we will be able to determine if any antibacterial compounds isolated from the plant could be present in the insect.

5.2 Results and Discussion

5.2.1 Thin Layer Chromatography analysis

The leaves of *D. viscosa* extracted separately with various solvents (acetone, EtOH, hexane, DCM, and EtOAc). To examine the chemical composition 100 μg of each extract were spotted on TLC plates and developed in different solvent systems. The yields achieved in mg/ml were the following: hexane = 17, dichloromethane = 66, ethyl acetate = 114, acetone = 150 and ethanol =187 (Table 5.1). This indicates that *D. viscosa* contains many polar compounds as there was an increase in the yield with the more polar extractants. The TLC chromatograms revealed that the extracts contain various constituents (Fig 5.1). The CEF solvent system separated more components followed by EMW whereas BEA separated least constituents. This implies that the extracts of *D. viscosa* have more components that are moderately polar (CEF) and polar (EMW) and fewer compounds that are non polar (BEA).

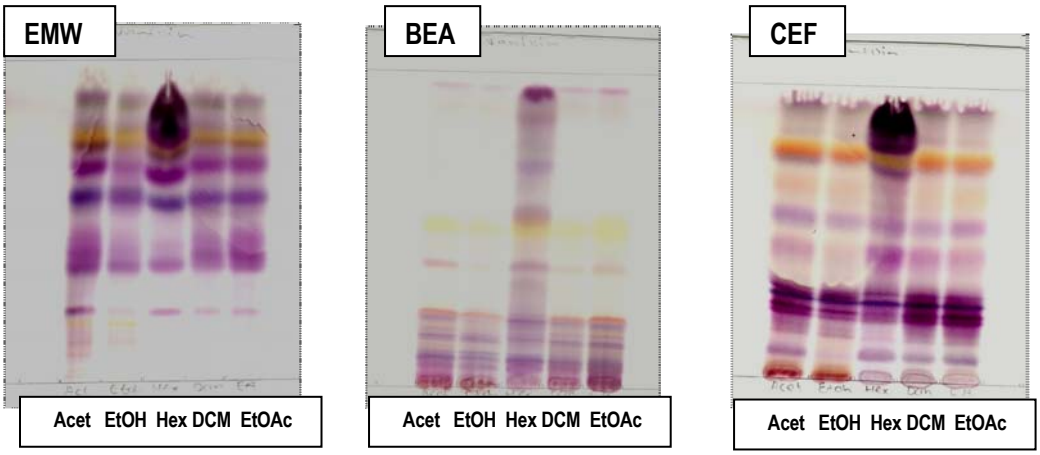


Figure 5.1: TLC chromatograms of *D. viscosa* crude leaf extracts developed in EMW, BEA, and CEF solvent systems. The solvents used for extraction are acetone, EtOH, hexane, DCM and EtOAc.

5.2.2 Bioautography assay

The results in Fig. 5.2 show that, EMW eluent system separated components that are active against *S. aureus*, *E. faecalis* and *E. coli*. On the other hand, *P. aeruginosa* was not growing well on TLC plates however, one component at Rf of 0.79 showed activity (data not shown). A different number of antibacterial compounds were separated by EMW, with acetone (5) >>>>ethyl acetate (4)>>>dichloromethane (3) >>ethanol (4)>hexane (1). Hexane extracts contained few active compounds, thus less activity was observed. Four components were active against *S. aureus* at R_f values of 0.79, 0.75, 0.37 and 0.25 respectively. Two components showed activity against *E. faecalis* at R_f values of 0.78 for compound 1 and 0.74 for compound 2. There was only one compound that was active against *E.coli* at R_f value of 0.85. The TLC chromatogram in Fig. 5.3 shows that the BEA eluent system was also able to separate 4 components that are active against *E.coli* at R_f values of 0.08, 0.15, 0.22 and 0.15 respectively.

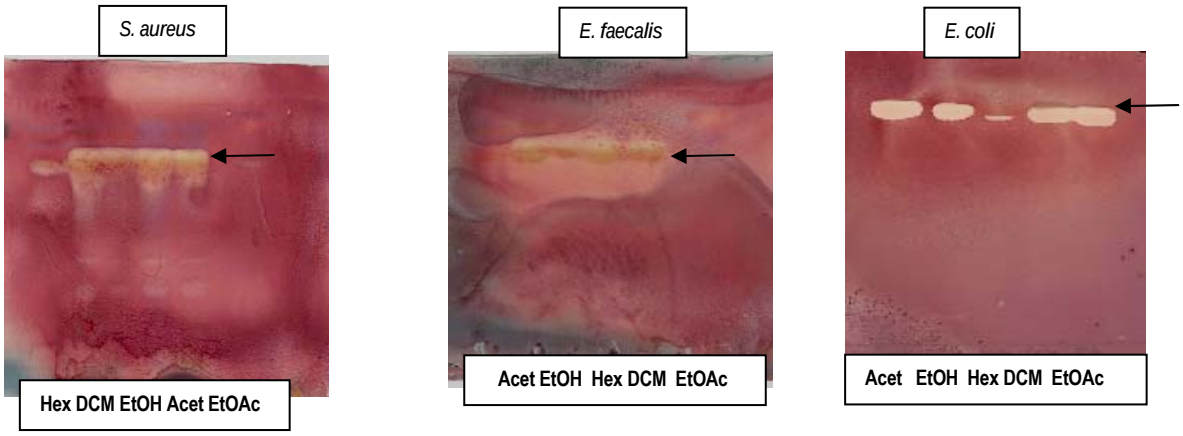


Figure 5. 2: The plant extracts were separately extracted in acetone, EtOH, Hex, DCM and EtOAc. The TLC chromatograms of *D. viscosa* extracts were all developed in EMW solvent system. The extracts (TLC plates from left to right) were tested against *S. aureus*, *E. faecalis* and *E. coli* and clear zones on the chromatograms indicated inhibition of growth.

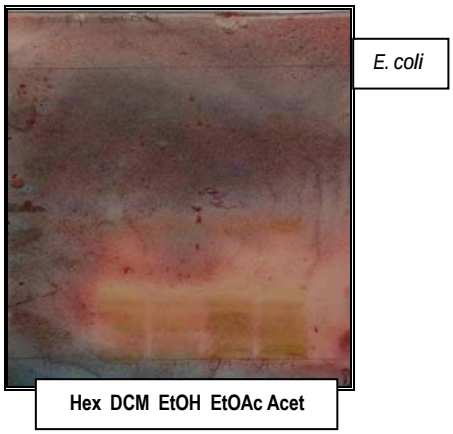


Figure 5.3: The extracts of *D. viscosa* were separately extracted with Hex, DCM, EtOH, EtOAc, and acetone. Bioautography chromatograms of *D. viscosa* extracts were developed in BEA eluent system and components tested for activity against *E. coli.* Clear zone indicate inhibition of growth.

5.2.3 Microdilution assay

The MIC results in Table 5.1 reveal the MIC value (i.e. the lowest concentration of the extract that inhibited growth of bacteria) of hexane extract tested on *E. coli* to be at 0.31 mg/ml. This value of 0.31 mg/ml is the highest inhibitory concentration compared to other values obtained from other four solvents (DCM, EtOH, EtOAc and acetone) that exhibited a lower MIC value of 0.08 mg/ml. This indicates that hexane extracted fewer components that are active against *E. coli*. The most sensitive microorganism tested was *E. faecalis* with the lowest MIC of them all, which is 0.02 mg/ml with acetone extract. Other solvents, that is, DCM, EtOH and EtOAc had similar MIC values of 0.04 mg/ml. Although hexane extract had an MIC value of 0.08mg/ml, the data still indicates that *E. faecalis* is the most sensitive strain among the organisms tested. On average, all solvent extracts of the leaves of *D. viscosa* inhibited growth of both *S. aureus* and *P. aeruginosa* at concentration of 0.28 mg/ml, with *E. coli* at 0.13 mg/ml and the lowest being 0.04 mg/ml against *E. faecalis*.

The results calculated for total activity (TA) are represented in Table 5.2. Eloff, (2000) describes total activity as the volume to which the bioactive compounds that are present in 1 g of the extract can be diluted and still inhibit growth of bacteria. The data for total activity indicates that acetone extract exhibited the highest total activity for all organisms tested ranging from 48.4-750 ml/g (Table 5.2). Hexane extracts showed the lowest total activity for all the bacteria tested ranging from 5.5 -21.3 ml/g (Table 5.2). The highest TA of 750 ml/g was obtained against *E. faecalis* which makes it to be the most sensitive microorganism of them all. This means that 1 g of *D. viscosa* leaf extract can be dissolved in the highest volume (750 ml) of acetone and still inhibit the growth of *E. faecalis*. These results agree with MIC results, whereby hexane has demonstrated higher MIC values of 0.31 mg/ml (Table 5.1) for *E. coli* and *S. aureus* (with TA of 5.5 ml/g, Table 5.2) and the lowest MIC value of 0.08 mg/ml (Table 5.1) for *E. faecalis* (with TA of 21.3 ml/g, Table 5.2).

From the literature, Getie *et al*., (2003) used only methanol extracts to determine the activity of *S. aureus* and *E. coli* by using the agar diffusion technique. Antibacterial activity was observed only with *S. aureus* (8 mm) whereas *E. coli* was resistant. Rojas et al., (1992) also detected antifungal and antibacterial activity of *D. viscosa* extracts from Mexican species with MIC less than or equal to 100 µg/ml. These results differs completely with our quantitative data obtained from MIC and TA which explains a lot about the strength of activity of *D. viscosa* leaf extracts. We have obtained antibacterial activity from all different solvent extracts but low activity was observed from hexane extracts. Furthermore, we have also observed strong activity against *E. faecalis* and *E. coli*, whereas the above authors did not obtain any activity against *E. coli*. The reason for them not obtaining activity from *E. coli* could be that methanol extracted more polar components that did not exhibit activity against *E. coli* as it is a Gram negative microorganism and is prone to resistant. Our data have demonstrated *E. faecalis* to be the most sensitive bacteria followed by *E. coli* then *S. aureus*. Although we were not successful to determine activity with *P. aeruginosa* by using bioautography, the MIC and TA gave us better results. The level of activity of *P. aeruginosa* was at the same level with that of *S. aureus*.

Table 5. 1: MIC values of *D. viscosa* leaf extracts

Solvents	**Amount Extracted (mg/g)**	% Yield	**Minimum Inhibitory Concentration (mg/ml)**				
			E. coli	*S. aureus*	*E. faecalis*	*P. aeruginosa*	**Average**
Hexane	17	1.7	0.31	0.31	0.08	0.16	0.21
DCM	66.2	6.62	0.08	0.16	0.04	0.31	0.14
EtOAc	114.0	11.4	0.08	0.31	0.04	0.31	0.18
Acetone	150	15	0.08	0.31	0.02	0.31	0.18
EtOH	186.5	18.65	0.08	0.31	0.04	0.31	0.18
Average	**106.7**	**10.67**	**0.13**	**0.28**	**0.04**	**0.28**	
Gentamicin			0.8 µg/mL	0.2 µg/mL	6.3 µg/mL	0.8 µg/mL	**2.0 µg/mL**

Table 5. 2: Total activity in ml/g of leaf extracts of *D. viscosa*

Solvent	**TA (ml/g)** *E. coli*	**TA (ml/g)** *S. aureus*	**TA (ml/g)** *E. faecalis*	**TA (ml/g)** *P. aeruginosa*	**Average**
Hexane	5.5	5.5	21.3	10.6	10.7
DCM	82.8	41.4	165.5	21.4	78.1
Ethyl Acetate	142.5	36.8	285	36.8	125.2
Acetone	187.5	48.4	750	48.4	258.5
Ethanol	233	60.2	466	60.2	204.8
Average	**130.3**	**38.4**	**338**	**35.4**	**135.5**

5.2.4 Antioxidant assays

Qualitative assay

The three solvent systems EMW, CEF and BEA separated the antioxidant constituents contained in the extracts (Fig. 5.4). DPPH, a stable purple free radical can be reduced to yellow diphenylpicryl hydrazine. Separated constituents of the *D. viscosa* extracts, turned yellow after spraying with 0.2% DPPH in methanol. The yellow bands on the purple TLC chromatogram background indicated that extracts from *D. viscosa* contains components that have antioxidant activity. All extracts were best separated by BEA solvent system. Most of the components separated by BEA eluent system reacted rapidly and bleached the purple colour background when sprayed with 0.2% DPPH in methanol (Fig. 5.4). The major antioxidant compounds in the extracts appeared at R_f of 0.14 and at the baseline in BEA and 0.81 in EMW eluents. DCM extracted another major component at R_f 0.48.

The results demonstrated that antioxidant compounds of various polarities can be isolated from the leaf extracts especially from ethyl acetate and acetone crude extracts. Analyses of the nature of the active compounds as revealed by vanillin-sulphuric acid spraying reagent demonstrated the presence of phenolic compounds (Stahl, 1969). Several flavonoids such as santin, aharin, penduletin, pinocembrin, 5-hydroxy-3, 6, 7, 4'-tetramethoxyflavone were reported from the genus *Dodonaea* (Sachdev and Kulshreshtha, 1983; Payne and Jefferies, 1973; Dawson *et al.,* 1966). This is in line with the previous study that linked antioxidant activity in higher plants to the presence of polyphenolic compounds (Wangensteen *et al.*, 2004).

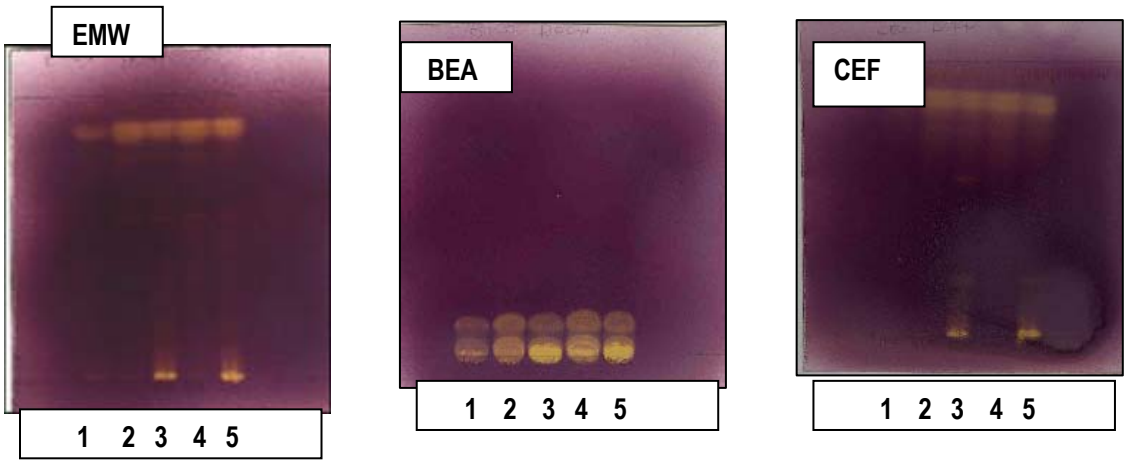


Figure 5.4: The crude extract of *D. viscosa* was separately extracted with Hex, DCM, EtOH, EtOAc and acetone (Lanes 1-5). The extracts were developed in EMW, BEA and CEF solvent systems and the yellow spot indicates components with antioxidant activities.

Quantitative assay

In the quantitative assay, there were big differences in the antioxidant activity of the selected crude extracts. The extracts had moderate activity compared to L-ascorbic acid used as standard which is a pure compound. As expected the more polar solvent extracts (EtOAc and acetone) were the most active (Table 5.3). The EC_{50} indicates extracts that are suitable as sources of potent antioxidant compounds. This is useful for selection of extracts for bioassay-guided fractionation to isolate the antioxidant compounds.

Table 5.3: EC_{50} of antioxidant activity of *D. viscosa* leaf extracts based on DPPH Spectrophotometric assay

Sample	**EC_{50} ± SEM (µg/ml)**
Hexane	NA
Dichloromethane	469 ± 8.8
Ethyl acetate	240 ± 3.4
Acetone	213 ± 4.5
L-ascorbic acid	1.96 ± 0.01

NA- No activity, hexane extract did not inhibit 50% of DPPH free radical even at the highest concentration (500 µg/ml) of the extract tested.

5.3 Conclusion

The leaf extracts of *D. viscosa* possess medicinal properties; the data are comparable with that of Getie *et al*., (2003) where they have reported antibacterial activity against various microorganisms. In our study, *D. viscosa* extracts showed antibacterial activity against E*. faecalis,* E*. coli*, *S. aureus* and *P. aeruginosa*. The antibacterial activity is supported by data of the microdilution assay and the bioautography. The minimum inhibitory concentration value as low as 0.02 mg/ml was obtained. Our findings suggest that the agar diffusion method can not be used alone to make conclusive results about the level of activity of plant extracts. Other method such as the microdilution assay and TA must be employed in order to have conclusive quantitative data. The extracts also had some antioxidative activities, as demonstrated by free radicals scavenging effect on DPPH. The fact that the plant also possesses both antioxidant and antibacterial activities make it a very useful plant, because the same compounds that had antibacterial activity have tested positive for antioxidant activity. This implies that the active constituents possess both antibacterial and antioxidant activity which may lead to an increase of activity by direct or indirect effects. Our results suggest that *D. viscosa* extracts could be useful in therapeutic treatment, but this has to be substantiated by *in vivo* experiments. Both antibacterial and antioxidant components were isolated using bioassay guided fractionation, details in chapter 6.

C H A P T E R 6

6. ISOLATION OF BIOACTIVE COMPOUNDS FROM *D. VISCOSA*

6.1 Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful tools available for determining the structure of both organic and inorganic species. NMR in conjunction with other analytical instruments such as ultraviolet (UV) and mass spectrometer (MS) can provide useful information for elucidating the structure of the isolated pure compounds. MS provides information in terms of molecular mass of the structural components of isolated compound; as such an accurate measure of the molecular weight of the compound can be obtained (Skoog and West, 1980). In this study, the crude leaf extract of *D. viscosa* was fractionated and purified by column chromatography on variety of supports and the structure of isolated compounds elucidated using NMR and MS.

6.2 Methodology

6.2.1 Chromatographic purification of the extracts.

Initially, 5 kg of dried material of *D. viscosa* was serially extracted with 3 x 5L of the following solvents: hexane, DCM, acetone and methanol respectively. The extracts were filtered with Whatman no. 1 filter paper and dried using rotary evaporator at a reduced pressure and a temperature below 40 °C. The residues were allowed to dry under a stream of air. The TLC analysis and bioautography were determined in order to select the extracts with a number of components of good bacterial and antioxidant activities.

Briefly, 30.1 g of DCM extract was loaded onto vacuum liquid chromatography (VLC) (95 X 480 mm) packed with silica gel 60 particle size 0.063-0.2 mm (70-230 mesh) (Fluka Chemika) . The column was eluted with 750 ml at a gradient system beginning with pure hexane (100%) through hexane-EtOAc (90:10, 80:20, 70:30, 50:50, 30:70, 20:80, 10:90, 0:100) and finally with EtOAc: MeOH (95:5, 90:10, 80:20, 70:30, 0:100). About 150 ml were collected in test tubes and eluents from test tubes that exhibited similar Rf values as indicated by TLC analysis upon spraying with vanillin-sulphuric acid and visualized under UV, were pooled together and a total of 15 fractions were obtained and dried. TLC analysis and bioautography were performed and fractions with good antibacterial activity were subjected to subsequent column chromatography (11 cm height x 10 cm diameter) over silica gel 60 (17cm x 4.9 cm) with 320 ml eluent (EtOAc: MeOH as outlined above). Fractions that were found to have similar R_f values as revealed by TLC chromatogram pattern were pooled together. The following fractions (fractions 3, 4+5, 6+ 7+8) were selected for further purification by additional chromatographic procedures. Some of these fractions contained only one compound by TLC. After removing the solvent structures were elucidated by NMR and MS. After the structure of the pure compounds was obtained the samples were recovered and used for biological assays. The

above procedure was applied to further purify 3.240 g of acetone crude extract. Fractions from tubes 24-26 were combined and further purified using Sephadex LH 20 pre-swollen with DCM:MeOH ratio (2:1) and the column was eluted with 50 ml of the DCM:MeOH mixtures and 10 ml of eluent was collected and air dried.

6.3 Results and Discussion

6.3.1 Crude extract obtained by VLC

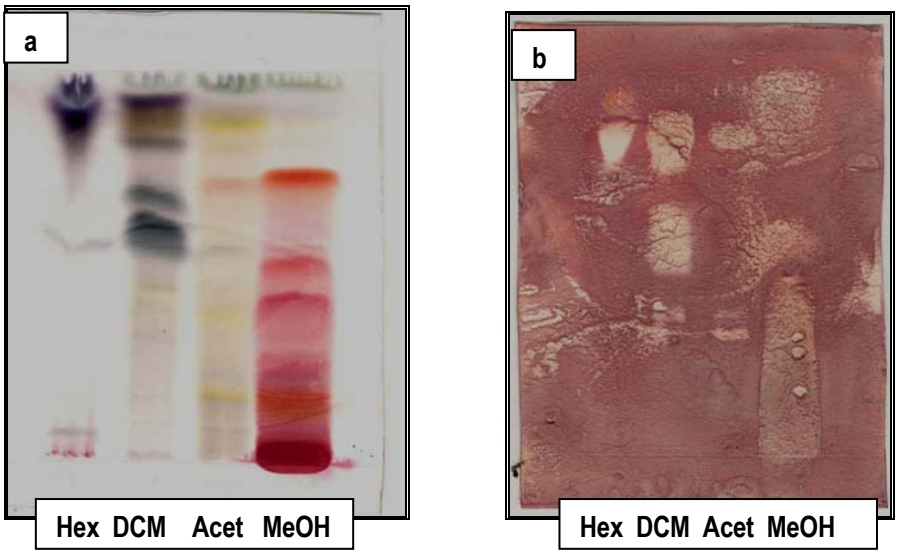


Figure 6. 1: TLC chromatograms separated by CEF eluent system: (a) shows the TLC profile of compounds separated by CEF solvent system followed by spraying with vanillin in sulphuric acid. (b) indicates bioautography of extracts against *S. aureus*.

6.3.2 Chromatographic purification

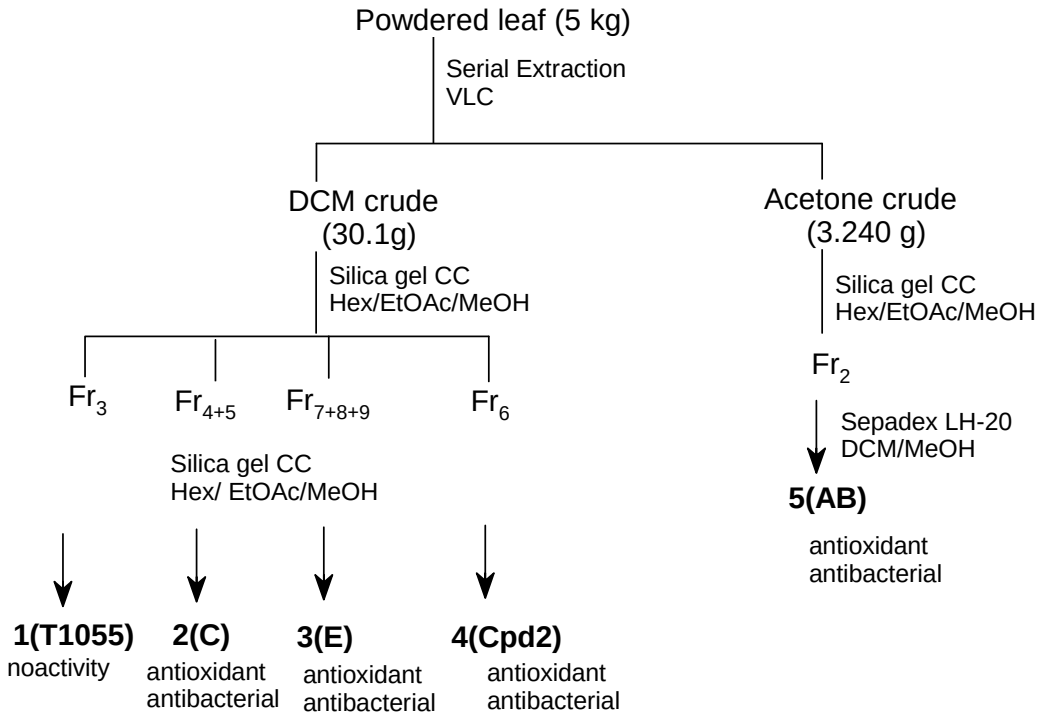


Figure 6.2: Schematic representation of isolation and purification of compounds from *D. viscosa* crude leaf extract.

6.3.1.1 Purification of DCM crude extracts

From serial extraction of *D. viscosa* crude material, four extracts were obtained, hexane, DCM, acetone and methanol (Fig 6.1**)**. Only DCM and acetone extracts were selected for further purifications because they showed more compounds with good activity against *S. aureus.* Fifteen fractions were obtained from the DCM extracts (Fig. 6.3) and to obtain pure compounds, fractions of similar TLC profile and activity were pooled together, evaporated and rechromatographed. Four column chromatography experiments were run separately and fractions (3; 4+5; 6; 7+8+9) were subjected to separate column chromatography packed with silica gel 60 and eluted with Hexane:EtOAc with the following increasing polarity (100:0; 90:10; 80:20; 70:30; 50:50; 30:70; 20:80; 10:90; 0:100). The effluents collected in test tubes (50 mL) were allowed to dry and upon drying some compounds crystallized forming pure compound whereas some were further purified using Sephadex LH20. The Sephadex LH20 column was eluted with DCM: MeOH at ratio of 2:1.

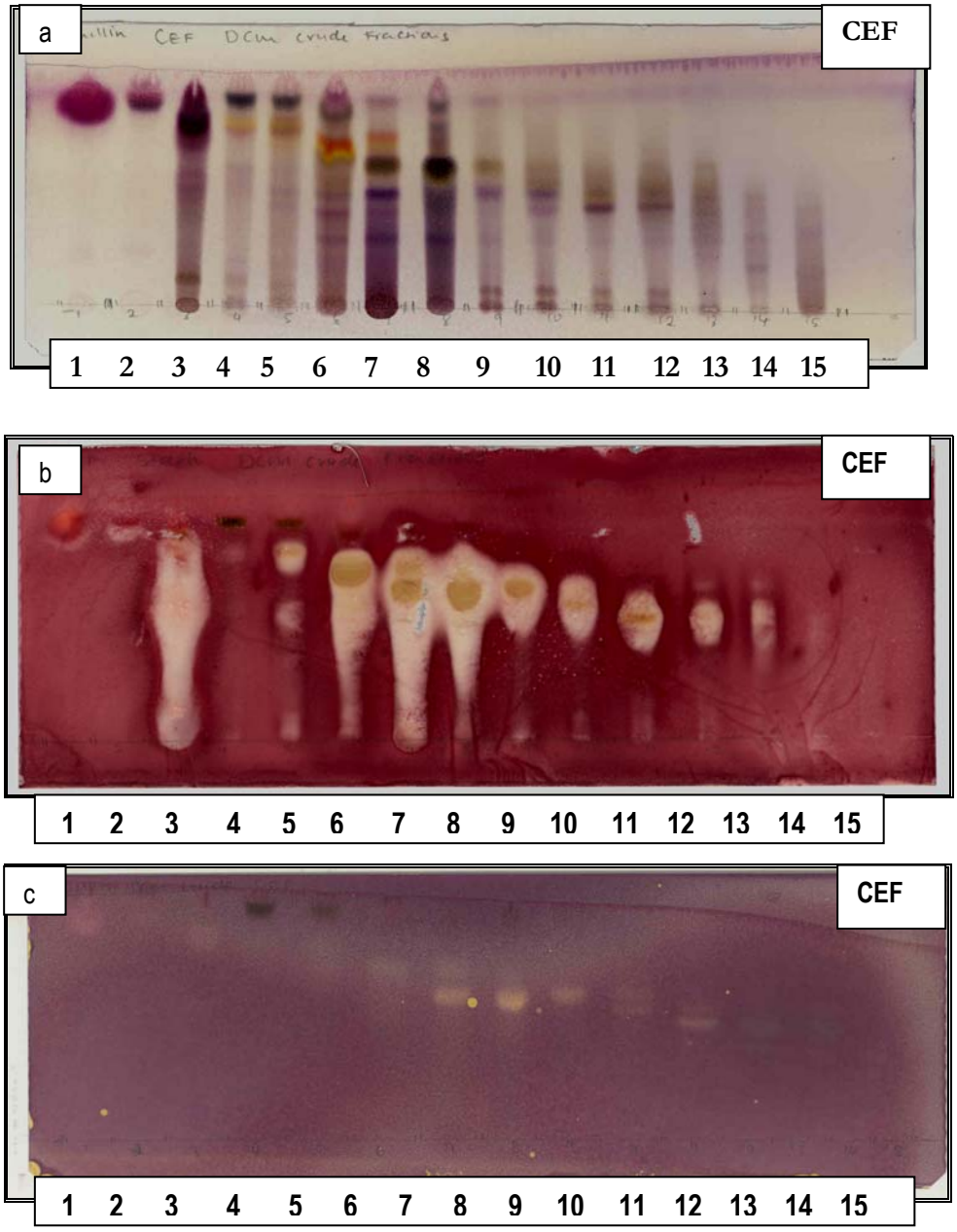


Figure 6.3: TLC chromatograms (a) TLC pattern of fractions obtained from DCM VLC and the extracts were separated by CEF solvent system followed by spraying with vanillin-sulphuric acid. (b) bioautography of VLC fractions against *S. aureus*, (c) TLC chromatogram of antioxidant activity after spraying with 0.2% DPPH in methanol.

Purification of fraction 3

Fraction 3 (0.89 g) was further purified by using column chromatography silica gel 60 and various fractions were collected into 50 ml test tubes and allowed to dry. The fractions that had similar TLC pattern were pooled together

resulting in 5 fractions (Fig. 6.4). Sample in test tube 3 formed yellow crystals (9 mg) upon drying and the pure compound was coded T1055 (Compound 1), which had R_f of 0.84 when separated with CEF eluent system.

Figure 6.4: TLC profiles of pooled fractions from column chromatography of fraction 3 eluted with CEF. Sample from test tube 3 formed a pure Compound 1.

Purification of combined fractions 4+5

Column chromatography on silica gel of combined fractions 4+5 (2.106 g) gave fractions of various TLC profiles **(**Fig. 6.5). Fractions from test tubes with similar TLC patterns were pooled together and allowed to dry under a stream of air. A pure compound in test tubes 48-60 formed yellow crystals (8 mg) upon drying and was coded C (Compound 2) with R_f of 0.79 when developed in CEF solvent system.

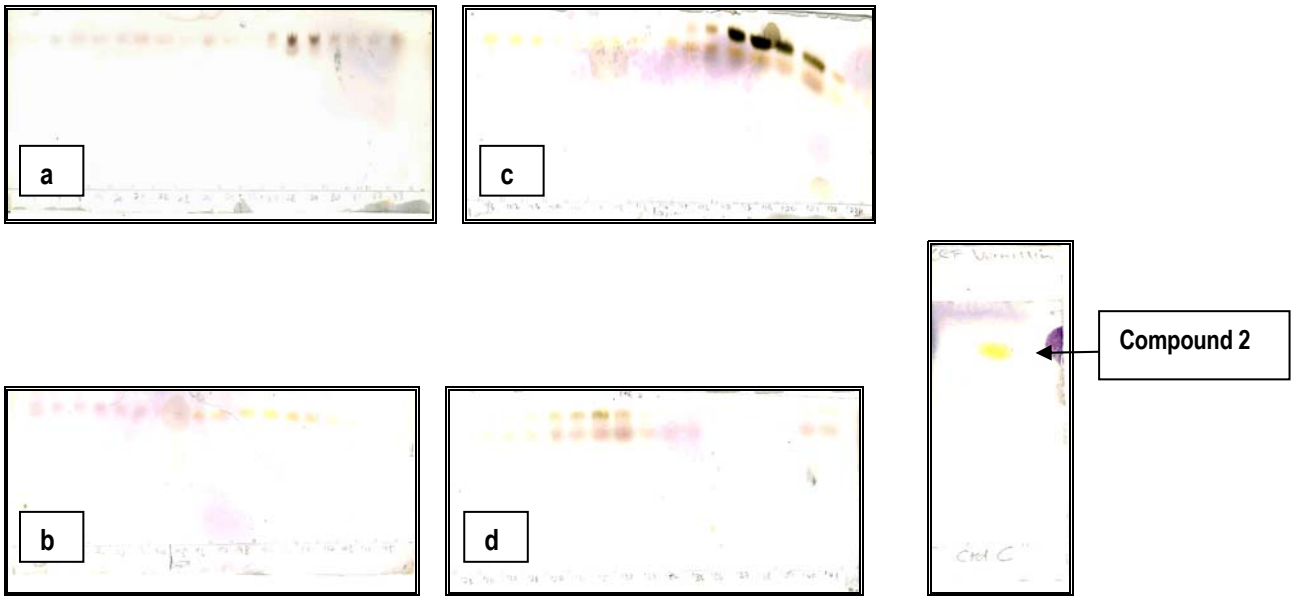


Figure 6.5: TLC chromatogram obtained from fractions 4+5 column chromatography separated by CEF. Fractions were collected from (a) test tube 1-30, (b) test tube 31-50, (c) test tubes 51-66, (d) test tube 67-78.

Purification of combined fractions 7+8+9

Combined fractions 7+8+9 (1.033 g) was subjected to column chromatography silica gel 60 (Fig. 6.6) and eluted with Hex: EtOAc to produce various fractions and the ones with similar TLC profiles were pooled together producing pure compounds upon drying. Eluents in test tubes 72-78 upon drying, formed pure compound (yellow powder) (25 mg) and was coded E (Compound 3) with R_f of 0.61 when developed in CEF solvent system.

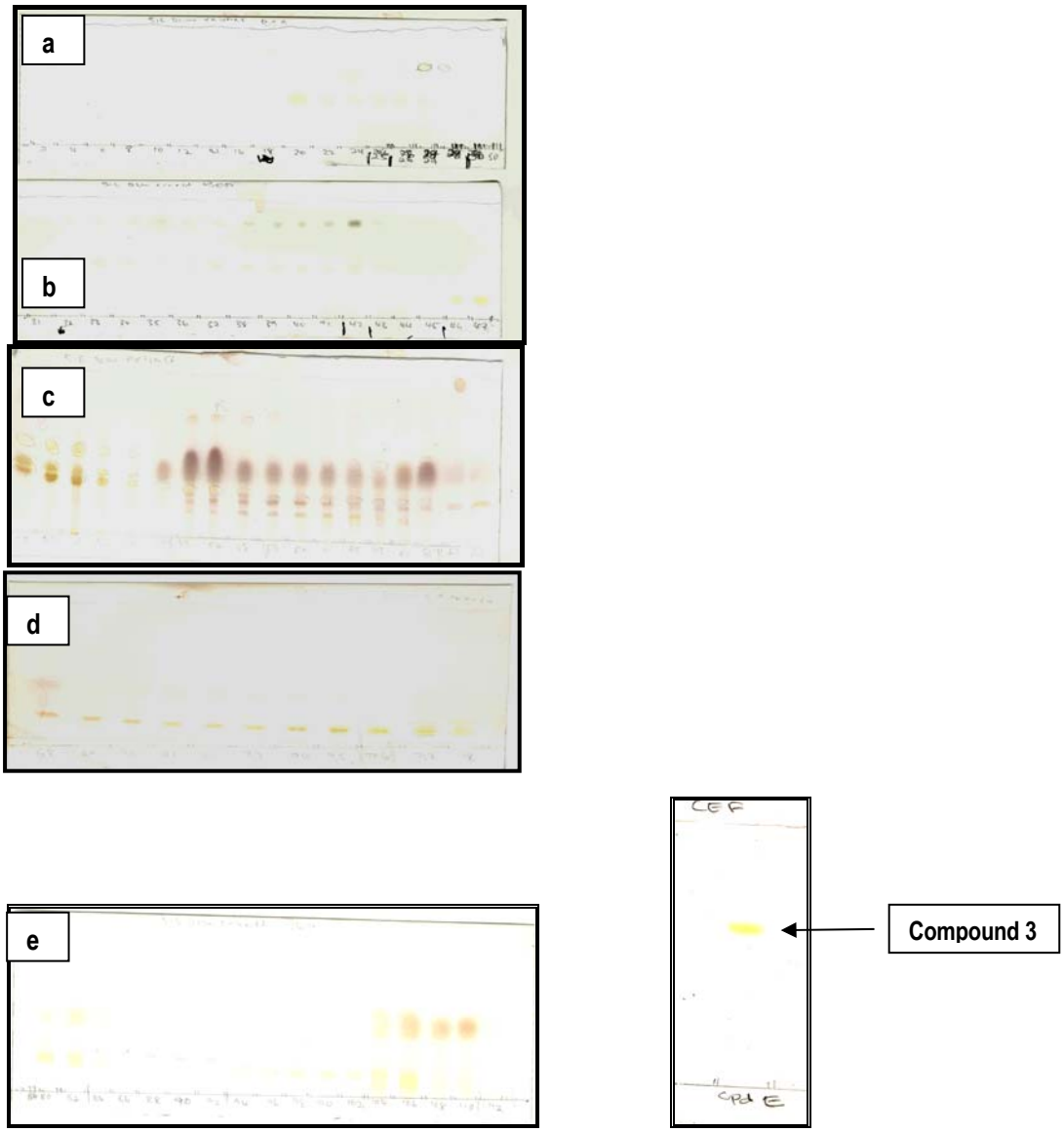


Figure 6.6: TLC chromatogram of fractions obtained from combined fractions 7+8+9 column chromatography separated by BEA eluent system. Fractions were collected from (a) test tube 1-28, (b) test tube 30-48, (c) test tubes 50-66, (d) test tubes 68-78, (e) test tubes 80-112.

Purification of fraction 6

Fraction 6 (1.8 g) was subjected to a small column and several fractions were obtained. Constituents of test tubes 23-25 upon drying crystallized; the compound was cleaned with Sephadex LH20 eluted with DCM: MeOH (2:1). The pure compound, a yellow powder (7mg) was coded Cpd2 (Compound 4) with R_f of 0.61 when developed in CE eluent system (Fig 6.7).

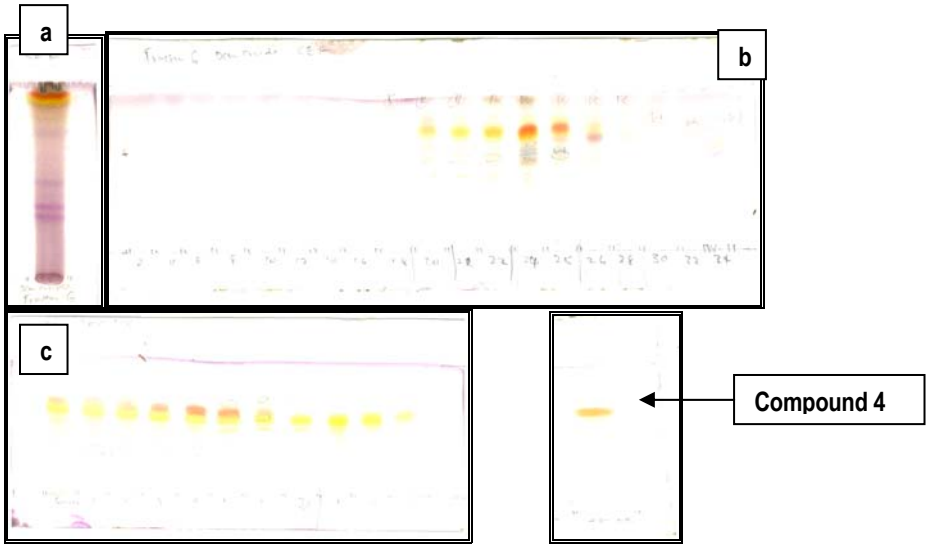


Figure 6.7: TLC chromatograms of fractions collected from silica gel column chromatography separated by CEF eluent system. (a) DCM crude extract; (b) fractions collected from test tubes 1-34 (c) further purification of fractions from tubes 23-25 using Sephadex LH20; (d) pure compound Cpd2 (compound 4).

6.3.1.2 Purification of acetone crude extract.

Acetone crude extract (3.240 g) was fractionated with column chromatography on silica gel 60 and tubes 24-26 were combined as they had the same TLC profile. The extract from tubes 24-26 was further subjected to a Sephadex LH20 chromatography eluted with 50 ml of DCM: EtOAc ratio (2:1). A pure compound (yellow powder) was obtained and coded AB (Compound 5) (Fig. 6.8) at R_f of 0.41 when developed in CE eluent system.

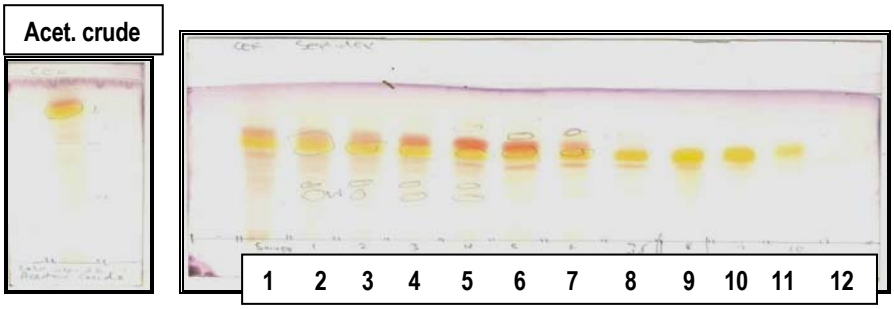


Figure 6.8: TLC profiles of acetone crude eluted on Sephadex LH20 column chromatography, fractions from test tubes 1-12 was obtained and pure Compound AB (compound 5) was obtained from test tube 8-11 by crystallization.

6.3.2 Structure Elucidation of the Isolated Compounds

6.3.2.1 Compound 1

The mass spectrum showed molecular ion peak at m/z = 329, [MH^+, 100%] base peak, corresponding to the molecular formula $C_{18}H_{16}O_6$ and also an intense peak at 285 (M-43) (Fig 6.10) corresponding to the standard flavonol C-ring contraction (Harborne,1994) for 3-methyl ether flavone. The ^1H NMR ($CDCl_3$) spectrum showed three methoxy signals at δ 3.84, 3.86 and 3.88 (3H each, s, OMe-3, 7 and 4'. There was presence of AA'BB' system due to ring B at δ 7.03 (2H, d, J= 9.0 Hz, H-3', H-5') and δ 8.08 (2H, d, J=8.7, H-2', H-6'). The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.66. ^{13}C NMR data are presented in Table 6.1. The spectral data are in close agreement with those reported in the literature (Table 6.1), the compound is therefore 5-hydroxyl- 3, 7, 4'-trimethoxyflavone (Fig. 6.9). This compound had previously been reported from many plant species e.g. *Siparuna apiosyce* (family Monimiaceae) (Leitao *et al*., 2000) and Aniba species (Rossi *et al*., 1997). There was a discrepancy in C-2 value and this was resolved by comparing our ^{13}C NMR data with several 3- OMe flavones (Horie e*t al*., 1998) and kaempferol (Markham, 1982). C-2 of 3- OMe flavone shifted down field to ≈ 155.

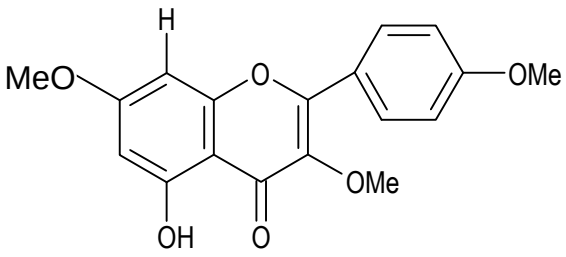
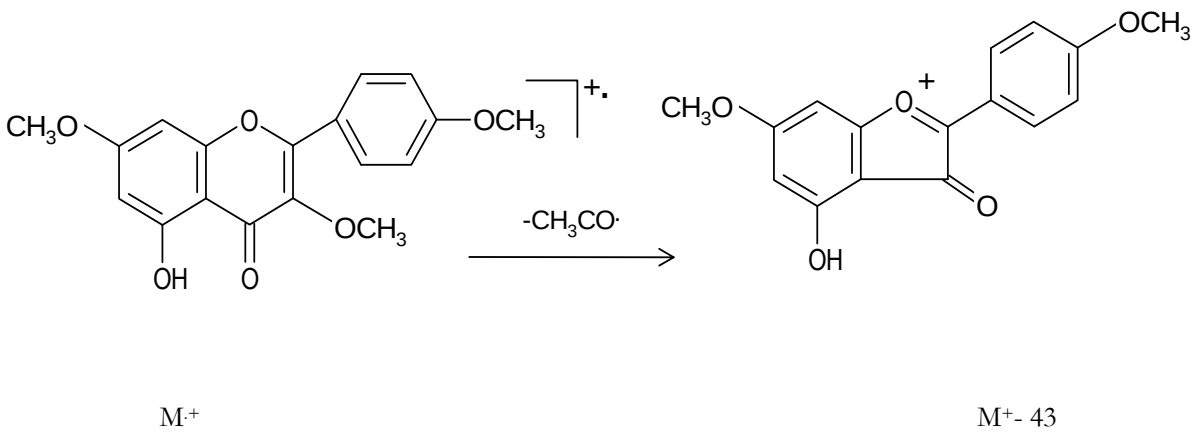


Figure 6.9: Structure of 5-hydroxy -3, 7, 4'-trimethoxyflavone.



M·+ M+- 43

Figure 6.10: Mass Spectrum Fragmentation of 5-hydroxyl-3, 7, 4'-trimethoxyflavone

Table 6.1: ^{13}C NMR data of Compound 1

^{13}C Assignment	Compound 1 ($CDCl_3$)	5-hydroxy-3,7,4' trimethoxyflavone ($CDCl_3$) (Rossi *et al.*,1997)
2	156.0	148.0*
3	138.8	138.9
4	178.8	178.9
5	162.0	161.7
6	97.8	97.8
7	165.4	165.4
8	92.1	92.2
9	156.7	156.8
10	106.0	106.0
1'	122.8	122.8
2', 6'	130.1	130.0
3', 5'	114.0	114.0
4'	161.6	162.0
OMe -4'	55.8*	55.8
OMe-7	55.4*	55.4
OMe-3	60.1	60.1

*Interchangeable

6.3.2.2 Compound 2

The mass spectrum showed molecular ion peak at m/z = 344, [$M^{+\cdot}$ 100%] base peak, corresponding to the molecular formula $C_{18}H_{16}O_7$ and also intense peaks at 329 [M-CH_3] and 301 [M-43] (Fig. 6.12) corresponding to 6-OCH_3 flavonol fragmentation (Markham, 1982) and standard flavonol C-ring contraction (Harborne, 1994) for 3-methyl ether flavone respectively. The ^{1}H NMR (acetone-d_6) spectrum showed three methoxy signals at δ 3.87, 3.88 and 3.90 (3H each, s, OMe-3, 6 and 4′) and there was presence of AA'BB' system due to ring B at δ 7.12 (2H, d, J= 9.6 Hz, H-3', H-5') and δ 8.11 (2H, d, J=9.3, H-2', H-6'). The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.97. ^{13}C NMR data are presented in Table 6.2. The spectral data are in close agreement with those reported in the literature (Table 6.2). The compound is therefore 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone (santin) (Fig. 6.11). Santin had earlier been reported from the leaf extract of *D. viscosa* (Sachdev and kulshreshtha, 1982; Abdel-Mogib *et al*., 2001).

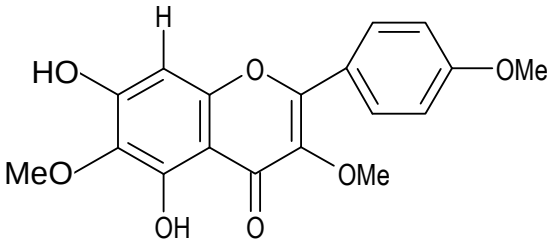


Figure 6.11: Structure of 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone

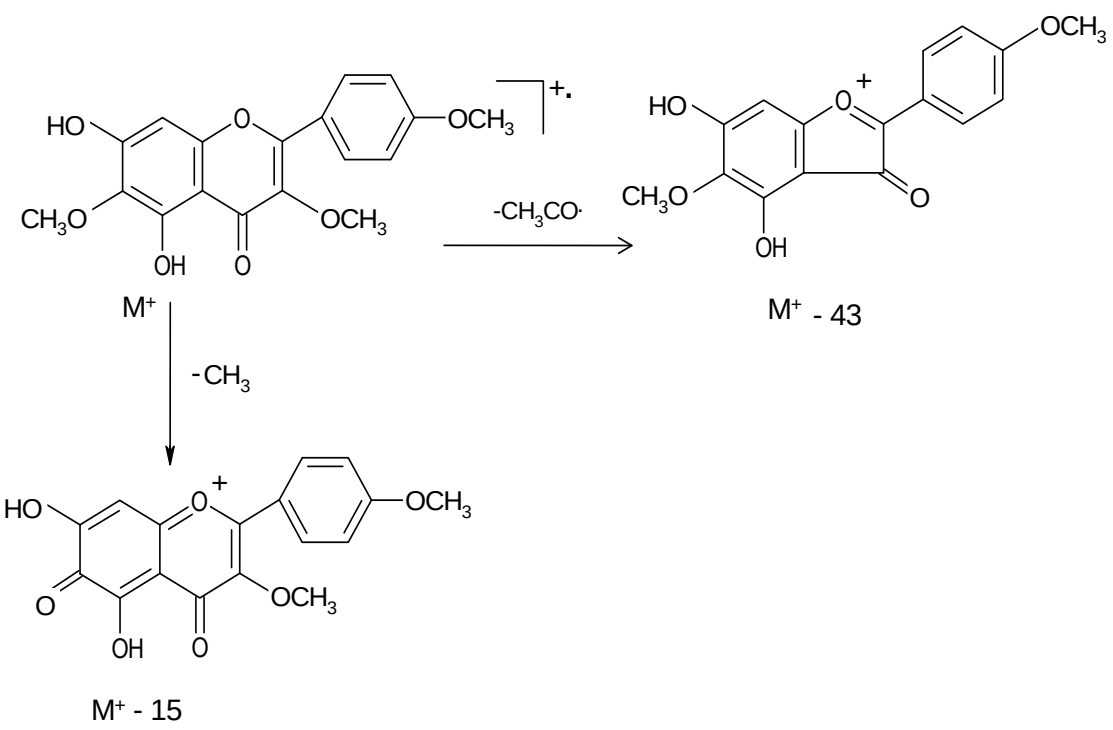


Figure 6.12: Mass Spectrum fragmentation of santin.

Table 6. 2: ^{13}C NMR data of Compound 2

^{13}C Assignment	Compound 2 (acetone-d_6)	Santin ($CDCl_3$) (Rashid *et al*., 1992)
2	157.1	156.2
3	138.8	138.7
4	179.2	179.6
5	152.5	151.8
6	131.0	130.1
7	153.0	152.2
8	93.83	93.1
9	156.0	155.9
10	105.0	106.5
1'	123.0	123.1
2', 6'	130.4	130.2
3', 5'	114.3	114.1
4'	162.1	160.2
OMe -4'	55.2	55.4
OMe-6	60.1	61.8
OMe-3	59.6	60.1

6.3.2.3: Compound 3

The mass spectrum showed molecular ion peak at m/z = 330 [M^+, 100%] base peak, corresponding to the molecular formula $C_{17}H_{14}O_7$ and an intense peaks at 315 (M-CH_3) and 287 (M-43) (Fig. 6.14) corresponding to the 6-OCH_3 flavonol fragmentation (Markham, 1982) and standard flavonol C-ring contraction (Harborne, 1994) for 3-methyl ether flavone respectively. The ^{1}H NMR (acetone-d_6) spectrum showed two methoxy signals at δ 3.86 and 3.87 (3H each, s, OMe-3 and OMe-6). There was presence of AA'BB' system due to ring B at δ 7.02 (2H, d, J= 9.0 Hz, H-3', H-5') and δ 8.04 (2H, d, J=9.0, H-2', H-6'). ^{13}C NMR spectral data were compared with that of santin in which rings A and C are intact and kaempferol in which ring C is intact. The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.98. The spectral data are in close agreement with those reported in Table 6.3. The compound is therefore 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone (Fig. 6.13). This compound was previously reported as a constituent from the genus *Dodonaea* such as from *D. viscosa* (Sachdev and Kulshreshtha, 1983), *D. attenuata* (Payne and Jefferies, 1973) and *D. angustifolia* (van Heerden *et al*., 2000).

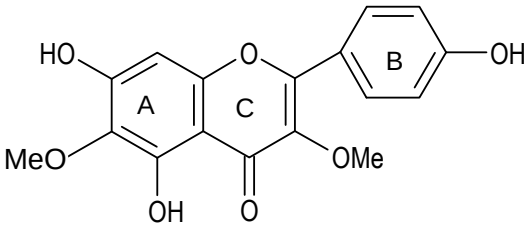


Figure 6.13: Structure of 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone.

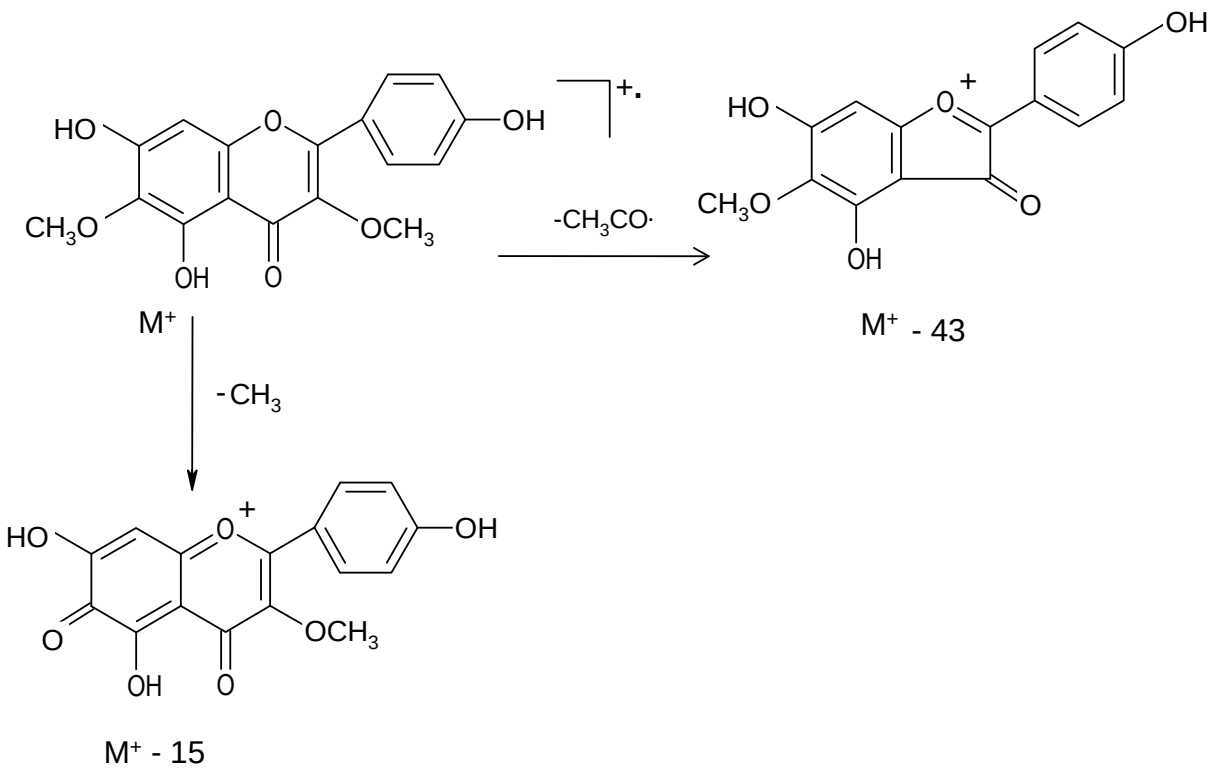


Figure 6.14: Mass Spectrum Fragmentation of 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone.

Table 6.3: ^{13}C NMR data of Compound 3

^{13}C Assignment	Compound 3 (acetone-d_6)	santin($CDCl_3$) (Rashid *et al*., 1992)	kaempferol (DMSO-d_6) (Markham, 1982)
2	157.6	156.2	146.8
3	138.8	138.7	135.6
4	179.8	179.6	175.9
5	153.6	151.8	160.7
6	131.8	130.1	98.2
7	153.1	152.2	163.9
8	94.4	93.1	93.5
9	156.9	155.9	156.2
10	106.3	106.5	103.1
1'	122.7	123.1	121.7
2', 6'	131.2	130.2	129.5
3', 5'	116.4	114.1	115.4
4'	160.9	160.2	159.2
OMe-6	60.7	61.8	--
OMe-3	60.2	60.1	--
OMe-4'	--	55.4	--

6.3.2.4: Compound 4

The mass spectrum showed molecular ion peak at m/z =301.01 {[M +H]$^{+\cdot}$ 57 %} corresponding to the molecular formula $C_{16}H_{12}O_6$. There was no fragmentation of ions probably due to absence of extensive methoxylation. The ^{1}H NMR (acetone-d_6) spectrum showed one methoxy signal at δ 3.89 (3H, s, OMe-4'). There was presence of AA'BB' system due to ring B at δ 7.10 (2H, d, J= 9.3 Hz, H-3', H-5') and δ 8.02 (2H, d, J=9.6, H-2', H-6'). The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.17. Also there was a presence of two doublets at 6.26 and 6.54 (1H each, J = 2.4 and 2.1 Hz respectively H-6, H-8). ^{13}C NMR is presented in Table 6.4. The spectral data are in close agreement with those reported in the literature (Table 6.4). The compound is therefore 3, 5, 7-trihydroxy-4'-methoxyflavone (4'-O-methylkaempferol) (Fig. 6.15). We are reporting for the first time isolation of 4'-O-methylkaempferol from *D. viscosa*, however, the compound has been previously isolated from legumes lentil, *Lens culinaris* (Family, Fabaceae) (Latha and Daniel, 2001).

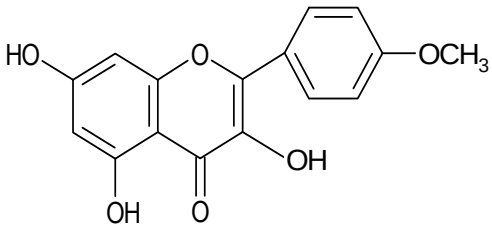
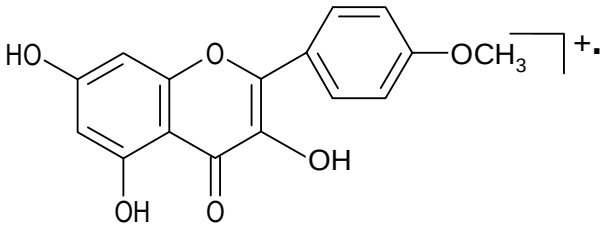


Figure 6.15: Structure of 4'-O-methylkaempferol**.**



$[M+H]^+$ = 301.01, 57%

Figure 6.16: Molecular ion peak of 4'-O-methylkaempferol.

Table 6.4: ^{13}C NMR data of Compound 4

^{13}C Assignment	Compound 4 (acetone-d_6)	4'-O-methylkaempferol (DMSO-d_6) (Harborne, 1982)
2	147.0	146.4
3	136.0	135.8
4	176.0	175.8
5	161.7	160.6
6	98.5	98.2
7	164.5	163.9
8	93.9	93.4
9	157.2	156.2
10	104.0	103.1
1'	123.7	123.2
2', 6'	129.6	129.0
3', 5'	114.2	114.0
4'	161.2	160.6
4'-OCH_3	55.3	55.3

6.3.2.5: Compound 5

The mass spectrum showed molecular ion peak at m/z =287.06 {[M +H]$^{+\cdot}$ 30%} corresponding to the molecular formula $C_{15}H_{10}O_6$. There was no fragmentation of ions probably due to absence of extensive methoxylation. The ^{1}H NMR (acetone-d_6) spectrum showed AA'BB' system due to ring B at δ 7.02 (2H, d, J= 9.0 Hz, H-3', H-5') and δ 8.16 (2H, d, J=9.0, H-2', H-6'). The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.17. Also there was a presence of two doublets at 6.26 and 6.53 (1H each, J = 2.4 and 2.1 Hz respectively H-6, H-8). ^{13}C nmr is presented in Table 6.5. The spectral data are in close agreement with those reported in the literature (Table 6.5). The compound is therefore 3, 4', 5, 7-tetrahydroxyflavone (kaempferol) (Fig. 6.17) and this compound was isolated previously from *D. viscosa* (Wollenweber, *et al*., 1986; Getie *et a*l., 2000) and from other plant species such as *Ginkgo biloba* (Family Ginkgoaceae) (Chin *et al*., 2000).

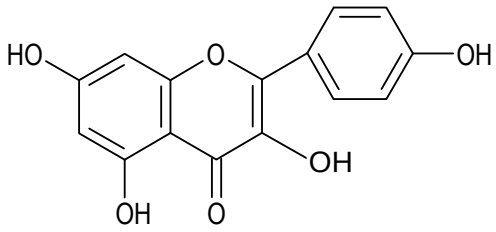
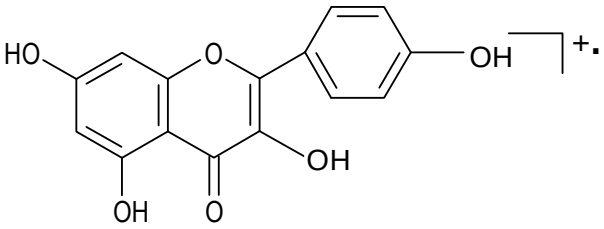


Figure 6.17: Structure of kaempferol



[M+H]$^+$ = 287.06, 30%

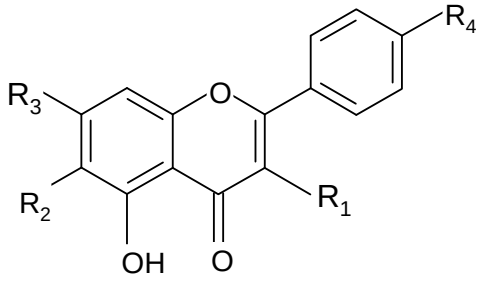
Figure 6.18: Molecular ion peak of kaempferol

Table 6.5: ^{13}C NMR data of Compound 5

^{13}C Assignment	Compound 5 (acetone-d_6)	kaempferol (DMSO-d_6) (Markham, 1982)
2	147.0	146.8
3	136.0	135.6
4	176.0	175.9
5	161.6	160.7
6	98.5	98.2
7	164.5	163.9
8	93.8	93.5
9	157.1	156.2
10	103.5	103.1
1'	122.6	121.7
2', 6'	129.8	129.5
3', 5'	115.7	115.4
4'	159.6	159.2

6.4 Conclusion

Five flavonoids (Fig. 6.19) were isolated from crude leaf extract of *D. viscosa*; 5-hydroxyl- 3, 7, 4'-trimethoxyflavone (Compound **1**); 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone (Compound **2**); 3, 6-dimethoxy-5, 7, 4'-trihydroxyflavone (Compound **3**); 4'-O-methylkaempferol (Compound **4**) and kaempferol (Compound **5**). We isolated for the first time one compound which is 4'-O-methylkaempferol from *D. viscosa*.



Compound Name	Number	R1	R2	R3	R4
5-hydroxy -3, 7, 4'-trimethoxyflavone	**1**	OMe	H	OMe	OMe
5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone	**2**	OMe	OMe	OH	OMe
5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone	**3**	OMe	OMe	OH	OH
4'-O-methylkaempferol	**4**	OH	H	OH	OMe
kaempferol	**5**	OH	H	OH	OH

Figure 6.19: Five isolated compounds from *D. viscosa* leaf extracts.

CHAPTER 7

7. BIOLOGICAL ACTIVITY OF ISOLATED COMPOUNDS FROM *D. VISCOSA*

7.1 Biological assays

For the bioassays procedures (Bioautography and MIC assays) of the isolated compounds, the procedure as described under Methodology in Chapter 2 was followed. The following two Gram-positive bacteria (*S. aureus* and *E. faecalis*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were used.

7.2 Results and Discussion

7.2.1 Biological assays

7.2.1.1 Bioautography assay

Compound 1

Compound 1 exhibited no antibacterial activity against all bacteria tested (data not shown).

Compound 2

At concentrations of 10 μg/ml, Compound 2 showed various level of bacterial inhibition. A clear zone of growth inhibition was highly evident with *P. aeruginosa*, less growth inhibition was observed on *S. aureus* whereas slight activity was observed with *E. coli* and *E. faecalis* (Fig 7.1).

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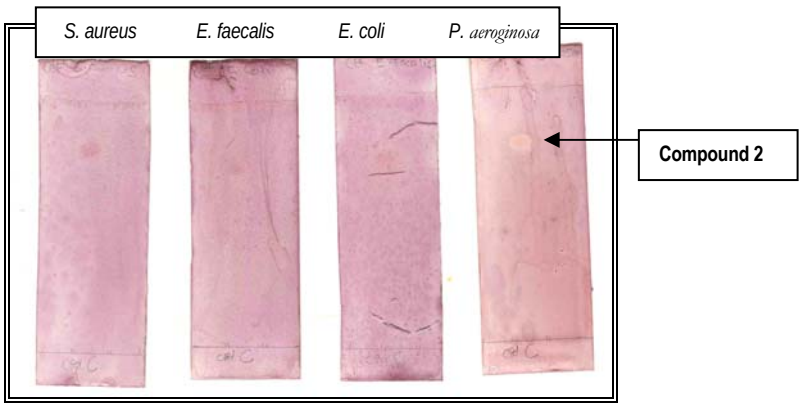


Figure 7.1: TLC chromatograms of Compound 2 against four test microorganisms, (an arrow points the area of growth inhibition).

Compound 3

Compound 3 (Fig. 7.2) showed a good activity against *E. faecalis* at concentrations of 5 and 10 μg. Good antibacterial activity was also found with *E. coli*, whereas *S. aureus* and *P. aeruginosa* were not sensitive when 10 μg was separated.

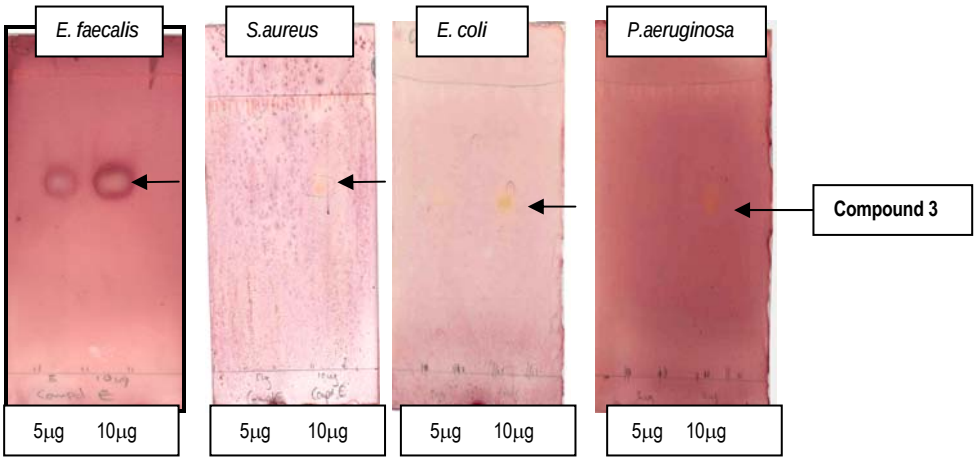


Figure 7.2: Bioautography of Compound 3 against four test microorganisms.

Compound 4

The bioautography results gave clear spots as zone of inhibition against *S. aureus* and *E. coli* (Fig 7.3). *P. aeruginosa* and *E. faecalis* did not grow well on the TLC plates (data not shown).

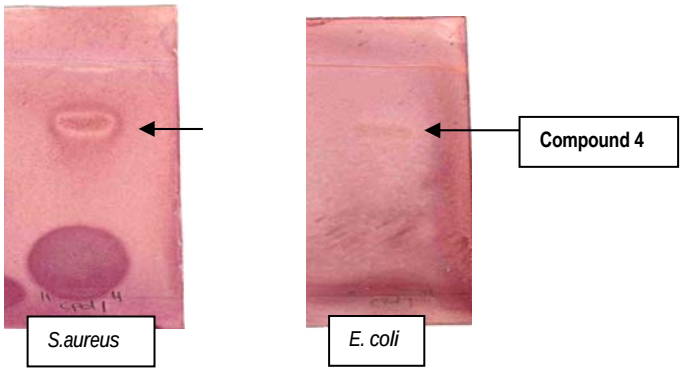


Figure 7.3: Bioautography activity of Compound 4 against *S. aureus* and *E. coli*.

Compound 5

There was a clear zone of inhibition against *S. aureus.* Clear yellow zones were less evident against the pink background for *E. coli* and *P. aeruginosa*. This implied that there was growth inhibition by Compound 5 against *S. aureus, E. coli* and *P. aeruginosa* (Fig. 7.4)

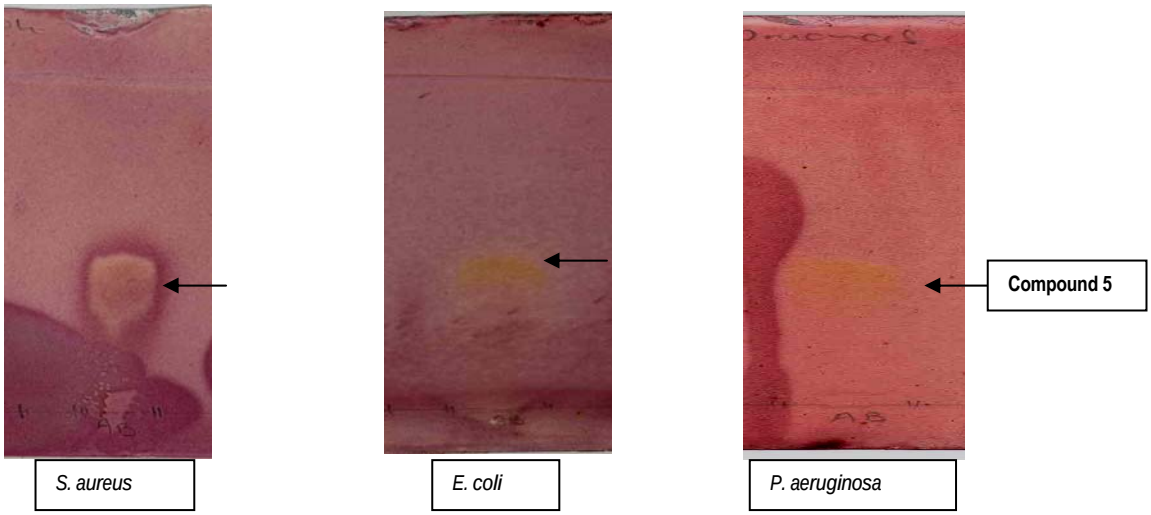


Figure 7.4: TLC chromatograms of Compound 5 activity against three test microorganisms.

7.2.1.2 Antioxidant assay

Qualitative assay

In the TLC autographic DPPH assay, Compound 1 did not show any antioxidant activity (results not shown). The TLC chromatograms in Fig 7.5 indicates that the compounds posses antioxidant activities. Compound 2 showed a clear yellow band 15 minutes after spraying with 0.2% DPPH in methanol depicting a good level of antioxidant activity. Compound 3 also tested positive for antioxidants showing a clear yellow band 5 minutes after spraying with DPPH. Compound 4 revealed a yellow band after 15 min whereas Compound 5 showed a yellow band immediately after spraying with DPPH. All the compounds except Compound 1 had antioxidant activities. The level of antioxidants in these compounds was quantified using a DPPH Spectrophotometer assay.

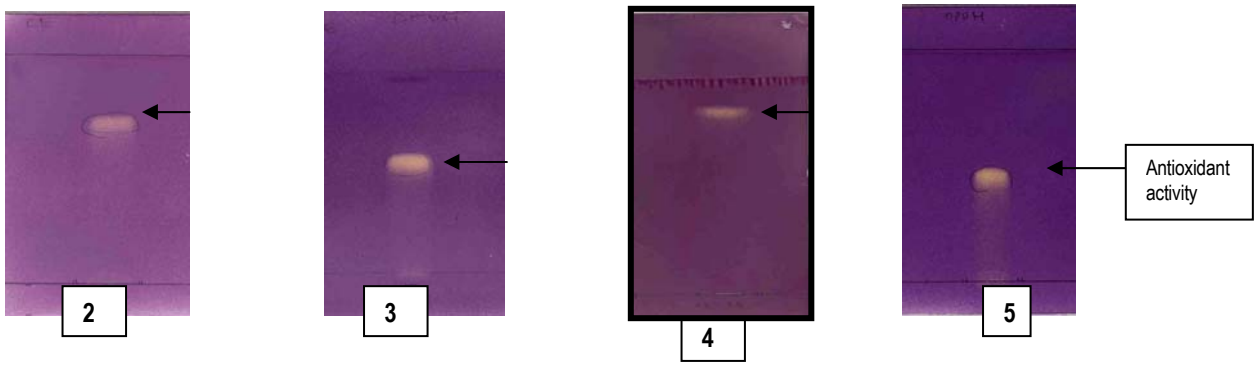


Figure 7.5:TLC chromatogram separated by CE indicating the level of antioxidant activity of Compound 2, 3, 4 and 5.

DPPH Spectrophotometric assay

Quantitative assay has revealed a varying degree of antioxidant potential of the compounds. Kaempferol without any methoxyl substitution was the most active. Compounds 1-4 are kaempferol derivatives with different methoxylation patterns. Of the four derivatives, compound 4 which is a 4'-OMe derivative was the most active. Compound 3 with 3-OMe and 6-OMe and also compound 2 with 3-OMe, 4'-OMe and 6-OMe did not inhibit 50% of DPPH free radical. At the highest concentration (200 µM) tested compound 2 and 3 had 19.06 and 8.23 percentage antioxidant activity (Table 7.1). It appeared that 3-OMe reduced activity significantly when compared with compound 4 with free 3-OH. This observation is in agreement with the earlier structural activity study (Op de Beck, 2003). The effect of 6-OMe on activity is not clear. Compound 1 was not quantified since it did not demonstrate activity in the qualitative assay apparently due to extensive methoxylation (3-OMe, 4'-OMe and 7-OMe). This observation suggests that 5-OH does play strong role in antioxidant activity of the investigated compounds since the level of antioxidant activity demonstrated by these compounds correlated in the number of free-OH groups in the rings. This is also in line with the earlier report on flavonoids (Rive-Evans *et al*., 1996). Our results have shown that only Compound 5 (kaempferol) and Compound 4 have demonstrated strong activity (EC_{50} = 35.06 ± 0.85 and 75.49 ± 1.76 µM respectively) but lower than L-ascorbic acid (EC_{50} = 13.55 ± 0.28 µM) used as a standard antioxidant agent (Table 7.2).

Table 7.1: Percentage antioxidant activity of Compounds **2-5**

Conc.(µM)	Mean absorbance (%) (3x experiments) (SD)			
	Compound 2	**Compound 3**	**Compound 4**	**Compound 5**
200.0	19.06 ±0.001	8.28 ± 0.002	95.22 ± 0.16	-
100.0	0.00	0.00	65.36 ± 7.63	92.37 ± 0.66
50.0	0.00	0.00	48.92 ± 1.97	62.83 ± 8.97
25.0	0.00	0.00	27.93 ± 3.42	49.25 ± 5.11
12.5	0.00	0.00	16.50 ± 3.45	27.87 ± 2.43

Table 7.2: EC_{50} of compound 4 and 5 from *D. viscosa* leaf extracts

Compound	EC_{50} ± SEM µM	Correlation coefficient (r^2)
4	75.49 ± 1.76	0.947
5	35.06 ± 0.85	0.952
L-ascorbic acid	13.55 ± 0.28	0.999

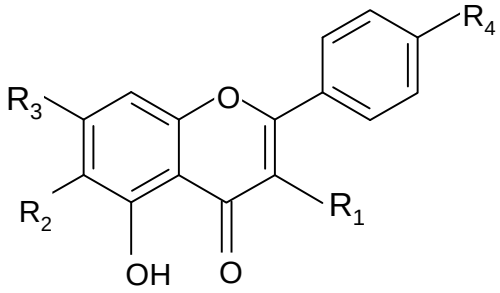
7.2.1.3 Microdilution assay- structure activity relationship

In the bioassay used (Eloff, 1988b) the initial concentration is reduced four fold. The MIC values of the isolated compounds were determined in triplicate using the four nosocomial bacterial pathogens (Table 7.3). In Table 7.3 the number of methoxy, hydroxyl and free hydrogen atoms on R1-R4 is also provided (also refer to Fig 7.6). The structure activity relationship differed for the different pathogens. This was not associated with Gram positive-Gram negative classification indicating that the effect was not cell wall related. In all cases a hydroxyl was required at R3 for antibacterial activity. An hydroxyl was required at R4 for activity with *P. aeruginosa* but this was apparently not the case with *S. aureus*. It does not seem as if substitution at R2 plays an important role. The data available did not make it easy to evaluate the contribution of substitution on R1 to the activity. Flavonoids are known for potent

multiple biological actions (Alcaraz *et al*., 2000). However, low antibacterial activity demonstrated by these compounds could be ascribed to methylation of the phenolic -OH groups in our compounds.

Table 7.3: MIC values in μg/ml of the isolated pure compounds determined in triplicate and a positive control. Structural information is also provided.

Pure Compounds	**Methoxy groups**	**OH groups**	**Free H atoms**	**MIC values (μg/ml)**			
				E. faecalis	*S. aureus*	*E. coli*	*P. aeruginosa*
1	3	0	1	>250 ± 0.00	>250 ± 0.00	>250± 0.00	>250 ± 0.00
2	3	1	0	62.5 ± 0.00	62.5 ± 0.00	62.5 ± 0.00	125 ± 0.00
3	2	2	0	31.25 ± 0.00	125 ± 0.00	26 ± 9.01	31.25 ± 0.00
4	1	2	1	23.44 ± 9.01	250 ± 0.00	125 ± 0.00	250 ± 0.00
5	0	3	1	31.25 ± 0.00	62.5 ± 0.00	15.63 ± 0.00	62.5 ± 0.00
Gentamicin				6.3 μg/ml	0.2 μg /ml	0.8 μg /ml	0.8 μg /ml



Compound Name	**Number**	**R1**	**R2**	**R3**	**R4**
5-hydroxy -3, 7, 4'-trimethoxyflavone	**1**	OMe	H	OMe	OMe
5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone	**2**	OMe	OMe	OH	OMe
5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone	**3**	OMe	OMe	OH	OH
4'-O-methylkaempferol	**4**	OH	H	OH	OMe
Kaempferol	**5**	OH	H	OH	OH

Figure 7.6: Srtucture of the five isolated compounds (Compound **1-5**) from *D. viscosa* leaf extracts.

7.3 Conclusion

According to the MIC results, Compound 1 possessed no activity against the tested microorganisms. Compound 2 exhibited a poor bacterial activity against all the tested microorganisms. Compound 3 showed a high level of activity against *E. coli*, *P. aeruginosa* and *E. faecalis*, however *S. aureus* was resistant. Only *E. faecalis* was sensitive towards Compound 4. However, Compound 5 exhibited a good level of activity against *E. coli* and *E. faecalis*. The most sensitive bacteria against isolated compounds were *E. coli* and *E. faecalis.* The structure activity relationship differed for the different pathogens. This was not associated with Gram positive-Gram negative classification indicating that the effect was not cell wall related. In all cases a hydroxyl was required at R3 for antibacterial activity. An hydroxyl was required at R4 for activity with *P. aeruginosa* but this was apparently not the case with *S. aureus*. It does not seem as if substitution at R2 plays an important role. The data available did not make it easy to evaluate the contribution of substitution on R1 to the activity.

From the qualitative antioxidant assay, all the compounds except for compound 1 had good antioxidant activity. After spraying with 0.2% DPPH in methanol, good level of antioxidant activity was revealed immediately with Compound 3 and 5, whereas with Compound 2 and 4; the antioxidant activity was observed after 15 min. Based on the literature, antioxidant potential of the compounds correlates to the number of free-OH groups in the rings. In this case the relationship did not hold well because Compound 4 had no free hydroxyl groups (Table 7.1) A quantitative DPPH spectrophotometric assay has demonstrated that only compound 4 and 5 have strong antioxidant activities. The presence of these antibacterial and antioxidant compounds although they have demonstrated moderate activities, they could provide rationale for the ethnomedicinal use of *D. viscosa* leaf extract in traditional medicine.

CHAPTER 8

8. GENERAL DISCUSSION AND CONCLUSION

In western countries, entomophagy is not highly appreciated. Consumption of insects is more common in Africa and Asia, where insects are widely consumed by the rural population. The problem with eating of insects is that many of educated people are ashamed of their culture and they eat insects secretly whereas some regard insects as food for the poor people. It is necessary to educate people and promote eating of insects as they are cheap source of protein and some families harvest and sell insects for income.

The main aim of this study was to investigate possible interaction between thongolifha (*E. delegorguei*) and its host plant *D. viscosa.* Because thongolifha feeds mainly on *D. viscosa* with known antibacterial activity, it may be possible that some compounds that are present in *D. viscosa* are also present in the insect. Thongolifha is an important cultural food of the vhaVhenda tribe and many families rely on selling of the insects for income. It was therefore necessary in this study to also determine the nutritional composition of thongolifha since it was never determined before.

8.1 E. delegorguei extracts

It is well documented that some insects ingest secondary metabolism compounds from plants and store them as part of its defence mechanism (Harborne, 1993). In our study, this was tested by comparing hexane, DCM, acetone and methanol extracts obtained from *D. viscosa and E. delegorguei* loaded onto TLC plates and developed in three eluent systems, EMW, CEF and BEA. Few similarities of compounds separated from both the plant and insect extracts were observed; implying that the sequestered compounds from host plant may be present in lower quantities in the insects extract, or the insect might not have sequestered components or have metabolised the sequestered compounds. The feeding mechanism of the bug from the host plant is by sucking the sap which is mainly the liquid from the leaves of the host plant. In that case they might not be much of the secondary plant compounds that are being ingested by the insect. Bioautography had revealed that two of the insect extracts compounds (Rf 0.79 and 0.66) had antibacterial against *S. aureus* and only one compound (Rf 0.66) showed activity against *E. faecalis*. The quantitative assay using the microplate dilution assay had MIC. 2.5 mg/ml indicating that the insect extracts had low antibacterial activity.

To determine the nutritional composition of *E. delegorguei,* ground material of the insect was extracted using standardised techniques. Thongolifha can be utilised as a protein supplement diet as it contains good quantity of protein (35. 2%) with high energy content of 2599 k.cal/100 g. The total mineral content of the thongolifha was at 1.2

g/100g and is a level comparable to other insects. Furthermore relatively high concentrations of B, Ba, Mn, Sr and Zn were detected in *D. viscosa* (Nagaraju and Karimulla, 2002) indicating that thongolifha obtained most of its minerals from its host plant.

The level of carbohydrate was at an acceptable level of 7.63 g/100 g. The insect contained high levels of fat (50.5%) and its amino acids content showed good levels of essential amino acids which varied from 0.82 mg/100 g (threonine) to 1.32 mg/100 g (valine). However to satisfy the daily nutritional need for the essential amino acids (phenylalanine and threonine), 680 and 3400 of thongolifha has to be consumed which can be quite a large number, and may be difficult to attain.

8.2 D. viscosa extracts

Methanol leaf extracts of *D. viscosa* possess antibacterial activity (Getie *et al*, 2003). However, their agar diffusion method did not provide any quantitative data regarding the level of activity of the extract against *S. aureus. E. coli* was found to be resistant. In this study, the antibacterial activity of *D. viscosa* crude leaf extracts was quantified by using serial dilution microplate assay and the total activity (TA) was also calculated. The microplate assay has confirmed that *D. viscosa* leaf extract had strong activity against *S. aureus* and even other test bacteria such as *E. coli*, *E. faecalis* and *P. aeruginosa* were susceptible to the extract MIC value (0.04-0.28 mg/ml). The data for total activity has indicated that acetone extract exhibited the highest total activity for all test microorganisms tested ranging from 48.4-750 ml/g. The antibacterial compounds extracted from 1 g of leaves could therefore be diluted up to 750 ml and still inhibit bacterial growth. *D. viscosa* extracts were also found to have reasonable antioxidant properties. Both the qualitative and quantitative DPPH methods have reported ethyl acetate and acetone extracts to have good level of antioxidant activities.

Based on the above findings with regard to *D. viscosa* extracts, a bioassay-guided fractionation silica gel chromatography was performed and four flavonoids were isolated from dichloromethane fraction of crude *D. viscosa* leaf extract: (5-hydroxyl- 3, 7, 4'-trimethoxyflavone (**Compound 1**); 5,7-dihydroxy-3, 6, 4'-trimethoxyflavone (**Compound 2**); 3,6-dimethoxy-5, 7, 4'-trihydroxyflavone (**Compound 3**); 4'-O-methylkaempferol (**Compound 4**). Only one compound, kaempferol (**Compound 5**) was isolated from the acetone crude leaf extract fraction. We report for the first time isolation of 4'-O-methylkaempferol from *D. viscosa,* this compound is not novel since it was isolated before from other plant species such as *Lens culinaris* (Latha and Daniel, 2001). Only three of the isolated compounds (i.e. **Compound 3, 4** and **5**) exhibited good antibacterial activities against *E. coli* (15.63-26.5 μg/ml), *E. faecalis* (23.44-31.25 μg/ml) and *P. aeruginosa* (31.25 μg/ml). Furthermore, the TLC qualitative assay using 0.2% DPPH in methanol has shown that the four isolated compounds except for **Compound 1** have good level of antioxidant activity. The level of activity was observed from immediately to up to 15 min after spraying with 0.2% DPPH in methanol. Based on the literature, antioxidant potential of the compounds correlates to the number of free-

OH groups in the rings. The DPPH spectrophotometer assay indicated that, 4-methoxykaempferol and kaempferol have strong antioxidant activity (EC_{50} = 35.06 ± 0.85 and 75.49 ± 1.76 µM respectively) but lower than L-ascorbic acid (EC_{50} = 13.55 ± 0.28 µM) used as a standard antioxidant agent. Although the isolated compounds **3**, **4** and **5** had moderate to good level of both antibacterial and antioxidant activities, it was difficult to establish clear structure function correlation between antioxidant and antibacterial activities of these compounds. Flavonoids are however known for potent multiple biological actions (Alcaraz *et al*., 2000).

8.3 Insect-plant interaction

For the insect extracts there were compounds with antibacterial activity against *S. aureus* (Rf 0.79 and 0.66) and *E. faecalis* (Rf 0.66) based on bioautography when separated with CEF solvent system. Some of the isolated antibacterial compounds from *D. viscosa* leaf extracts had an Rf 0.79 (5,7-dihydroxy-3,6,4'-trimethoxyflavone) and Rf 0.61 (5,7,4'-trihydroxy-3,6-dimethoxyflavone) when separated with CEF eluent system. This indicates that thongolifha may have sequestered the two compounds from *D. viscosa* leaves, but this possibility has to be confirmed. This coclusion may be speculative, but there were only two antibacterial compounds in the insect extract. The close correlation of Rf values suggests that they may be identical to compounds isolated from the plant extract. Unfortunately bioautography is a difficult process that does not work with all TLC solvent systems otherwise it would have been easy to compare the Rf values of the antibacterial compounds of the insect extract co-chromatographed with the isolated compounds using other TLC solvent systems.

In conclusion, thongolifha has good protein content and it is therefore not just a traditional delicacy for the vhaVenda tribe, it contains a good source of protein, minerals and vitamins. It is also a good diet supplement and it provides a valuable source of income for the harvesters and therefore, sustainable harvesting is necessary. Although our study did not reveal any antibacterial activities of the insects, it contains a chemical defensive substance that can be toxic to human eyes. The insects may have medicinal value in folk medicine that is not published anywhere in the literature. The crude leaf extracts of the host plant *D. viscosa* on the other hand, possess good level of antibacterial and antioxidant activities. There are two compounds obtained from insect extracts that may be the same as the isolated compounds 5,7-dihydroxy-3,6,4'-trimethoxyflavone and 5,7,4'-trihydroxy-3,6-dimethoxyflavone from *D. viscosa*, implying the possibility of the insect to have sequestered the two compounds from *D. viscosa* leaves.

Investigation into the structure relationship activity of isolated compounds did not yield a clear structure function correlation. However, the presence of these antioxidant and antibacterial compounds although demonstrating moderate level of activities could provide rationale for the ethnomedicinal use of *D. viscosa* leaf extract in traditional medicine

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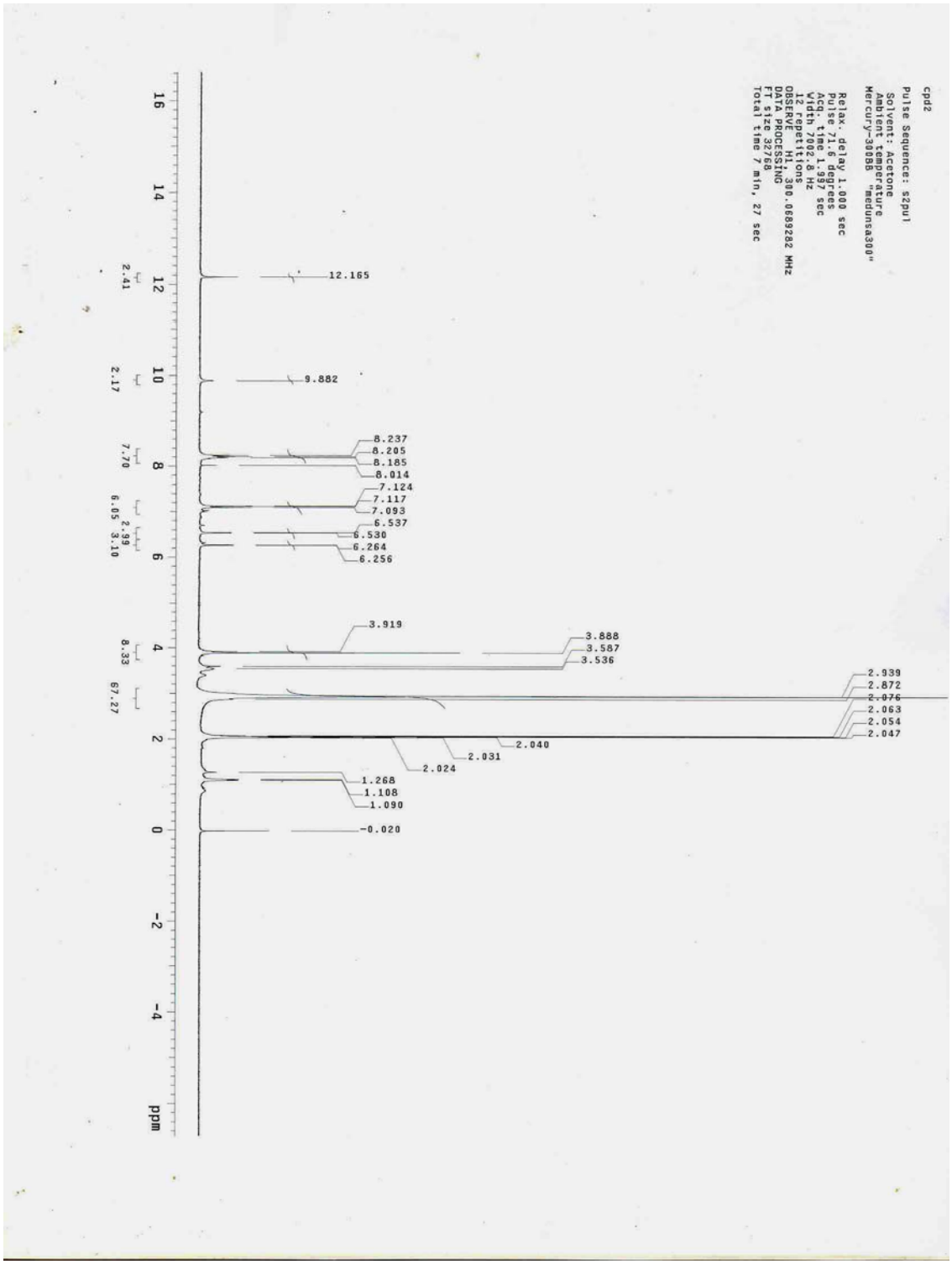
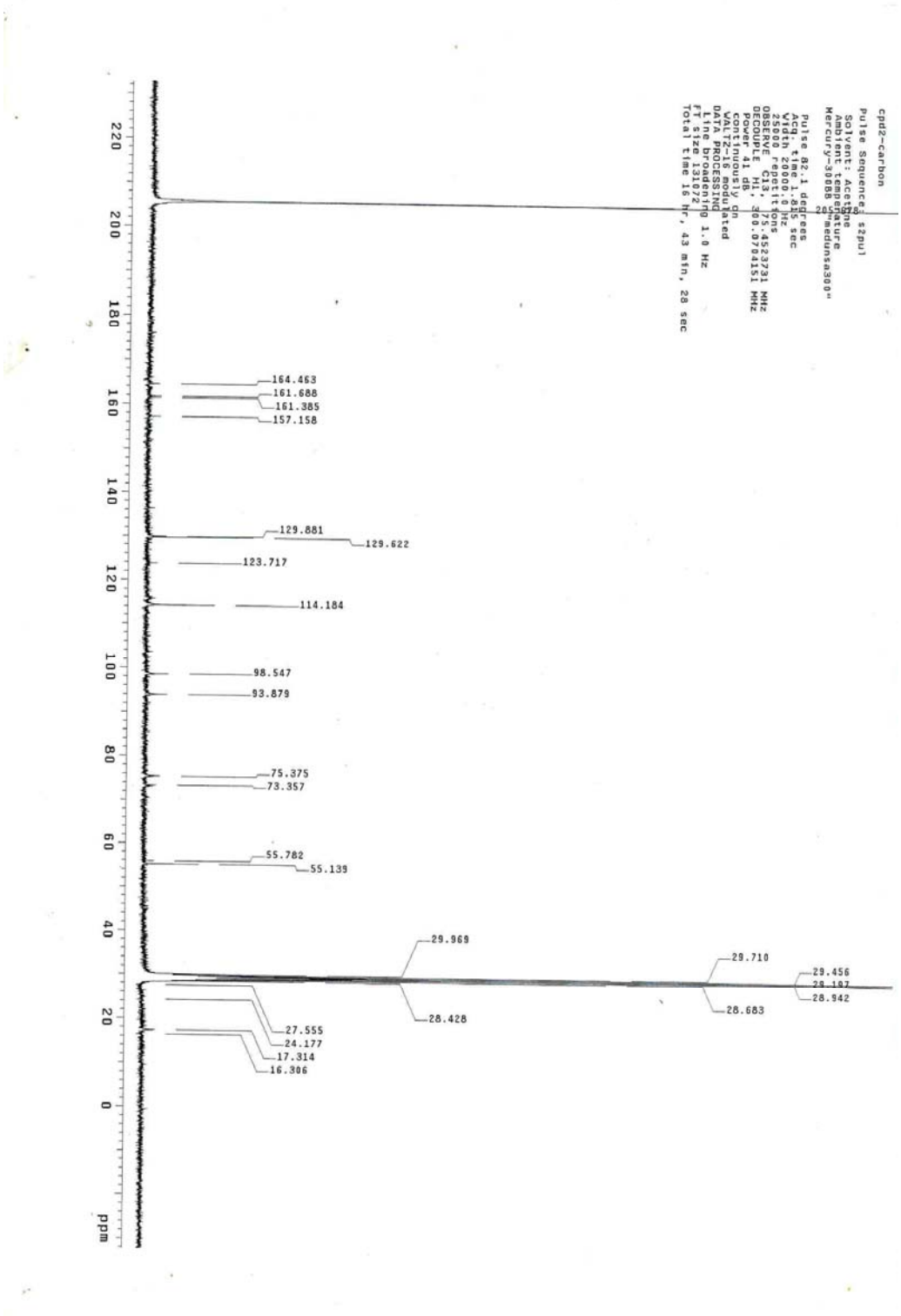
Appendix

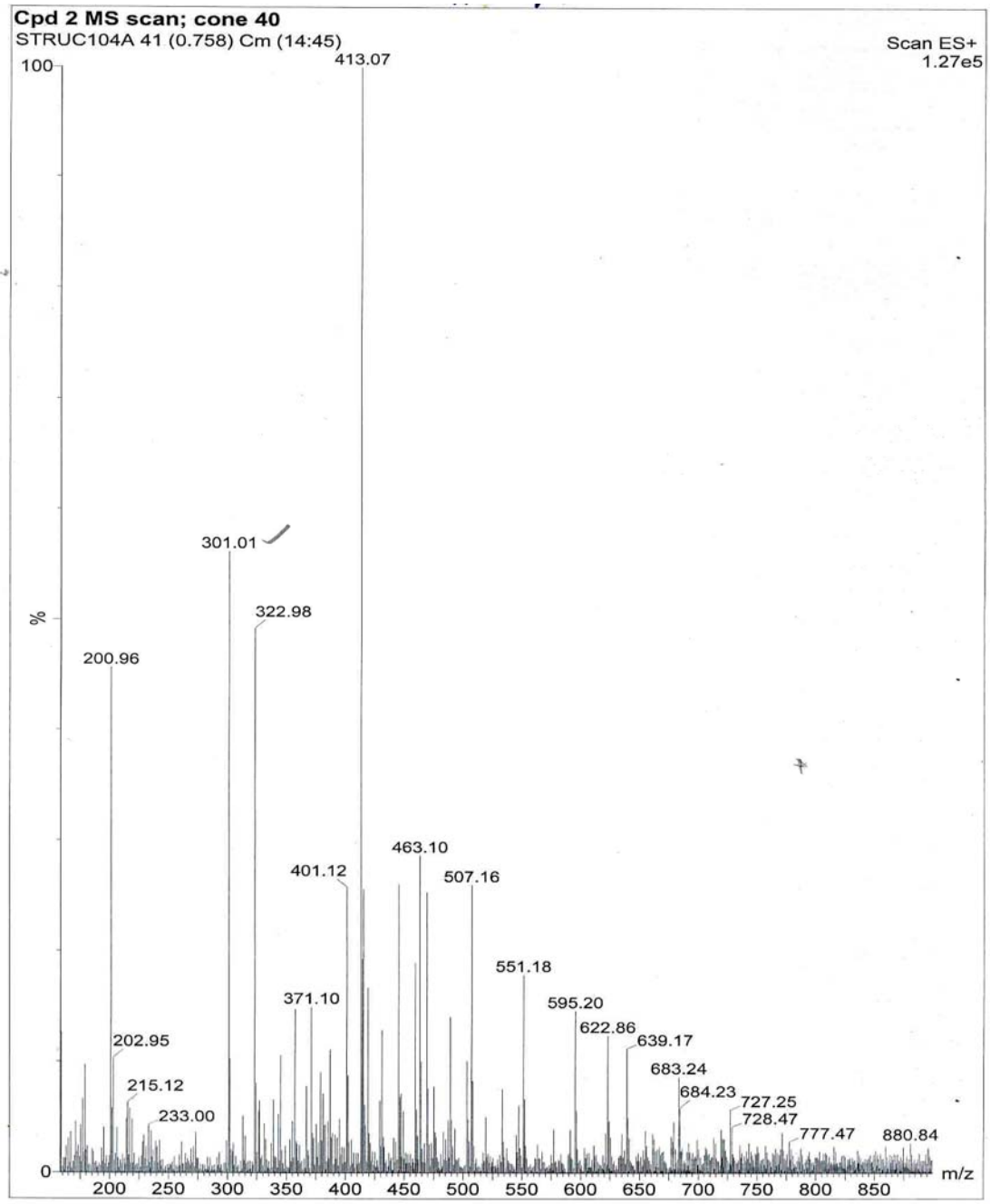
^{1}H NMR Spectrum of Compound 4 (4'-O-methylkaempferol)



^{13}C NMR Spectrum of Compound 4 (4'-O-methylkaempferol)



Mass Spectrum of Compound 4 (4'-O-methylkaempferol)



PRO€INVEST

Grant Application Form

Call for Proposals 2006

Demand-Driven activities - Intermediary Organisations

8.ACP.TPS.108

Name of applicant:	Association for African Medicinal Plants Standards
Type of activity	☐ Public Private Dialogue ☒ Capacity Building ☐ Mission to the EU ☐ Mission to or in ACP ☐ Company Twinning

Dossier N°	

(for official use only)

CONCEPT NOTE APPLICATION - Assocuaition for African Medicinal Plants Standards

1. Summary of the action

1.1 Brief description of the proposed action.

The Association for African Medicinal Plants Standards (AAMPS) is a recently formed trade association consisting of many of Africa's leading organisations involved in the identification, research, development, manufacture and trade in herbal medicines and related products. Since its inception AAMPS has been preparing and promoting trade standards and regulations for the cultivation, manufacture and use of 50 of Africa's most important medicinal plants. This information will be used as the starting point for the preparation of an African Herbal Pharmacopoeia (see www.aamps.net).

This proposed action is a capacity-building exercise designed to strengthen the financial, administrative and technical skills of the association to enable it to carry out these complex and important tasks.

Without the development and promotion of such standards, the development of safe and efficacious herbal drugs and dietary supplements for national, regional and international sale will not be possible. Africa currently accounts for less than 5% of the turnover of this multibillion dollar industry. The fact that sub-Saharan Africa [c. 59,800] and the Indian Ocean Islands [c 12,000] hold nearly 30% of the world's estimated 240,000 plant species diversity clearly illustrates the discrepancy in current trade.

AAMPS has members from across the whole of Africa as well as from Europe, Asia and North America. In order to expand its membership network and enhance its revenue, it urgently needs to strengthen its capacity to recruit members and raise other forms of income so that the organisation can become self sustaining.

The proposed action includes

A Preparing a detailed 5 year business plan outlining strategic planning, focussing on potential sources of revenue, financial sustainability for AAMPS, recommendations on membership recruitment, administration and international promotion.

B Organising an international workshop to discuss and approve the business plan and to authenticate and promote the 50 herbal profiles currently being prepared by AAMPS in order to increase the revenue of AAMPS, either through the sale of this information or through increasing membership.

C Upgrading and expanding of the AAMPS website and the preparation of a range of promotional materials designed to increase the number of members of AAMPS and to enhance communications and a sense of identity between existing members of this organisation.

D Investigating other opportunities for fundraising, e.g., by a royalty paid by subcontractors for quality control or research projects commissioned by industry and allocated by AAMPS.

AAMPS is registered in Mauritius and holds the intellectual property rights to all the documentation prepared under its auspices. This action will complement interventions already supported by CDE and CTA which have helped finance the selection, research, laboratory testing and preparation of the final drafts for the 50 most important African medicinal plant species standards to be completed by the end of the first half of 2007.

Through an efficient AAMPS this project would

- encourage the trade in African medicinal plants across international borders,
- lead to substantial value addition of medicinal plants,
- create jobs for people collecting or growing medicinal plants,
- alleviate poverty of poor rural communities,
- encourage the effective use of unexploited medicinal plants worldwide and
- increase the human defence against diseases.

AAMPS members have many skills that are important to this project, but there is limited administrative and managerial skills and to a certain extent also in contacts with other role players in Africa. Our partner, the Centre for Research Information Action in Africa Southern Africa Developing and Consulting CRIAA SA-DC (see page 4) would play a critically important role in enhancing AAMPS' managerial and administrative skills and resources.

2. Relevance:

2.1 Relevance of proposal to the needs and constraints of the target countries.

The lack of technical specifications and quality control standards for African Medicinal Plants and extracts was one of the major constraints identified at the Commonwealth/CDE Medicinal plants conference held in Cape Town, South Africa in December 2000. This discourages consideration of African medicinal plants usage if there is no trustworthy printed information on efficacy and safety available. This also makes it difficult for local or overseas buyers to compare batches of products from different places or from year to year. This is in marked contrast to other major regions of the world, e.g. China and India, where traditional formulations have been recorded and evaluated at both the local and international level.

If African herbals are to enter the formal herbal medicine market, formulation of internationally acceptable information on the identity, safety, efficacy, and quality control is *sine qua non* for the expansion of trade in this sector.

These product specifications combine some of the information usually found in scientific plant monographs (e.g. ESCOP, WHO etc.), trade specifications, quality control data and form the foundation for an African Herbal Pharmacopoeia. Information for the selected African medicinal plant species is not yet available in these scientific plant monographs.

The proposed standards will constitute reliable technical profiles and will address a void which currently exists in this market. The standards would also act as a catalyst for quality control and quality assurance programmes for African Medicinal Plants. They will form a reference base that companies which trade in these products could use to establish their own in-house specifications.

2.2 The problems to be resolved and the needs to be met.

While major efforts are underway to research, test and draft the 50 herbal profiles using consultants drawn from right across Africa and supported by advisors from the European Union, AAMPS itself suffers from lack of revenue and administrative support. In order to increase the number of members and to enhance the reputation of the organisation, a series of actions are required as listed below.

2.3 The actors (final beneficiaries, target groups) involved.

- African companies involved in the production and sale of herbal products both within Africa and as export commodities. (AAMPS members currently include around 5 such companies and there are in the order of 50 companies in Africa involved in the manufacture and sale of such products.)
- Herbal practitioners and clinics using traditional medicines or modern plant based dietary supplements. There are thousands of such clinics throughout Africa.
- Ministries of Health and associated state healthcare facilities.
- European, Asian and North American companies involved in the import, processing, distribution and use of African herbal ingredients and semi-finished products.
- Research and development centres interested in developing new products using African materials.
- Regional and international organisations involved in primary health care and in the planning and strategic management of African health programmes (WHO, OAU, etc.).

2.4 Objectives and expected results

a) Writing and evaluating a 5 year business plan to develop a thriving and sustainable organization.
b) Upgrading of the website to improve networking and communication and allow membership recruitment to be organised on line, publications and other information to be sold and downloaded and sponsored links to be developed.
c) Organisation of an international meeting and exhibition to which leading stakeholders involved in quality assurance and quality control of African herbals would be invited. This meeting would be used to evaluate the business plan, to raise funds through membership, sponsorship and the sale of data.

2.5 The added value of the action.

The profiles prepared will identify areas where research is required on the selected species. This will make it easier for scientists to identify and motivate potential funding on projects which address gaps in the profiles.

AAMPS also envisages that at some stage the database will be accessible to all approved members thus establishing a living database that can continually be upgraded under supervision of the webmaster or original author of the profile.

The cultivation of medicinal plants in Africa will not only increase the quality of primary health care, but also lead to the creation of jobs and export opportunities with the associated alleviation of poverty.

3. Methodology and Sustainability:

3.1 The main project activities

A Preparing a detailed 5 year business plan outlining potential sources of revenue and expenditure and identifying the best way to achieve sustainability for AAMPS. The plan will also make recommendations on membership recruitment, administration and international promotion.

B Organising an international workshop to approve the business plan and to authenticate and promote the 50 herbal profiles currently being prepared by the association thereby increasing the revenue of AAMPS through the sale of this information or through the raising of membership fees.

C Upgrading and expanding of the association's website and the preparation of a range of promotional materials designed to increase the number of members of AAMPS and to enhance communications and a sense of identity between existing members of this organisation.

3.2 The main implementing partners, length of relationship and involvement in the project

The main implementing partner is the Centre for Research Information Action in Africa Southern Africa Developing and Consulting. CRIAA SA-DC was one of the key organisations who helped set up AAMPS. They will advise us on all aspects related to administration of AAMPS and will be a key player in organising the Workshop (see http://www.criaasadc.org).

University and Research organisations as well as private companies involved in the cultivation, processing and trade in herbal raw materials and products will play a role and were involved from the beginning in the establishment of AAMPS. These include:

The Phytomedicine Programme, University of Pretoria, South Africa: This programme has 5 staff, and more than 35 masters, doctoral students and post doctoral fellows. It has been identified as a developed Research Niche Area by the South African National Research Foundation. The programme has a research and consultancy budget of c €400 000 per year. They have already successfully carried out contracts for both CDE and CTA.

Dept. of Chemistry, University of Mauritius, Reduit, Mauritius: This department has already successfully carried out contracts for CDE, IPGRI, CIDA and SIDA.

Parceval Pharmaceuticals, South Africa: One of Africa's leading growers and processors of medicinal plants and plant extracts with exports exceeding €2 million per year. They have a joint venture with a leading EU pharmaceutical company and have pioneered the export of value-added herbals in Africa.

African Artemisia, Tanzania: Another major pioneer in the African herbal industry involved in growing and processing natural products for export and local market. This company has already received technical assistance from CDE over many years. Turnover exceeds €1 million per year.

Biomox Pharmaceuticals Pty Ltd, South Africa: A leading herbal pharmaceutical company in South Africa producing herbal medicines for some 65 smaller herbal medicine companies in southern Africa and also for major pharmaceutical companies like Merck Pharmaceuticals.

3.3 Achieving sustainability

AAMPS is particularly concerned about the need to develop its own sources of income and to achieve sustainability as rapidly as possible. The main ways in which the organisation earns revenue is by levying of membership fees, sale of detailed technical profiles to non-members, inspection and certification services, special publications, sponsorship of events and conference and workshop fees. The business plan which will be developed as part of this action will help refine and expand on these activities and improve AAMPS chances of sustainability.

3.4 Possible multiplier effects of project

This will provide the basis for legislation to include herbal preparations into health care programmes, it will enable local pharmaceutical companies to achieve uniform quality products, give confidence to importers of African herbals that products meet minimum quality standards, encourage local and international companies to use more Africa herbal ingredients, provide an independent African organisation that can advise government and industry on matters of safety and efficacy and encourage increased cultivation, jobs and trade in 50 of Africa's most important medicinal plants.

It is not surprising that since the publication of the Centurion Declaration in 2003, which led to the formation of AAMPS, no less than 15 magazine articles have been published and 10 conference presentations were made confirming the importance of AAMPS to the African health care and pharmaceutical industry.

4. Expertise and operational capacity:

4.1 The experience of your organisation in project management?

AAMPS is a new organisation and hence has limited experience in project management. However, its partner has extensive experience in this field [See http://www.criaasadc.org].

The chairman of the board of AAMPS has more than a decade of experience managing the 8 National Botanical Gardens in South Africa and the Research Directorate of the National Botanical Institute in South Africa with a staff of some 600 including more than 100 scientists. He recently wrote a business plan, managed and completed a €300,000 research project within two years. He has completed several management courses and has managed the first phase of the AAMPS project with funding from CDE and CTA successfully.

Prof Ameenah Gurib-Fakim from the University of Reduit in Mauritius is currently Deputy Provost Chancellor of the University and handles many management matters in addition to several research grants and consultancy contracts every year.

The other associates have multinational businesses which include complex technical and management tasks.

4.2. What is the experience of your organisation and your partner(s) on the issues to be addressed?

AAMPS's members were selected because they are the experts in the field of the identification, analysis, cultivation, processing, quality assurance and marketing of African medicinal plants. The members and associates of AAMPS between them represent probably the most expert group of people in the field of African medicinal plants. Their technical skills are substantial.

AAMPS members, however, lack experience in the organisation and running of pan-African trade associations. The proposed actions are designed to rectify this weakness and give AAMPS the administrative strengths it needs to effectively run its technical programmes.

If AAMPS is not properly managed and does not have sufficient funds to organize itself, it would be impossible to attain its admirable aims.

AAMPS has limited managerial and administrative skills. Our partner, the Centre for Research Information Action in Africa Southern Africa Developing and Consulting [CRIAA SA-DC] (www.criaasadc.org) will play a very important role in this context. Their purpose is to support sustainable and democratic development through:

- Strengthening the capacities of national, regional and local operating bodies including government departments, local authorities, non-governmental organisations and producers' organisations in the private sector (including co-operatives)
- Contributing to the capacity of marginalised communities or members of society to improve their livelihoods.
- CRIAA SA-DC in Namibia undertakes applied research and consultancies in programme and project appraisal, monitoring, evaluation and management in the following sectors:
    - Agricultural and rural development, post-harvest research and development of botanical resources linking producers and markets
    - Primary producers' issues - from natural resource management to sustainable utilization
    - Informal sector, small-scale industry, technology and skills development
    - Producers' organisation capacity building and co-operative development

The last three items indicate that their expertise is closely related to AAMPS objectives. This is why they were also represented at the meeting when AAMPS was conceived (www.aamps.org).

From their website (www.criaasadc.org) it can be seen that CRIAA SA-DC has many collaborators all over the world and that they have obtained funding from many international funding agencies. Their experience in helping manage an organisation like AAMPS will be invaluable.

FULL APPLICATION FORM

I. THE ACTION

1. DESCRIPTION

1.1 Title

Institutional strengthening of the Assocuaition for African Medicinal Plant Standards

1.2 Location(s)

Africa-wide especially Botswana, Ethiopia, Ghana, Kenya, Madagascar, Mali, Mauritius, Namibia, Nigeria, South Africa, Tanzania and Uganda.

1.3 Cost of the action and amount requested from the Contracting Authority

Total eligible cost of the action	Amount requested from Pro€Invest	% of total eligible cost of action
EUR 124,931.52	EUR 83,704.12	67%

1.4 Summary

Duration of the action	10 months
Objectives of the action	Overall objective(s) This proposed action is a capacity building exercise designed to strengthen the financial, administrative and technical skills of AAMPS to assist to carry out its main function which is to select, prepare, edit, publish and disseminate quality assurance standards for medicinal plants and herbal medicines and to develop support services to ensure that these standards are used nationally, regionally and internationally. Specific objective To prepare a business plan, an interactive web site and to finalise the publication of trading standards for 50 of Africa's leading herbals to provide the foundation for the sustainable development of this Africa wide specialist trade association.
Partner(s)	Centre for Research, Information, Action in Africa: Southern Africa Development and Consulting (CRIAA SA-DC), Windhoek, Namibia
Target group(s)	Regulators, manufacturers, researchers into African medicinal plants
Final beneficiaries	Growers, traders, healers and consumers of herbal medicines
Estimated results	The widespread dissemination of the first Africa-wide set of medicinal plant trading standards, the strengthening of management and finance
Main activities	The proposed action includes A Preparing a detailed 5 year business plan outlining strategic planning, focussing on potential sources of revenue, financial sustainability for AAMPS, recommendations on membership recruitment, administration and international promotion. B Organising an international workshop to discuss and approve the business plan and to authenticate and promote the 50 herbal profiles presently being prepared by AAMPS in order to increase the revenue of AAMPS either through the sale of this information or through the raising of membership fees.

	C Upgrading and expansion of the AAMPS web site and the preparation of a range of promotional materials designed to increase the number of members of AAMPS and to enhance communications and encourage a sense of identity between existing members of this organisation. D Investigate other opportunities of fundraising e.g. by a royalty paid by subcontractors for quality control or research projects commissioned by Industry and allocated to subcontractors by AAMPS. AAMPS is registered in Mauritius and holds the intellectual property rights to all the documentation prepared under its auspices. This action will complement interventions already supported by CDE and CTA, which have helped finance the selection, research, laboratory testing and preparation of the final drafts for the 50 most important African medicinal plant species standards which will be completed by the end of the first half of 2007. Through an efficient AAMPS, this project would encourage the trade in African medicinal plants across international borders, lead to substantial value addition of medicinal plants, create jobs for people collecting or growing medicinal plants, alleviate poverty of poor rural communities, encourage the effective use of unexploited medicinal plants worldwide and increase the human armoury against diseases.

1.5 Objectives

This proposed action is a capacity-building exercise designed to strengthen the financial, administrative and technical skills of the association to carry out its main function, which is to select, prepare, edit, publish and disseminate quality assurance standards for medicinal plants and herbal medicines and to develop support services to ensure that these standards are used nationally, regionally and internationally.

Without the development and promotion of such standards, the development of safe and efficacious herbal drugs and dietary supplements for national, regional and international sale will not be possible. Africa presently accounts for less than 5% of the turnover of this multibillion dollar industry but accounts for some 30% of the world's plant biodiversity.

AAMPS already has members from all across Africa as well as from Europe, Asia and North America. In order to expand its membership network and enhance its revenue, it urgently needs to strengthen its capacity to recruit members and raise other forms of income so that the organisation can become self-sustaining.

1.6 Justification

The Pro€invest BDS programme has as its purpose the promotion, on a regional basis, of sustainable and environmentally friendly investment and inter-enterprise co-operation agreements (I&ICAs) in key sectors to increase the competitiveness of the ACP economies.
The AAMPS programme fits ideally into this concept as it aims to provide:

1. Employment opportunities for those in the collection, processing and exporting of herbals
2. Export revenue for those cultivating, collecting and distributing herbals
3. Improved health and welfare to consumers of herbal medicines (safety and efficacy)
4. Income generation for those growing, manufacturing & selling herbal products
5. Regional pan African co-operation in a sector where there is hardly any co-operation
6. Greater effectiveness for Africa's only trade association devoted to quality assurance of African herbal products
7. Greater harmonisation of African trading standards in the field of herbal extracts and phyto-medicines
8. Improved access to EU and other international markets through the development of internationally recognised trading and quality control standards

1.6.1 Identification of perceived needs and constraints in the target countries, in particular in the region(s) concerned.

One of the major constraints identified at the 2001 Commonwealth/CDE medicinal plants conference held in Cape Town was the lack of suitable technical profiles and quality control standards for African medicinal plants and extracts. This makes it extremely difficult for buyers, whether local or overseas, to compare batches of product from different places or from year to year. This is in marked contrast with other major regions of the world like China and the Indian subcontinent where traditional formulations have been recorded and evaluated both at local and national level. Consequently the level of world trade in Indian and Chinese medicinal plants and extracts is far more extensive than those of the African region.

Furthermore, without some well-documented information on the safety, efficacy and phytochemical characteristics of different compounds it is difficult for external buyers to make any accurate assessment of the likely utility or value of some new raw materials and extracts of African origin.

1.6.2 Description of the target group(s) and final beneficiaries and estimated number

- AAMPS existing members: 20
- AAMPS potential members: approximately 300
- Regulatory and official organisation involved in quality assurance for herbals: 500
- Scientists and researchers working on African medicinal plants and herbal medicine: 5000
- African herbal product manufacturers and traders: 2000
- European importers and formulators of African herbal products: 200
- African traditional healers:100000

1.6.3 Reasons for the selection of the target group(s) and identification of their needs and constraints. How does the Action contribute to the needs of the target group(s) and final beneficiaries?

The formation of AAMPS was a spontaneous action of a group of Africa's leading experts in the field of medicinal plants and herbal medicines' who met in Centurion in 2005 and prepared the Centurion Declaration which provides the "manifesto" for the organisation:

> We the undersigned, with a view to improving the health, welfare and livelihood of the people's of Africa, hereby declare:
>
> - To establish an Association with a registered office in Mauritius to support the African herbal industry and regulatory authorities by developing quality control and quality assurance standards for African medicinal plants and herbal medicines.
> - To offer membership of the newly formed association to any individual or organisations dedicated to the establishment of such standards and to the creation of an African Herbal Pharmacopoeia
> - To jointly review and promote the 23 African herbal profiles presently being prepared under the leadership of the Phytomedicine Programme, University of Pretoria. These herbal profiles include plants of African origin which are considered of regional and international importance that can be sustainably sourced in Africa.
> - To raise funds to prepare and disseminate a further 30 African herbal profiles selected by the founding members of the association at the Centurion Lake Hotel review meeting on May 2005.
> - To prepare and publish an African Herbal Pharmacopoeia based upon the c. 50 herbal profiles and to promote its use nationally and internationally
> - To help obtain international validation for these herbal standards and the subsequent herbal pharmacopoeia and to lobby health authorities throughout Africa to use such standards as the basis for licensing safe and effective herbal medicines in Africa
> - To promote capacity building in Africa for the establishment of regional training centres for certification, compliance and quality control of herbal medicines.
> - To promote the safe, sustainable national and international trade in the 50 profiled African medicinal plants
> - To carry out any other activities deemed by the members of the association as required to further its objectives.

1.7 Detailed description of activities

A Publication of the final 50 herbal profiles designed to help companies and organisations grow, process and prepare products according to the AAMPS standards (GAP, GMPs etc.). Some materials will be made available in electronic format only, others will be printed and available electronically.
This unique collection of quality assurance standards will be the baseline product for all other services and activities of AAMPS. Once these standards are prepared, AAMPS will aim to develop these into a truly African Herbal Pharmacopoeia which can form the basis of drug legislation throughout the region.
This work will be undertaken by AAMPS' own editorial committee.

B Upgrading and expansion of the association website (see www.aamps.net) and the preparation of a range of promotional materials designed to increase the number of members of AAMPS and to enhance communications and a sense of identity between existing members of this organisation. A basic website has already been prepared by AAMPS to alert members and potential members to the existence of this new association. If this proposal is approved, AAMPS aims to substantially upgrade the website so as to make it the main "shop window" for the organisation and moreover, a platform where members can interact with each other.
The public access part of the website will include all the information and forms necessary to join AAMPS. It will include some public access information on the organisation and simple presentations of each of the 50 herbal profiles prepared.
There will be a further area for sponsored products and services and items for sale.
The membership only area will be accessed by a password. This will enable members to download the full set of standards and any updates, provide a discussion area where members can interact as well as a bulletin board and newsletter.
This work will be done by a specialist website developer working closely with AAMPS management team.

C Preparation of a detailed 5 year business plan, outlining potential sources of revenue and expenditure and identifying the best way to achieve sustainability for AAMPS. The plan will also make recommendations on membership recruitment, administration and international promotion.
It is critical that AAMPS becomes a self-sustaining organisation within 2 years. The members are confident that this can be done if a well-structured business plan is prepared which provides the road map for the organisation for the next 5 years.
AAMPS has many advantages over other organisations in this respect because:

- It is a very specialised and focused organisation concentrating on the selection, preparation, dissemination, enforcement of quality control standards and the support training and promotion services needed to implement such standards.
- Apart from membership income AAMPS intends to have a wide range of saleable services for members as well as non members. These include a) sale of standards, b) certification and authentication services, c) specialist consultancy services, d) training workshops on enforcement of standards, e) laboratory testing and analysis, f) herbal photo library, g) text books.

The business plan specialist who will advise AAMPS on the business plan has a great deal of experience in setting up and running trade associations as well as extensive knowledge of project management in Africa. This plan will be prepared by a consultant working closely with the AAMPS management as well as CRIAA SA-DC. Our lead partner CRIAA SA-DC, an association of consultants and specialist companies working in the environmental and natural product field, also has an excellent background in this area. The consultant will visit or consult with AAMPS Board members prior to preparing this plan.

D Organising an international workshop in Windhoek, the headquarters of our main partner. CRIAA SA-DC will draw upon their widespread expertise in the management of both training and promotional workshops and international conferences. The workshop will have six main functions:

- To authenticate and promote the 50 herbal profiles currently being prepared by the association.

- To evaluate and approve the proposed business plan to allow finalisation for the subsequent membership drive and marketing campaign in 2008.
- To evaluate and approve the revised website and electronic communications system.
- To allocate tasks and responsibilities and approve any structural and organisational changes recommended in the business plan.
- To evaluate and approve the final profile drafts and to begin to disseminate them electronically and by other means.
- To draw world-wide attention to achievements of AAMPS, gain recognition by COMESA, ECOWAS and international WHO, WTO advisory bodies in the field of standards.
- To discuss any possible changes to AAMPS constitution if required by the 5 year business plan.

The organization will be the prime responsibility of CRIAA SA-DC our partners. They have extensive experience of holding both national and international conferences and workshops and staff who can handle the logistical issues.

1.8 Methodology

1.8.1 Methods of implementation and reasons for the proposed methodology

- Commission experts, consult on business plan, prepare draft business plan
- Draft TOR for revamp of website, revamp of website
- Draft TOR for review & edit of profiles, edit and review final profile drafts, visit key regional AAMPS research centres, crosscheck laboratory analysis
- Prepare consultation workshop, hold workshop
- Prepare workshop accounts, compile interim report to Pro€invest
- Revise & finalise business plan
- Prepare and print final profiles, launch profiles and other data on website
- Prepare final accounts, prepare final report
- Build up membership drive, develop accreditation service
- Sell profiles, update living data base, upgrade profiles to legal status
- Hold GMP/GAP training workshops
- Hold laboratory analysis training workshops

1.8.2 Not applicable

1.8.3 Where the action is the prolongation of a previous action, explain how the action is intended to build on the results of this previous action

AAMPS has already been a recipient of both CDE and CTA funding to select, research, review and prepare the first 21 herbal profiles during 2004/2005. Moreover, CDE has approved funds for the research and preparation of the remaining c. 30 profiles. The Pro€invest project, while not concerned with the research and preparation of the remaining profiles, will directly support these earlier initiatives by providing the resources required to review, print and publish these materials and a framework to disseminate the results worldwide.

1.8.4 Where the action is part of a larger programme, explain how it fits or is coordinated with this programme. Please specify the potential synergies with other initiatives, in particular from the EC

Once the Pro€invest project is completed AAMPS will be able to embark on a range of coordinated initiatives designed to

- gain international recognition for their standards,
- assist companies and regional support organisations achieve these standards
- expand and broaden these standards so they can become a true African Pharmacopoeia

1.8.5 Procedures for follow up and internal/external evaluation

The aim of the business plan, website and finished profiles is to provide the successful foundation and tools needed to recruit members, sell products and obtain other forms of financial support. A major programme to promote AAMPS will begin once the Pro€invest project is completed (see Action plan). We have budgeted for an external financial audit of the project.

The workshop itself will be a review meeting where many AAMPS members and other experts will review the business plan, draft profiles and electronic database and advise on how it can be upgraded and improved.

1.8.6 Description of the role and participation in the action of the various actors (local partner, target groups, local authorities, etc.), and the reasons for which these roles have been assigned to them.

AAMPS management board, especially the Chairman and the Treasurer, will together be the main coordinators of this project. They will recruit and monitor consultants, supervise the editing and publication of the profiles and visit the members and the key support organisations to ensure that AAMPS builds a strong Africa-wide framework for the future.

AAMPS members and associates are already involved in the preparation of the draft profiles and the dissemination of the AAMPS philosophy. Once again, they will be expected to act as an editorial board for the new materials and five of them will be selected as specialist speakers at the Windhoek evaluation workshop.

AAMPS short term consultants will be specialists in the field of African medicinal plants who know the aims and objectives of AAMPS. All contracts are for less than 15 days, see list of team members.

CRIAA SA-DC management board and staff will play an important role in the project by taking primary responsibility for organisation of the evaluation workshop. They will also assist AAMPS on all matters of accounting and management of the project, lending it their long years of experience of handling overseas aid funds.

AAMPS already has strong links with many **major research and development organisations,** such as the University of Pretoria, the University of Mauritius and the University of Antwerp. These organisations will all be providing technical support to the project and play a vital role in increasing the credibility of AAMPS worldwide.

1.8.7 Team proposed for implementation of the action (by function: there is no need to include the names of individuals here)

Team	**Experience**	**Man days**
AAMPS / Pro€invest Project Coordinator – To coordinate all aspects of the project and to liaise with Pro€invest and all other official bodies involved in project.	Extensive project management experience	28 (AAMPS budget contribution)
AAMPS Treasurer – to keep track of all financial matters relating to this project and prepare necessary financial accounts – liaise with auditors.	Basic bookkeeping/accounts	21 (AAMPS budget contribution)
AAMPS Project Secretary	Administrative, logistical and secretarial skills	42 (AAMPS budget contribution)
CRIAA SA-DC Management Board Executive Committee (ExCo) members – As main partner in the project to assist AAMPS in all aspects of the project especially the planning and organisation of the workshop.	Extensive project management experience	28 (CRIAA budget contribution)
CRIAA SA-DC administrative & finance manager – To assist CRIAA SA-DC and AAMPS coordinator with all aspects of the project financial administration and particularly to those related to hosting of the workshop.	Experience of financial administration and organising international workshops and seminars	42 (CRIAA budget contribution)
Business Plan expert – will review existing operations of AAMPS and prepare a viable 3 year business plan including all aspects of fundraising and income generation, present the plan at workshop and incorporate member feedback.	Trade association Natural Product Industry African Plant research Fund raising and promotion	12 (Contractors)
IT specialist – upgrades existing web site to include password access, newsletter and advertising facilities and ecommerce opportunities.	Web site development Ecommerce Natural product industry	15 (Contractors)

Uploads all the herbal profiles so the site can become a living database of African herbals.		
Plant Chemist – will review all the samples submitted as part of the trade specification draft documents and assess the quality and authenticity of the samples provided.	Phytochemistry Laboratory analysis Botanical identification	15 (Contractors)
West African Anglophone speaker – prepare presentation on status of herbal quality assurance standards in region.	Knowledge of herbal drugs regulations and standards	3 (Contractors)
West African Francophone speaker – ditto.	ditto	3 (Contractors)
East African speaker – ditto.	ditto	3 (Contractors)
Southern Africa Speaker – ditto.	ditto	3 (Contractors)
EU speaker – ditto.	ditto	3 (Contractors)

1.8.8 Main means proposed for implementation of the action (equipment, tools…)

The only specialist equipment required to complete the action will be analytical laboratory equipment in order to crosscheck the results* provided in the draft final profiles. Access to specialist library facilities may also be needed. Both are being provided by the University of Pretoria or other associated AAMPS facilities as a contribution in kind.

* Note that this intervention does not involve detailed biochemical analysis but the crosschecking of results prepared as part of previous research work not funded under the AAMPS Pro€invest intervention.

1.9 Duration and action plan

The duration of the action will be 10 months. Planned activities (in months):

Activity	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Implementing body**
Commission Experts	■	■									AAMPS
Consultations on Business plan		■	■	■							AAMPS
Preparation of draft business plan				■	■	■	■				AAMPS
Draft TOR for revamp of website	■	■									AAMPS
Revamp of website		■	■	■	■	■					AAMPS
Draft TOR for review & edit of profiles	■	■									AAMPS
Visit key regional AAMPS research centres				■	■						AAMPS
Edit and review final profile drafts		■	■	■	■						AAMPS
Crosscheck laboratory analysis				■	■						AAMPS
Preparation of consultation workshop			■	■	■						CRIAA SA-DC
Holding of workshop						■					CRIAA SA-DC
Preparation of workshop accounts							■				CRIAA SA-DC
Interim report to Pro€invest							■				AAMPS
Revise & finalise business plan								■	■		AAMPS Consultants
Prepare and print final profiles								■	■	■	AAMPS
Launch profiles and other data on website								■	■	■	AAMPS
Prepare final accounts									■	■	CRIAA/AAMPS
Prepare final report									■	■	CRIAA/AAMPS

Activities planned for the following 5 years (in semesters):

Activity	3	4	5	6	7	8	9	10	Implementing body
Membership drive	■	■	■						AAMPS
Sales of profiles		■	■	■					AAMPS
Develop accreditation service		■	■	■	■	■	■	■	AAMPS/CRIAA
Updating of living data base		■	■	■					AAMPS/other specialist agencies
GMP/GAP training workshops			■		■		■		AAMPS/CRIAA/UNIDO/ICS
Lab analysis training workshops				■		■			AAMPS/UNIDO/ICS
Upgrading of profiles to legal status		■	■	■	■	■	■	■	AAMPS, WHO, national regulators

2. EXPECTED RESULTS

2.1 Expected impact on target groups/beneficiaries

2.1.1 the situation of target groups/beneficiaries

The first set of accurate, up-to-date and comprehensive quality control standards will be made available not just to AAMPS members but also manufacturers, healers, traders, research staff involved with African medicinal plants and herbal medicine worldwide.

2.1.2 the technical and management capacities of target groups and/or any partners where applicable.

AAMPS will be much better equipped to serve its members and to generate its own sustainable income flow once this project has been completed. The business plan will form the road map for the next 2 years of the work for AAMPS. The review meeting will help bring together people from all over Africa who otherwise do not get an opportunity to develop common strategies for the African herbal industry.

2.2 Concrete outputs

- Publication of 50 herbal profiles / trading standards of the top African herbals
- Completion of an interactive living data base of these and other plants
- Completion of an upgrade of the Association web site as the main shop window for AAMPS
- Completion of 5 year action orientated business plan which will act as the road map for AAMPS

2.3 Multiplier effects

This project has excellent opportunities for replication, by

a) expanding the number of herbal profiles prepared (there are at least 400 key species in Africa),
b) beginning to prepare standards for multi herb formulations and other finished products,
c) organising a series of regional training workshop to help companies and organisation reach the required standards,
d) building an Africa-wide network of herbal testing services drawing upon the very best of Africa's scientific expertise in this field,
e) preparing the first African Herbal Pharmacopoeia,
f) undertaking a continent-wide lobbying programme to get herbal products based upon these safety standards incorporated into the primary health care systems of African countries,
g) organising international conferences to promote African herbals throughout the world.

2.4 Sustainability

2.4.1 The financial aspect (how will activities be financed when the grant ends?)

The business plan, which will be prepared as part of this proposal, will review this issue in depth. Meanwhile the main areas for financial sustainability for AAMPS are

1) Membership fees
2) Sale of profiles and other technical information to non members
3) Sale of certification and product-testing services

4) Specialist consultancy services in Good Agricultural Practices (GAP), Good Manufacturing Practices(GMP) and Good Laboratory Practices (GLP) for herbals and phytopharmaceuticals
5) Organising of specialist international conferences and sale of conference proceedings on quality assurance and related matters
6) Sale of consumer products like medicinal plant calendars, photographs , illustrated booklets
7) Grants from agencies such as CDE, Gates Foundation, UNIDO, National Governments

2.4.2 Institutional level (Will structures allowing the activities to continue be in place at the end of the action? Will there be local "ownership" of action outcomes?)

Although AAMPS is a new organisation, its membership consists of some of Africa's most experienced scientists and businessmen in the field of natural medicine. With the help of this grant and our very experienced partners CRIAA SA-DC we aim to "weld" AAMPS into a truly Africa wide association with real international "clout" and influence. (Our model is similar to that of ESCOP – European Scientific Cooperative on Phytotherapy (http://www.escop.com/) which has now become seen by the EU and national governments as the definitive source of knowledge and advice on the safety and efficacy of herbal products)

2.4.3 Policy level where applicable (What structural impact will the action have - e.g. will it lead to improved legislation, codes of conduct, methods, etc?)

AAMPS has a specific remit to change national and regional policy in the sphere of herbals and natural medicine. It aims to provide the scientific information needed by government and international agencies to make clear judgements about the pros and cons of herbal medicine. It will encourage integration of herbal medicine and traditional healing into the health system .Currently, despite the fact that 90% of Africans rely on herbal medicine, governments in only a handful of African countries give official recognition and support to this sector. AAMPS aims to change this.

2.5 Logical framework

not required as budget is under 100,000 euros

3. BUDGET FOR THE ACTION

N°	Type of costs	Unit	Unit cost (Euros)	Quant.	Total cost
1	**HUMAN RESOURCES**				
1.1	**Contracted expert Fees**				**16.770,00**
1.1.1	Business Plan expert	Man/day	410,00	12,00	4.920,00
1.1.2	IT specialist	Man/Day	320,00	15,00	4.800,00
1.1.3	Analytical chemist/Laboratory specialist	Man/day	320,00	15,00	4.800,00
1.1.4	West Africa speaker - anglophone	Man/day	150,00	3,00	450,00
1.1.5	West Africa speaker - francophone	Man/day	150,00	3,00	450,00
1.1.6	East African speaker - anglophone	Man/day	150,00	3,00	450,00
1.1.7	Southern African speaker - anglophone	Man/day	150,00	3,00	450,00
1.1.8	European speaker - regulatory affairs	Man/day	150,00	3,00	450,00
1.2	**Full cost estimate for beneficiary's staff allocated to the project**				**31.325,00**
1.2.1	Chairman	Man/day	375,00	28,00	10.500,00
1.2.2	Treasurer	Man/day	375,00	21,00	7.875,00
1.2.3	Secretrary	Man/day	75,00	42,00	3.150,00
1.2.4	CRIAA SA-DC Management Board Executive Committee (ExCo) members	Man/day	350,00	28,00	9.800,00
1.2.5	CRIAA SA-DC Administrative and Financial Manager	Man/Day	75,00	42,00	3.150,00
1.3	**ACP Participants' contribution**				**2.400,00**
	4 participants x 3 days	Man/day	200,00	12,00	2.400,00
	TOTAL 1				**50.495,00**
2	**PER DIEMS AND OTHER FIXED COSTS**				
2.1	**Per diem for external experts**				**2.754,00**
2.1.1	Country : Mauritius	day	201,00	4,00	804,00
2.1.2	Country : South Africa	day	150,00	5,00	750,00

2.1.3	Country : Windhoek 3 days x 5 speakers	day	80,00	15,00	1.200,00
2.2.	**Per diem for organisers (benificiary and partners)**				**4.172,00**
2.2.1	Country : Ghana	day	167,00	2,00	334,00
2.2.2	Country : South Africa	day	150,00	10,00	1.500,00
2.2.3	Country : Namibia 2 people	day	80,00	10,00	800,00
2.2.4	Country : Mauritius	day	201,00	2,00	402,00
2.2.5	Country : Mali	day	236,00	2,00	472,00
2.2.6	Country : Kenia	day	166,00	4,00	664,00
2.3	**Per diem ACP Participants (IOs only) 4 people**	**day**	**80,00**	**12,00**	**960,00**
2.4	**Telecommunications and mail - 2 offices**	**sum/month**	**250,00**	**10,00**	**2.500,00**
2.5	**Office supplies and consumables - 2 offices**	**sum/month**	**150,00**	**10,00**	**1.500,00**
	TOTAL 2				**11.886,00**
3	**REIMBURSABLE COSTS**				
3.1	**Transport costs for external experts**				**10.450,00**
3.1.1	London-Mauritius-London - 1 ticket business plan expert	trip	1.000,00	1,00	1.000,00
3.1.2	London-Johannesburg-Windhoek-London - 2 tickets business plan	trip	1.400,00	2,00	2.800,00
3.1.3	West Africa speaker - anglophone	trip	1.500,00	1,00	1.500,00
3.1.4	West Africa speaker - francophone	trip	1.500,00	1,00	1.500,00
3.1.5	East African speaker - anglophone	trip	1.500,00	1,00	1.500,00
3.1.6	Southern African speaker - anglophone	trip	750,00	1,00	750,00
3.1.7	European speaker - regulatory affairs	trip	1.400,00	1,00	1.400,00
3.2	**Transport costs for organisers (beneficiary and partners)**				**7.000,00**
3.2.1	Johannesburg-Windhoek-Johannesburg	trip	250,00	6,00	1.500,00
3.2.2	Berlin-Johannesburg-Windhoek-Berlin	trip	1.400,00	2,00	2.800,00
3.2.3	Johannesburg-Accra-Bamako-Johannesburg	trip	1.500,00	1,00	1.500,00
3.2.4	Johannesburg-Mauritius-Johannesburg	trip	600,00	2,00	1.200,00
3.3	**Transport costs for participants from invited IOs + Public Authorities**				**4.800,00**
3.3.1	Bamako-Windhoek-Bamako - 1 person	trip	1.600,00	1,00	1.600,00
3.3.2	Johannesburg-Windhoek-Johannesburg - 2 people	trip	250,00	2,00	500,00
3.3.3	Kampala-Jbrg-Windhoek-Kampala	trip	1.200,00	1,00	1.200,00
3.3.4	Abuja-Winhoek-Abuja - 1 person	trip	1.500,00	1,00	1.500,00
3.4	**Transport costs for participants from invited enterprises**				**0,00**
3.5	**Organisation costs**				**25.665,00**
3.5.1	Documents, leaflets, reports: editing, layout and print of 50 herbal profiles		300,00	50,00	15.000,00
3.5.2	Internet site, CD-Roms, rebuild and upgrade to ecommerce operation		2.875,00	1,00	2.875,00
3.5.3	Room hire, furnishing, posters, catering, rental of workshop space		2.000,00	1,00	2.000,00
3.5.4	Equipment rental, local transport organised for participants during event		750,00	1,00	750,00
3.5.5	Temporary personnel (translators, interpretors, secretaries, hushers,...)		90,00	56,00	5.040,00
	TOTAL 3				**47.915,00**
4	**OTHER COSTS**				
4.1	Auditing fees		1.000,00	1,00	1.000,00
4.2	Financial costs (bank charges)		250,00	1,00	250,00
4.3	Other : (please specify) B ook keeping				
	TOTAL 4				**1.250,00**
	SUBTOTAL DIRECT PROJECT COSTS				**111.546,00**
5	**OVERHEADS**				
5.1	fixed percentage (maximum 7%) of total direct eligible costs (items 1-4)	%	7,0%	111.546,00	7.808,22
6	**CONTINGENCIES(*)**				
6.1	fixed percentage (maximum 5%) of total direct eligible costs (items 1-4)	%	5,0%	111.546,00	5.577,30
	TOTAL COSTS				**124.931,52**

4. EXPECTED SOURCES OF FUNDING

	SOURCE OF FUNDING	Amount in Euro	% of total
	Beneficiary and partners contribution	33.725,00	26,99%
	Essential Nutrition UK	3.750,00	3,00%
	Parceval Pharmaceuticals	3.752,40	3,00%
	PROINVEST contribution sought (max 67 % of total cost)	83.704,12	67,00%
	TOTAL	**124.931,52**	**100%**

In-kind contributions

This project could not be completed without access to the following resources, which are being provided by AAMPS and CRIAA SA-DC members free of cost

- Library facilities
- Unpublished research data
- Collection and provision of botanical specimens
- Specialist analytical equipment
- Administrative support financed by own organization
- Knowledge and expertise gained over many decades

II. THE APPLICANT

1. IDENTITY

Full legal name :	Association for African Medicinal Plants Standards
Acronym :	AAMPS
Legal Entity Sheet number	Not applicable
Nationality:	Mauritian
Legal status	Trade association, Co. Ltd.
Official address:	c/o University of Mauritius, Reduit, Mauritius
Postal address:	c/o University of Mauritius, Reduit, Mauritius
Telephone number:	+230-4541041, ext. 1470
Fax number:	+230-4549642
E-mail of the Organisation:	info@aamps.org
Website of the Organisation:	www.aamps.org
Contact person for this action :	Prof. Kobus Eloff
Contact person's email address :	kobus.eloff@up.ac.za

2. BANK DETAILS

AAMPS Co. Ltd,
State Bank of Mauritius,
Reduit Branch, Reduit, Mauritius
Swift: STCBMUMU
Account # 62030100095508

AAMPS
Hypovereinsbank
Berlin, Germany
Swift: HYVEDEMM488
IBAN DE52100208906019938 48
Account # 601993848

3. DESCRIPTION OF APPLICANT

3.1 When was your organisation founded and when did it start its activities?

AAMPS was established with the signing of the Centurion declaration in May 2005. The Association CRIAA SA-DC was founded in 1996 and started its activities in Namibia in 1997.

3.2 What are the main activities of your organisation at present?

- Compilation of information on African medicinal plants
- Compilation of information on growers, traders and processors/manufacturers of African herbs
- Compilation of information on African regulatory systems
- Compilation of plant profiles and dissemination of this information
- Membership drive
- Development of quality test standards/methods
- Preparation of certification standards

CRIAA SA-DC undertakes in Namibia and in the SADC Region project management, applied research and consultancies in the following sectors:

- Agricultural and Rural Development, Post-Harvest Research and Development

- Natural Products Commercialisation
- Primary Producers' Issues - from Natural Resource Management to Sustainable Utilisation
- Small-Scale Industry, Technology and Skills Development
- Producers' Organisation Capacity Building and Co-operative Development

3.3 List of the management board / committee of your organisation

AAMPS

Name	Profession	Nationality	Position	Years on the board
Mr. Kobus Eloff	University Professor	RSA	Chairman	1.5
Mr. Thomas Brendler	Consultant	GER	Secretary/Treasurer	1.5
Mrs. Amenah Gurib-Fakim	University Professor	MRU	Director	1.5
Mr. Ben-Erik van Wyk	University Professor	RSA	Director	1.5
Mr. Ermias Dagne	University Professor	ETH	Director	1.5
Mrs. Marian Addy	University Professor	GHA	Director	0.5
Mr. Victor Attafua	Trader	GHA	Alternate Director	0.5
Mr. Ulrich Feiter	Manufacturer	RSA	Alternate Director	0.5

CRIAA SA-DC

Name	Profession	Nationality	Position	Years on the board
Mr Michel Mallet	Agricultural engineer	FRA	Executive Director	9
Mr David Cole	Sociologist	RSA	Deputy Executive Director	6
Mrs Josiane Leclercq	Entrepreneur	FRA		9
Ms Selma El Obeid	Agronomist	FRA		5
Ms Saskia Den Adel	Social Anthropologist	NED		4
Ms Jennifer Gatsi	Gender Programme Co-ordinator	ZBW		2
Mr Benoit Allanic	Urban Planner	FRA		9
Mr Roger Gamond	Technologist	FRA		5

4. CAPACITY TO MANAGE AND IMPLEMENT ACTIONS

4.1. Experience of similar actions

4.1.1 the object and location of the action

Preparation of first set of herbal profiles from South, Western and Eastern Africa

4.1.2 the results of the action

The final draft standards were prepared and evaluated by an international editorial committee, which met in South Africa to review the materials also launching of first basic web site

4.1.3 your organisation's role (lead manager or partner) and its degree of involvement in the action

Key AAMPS members including the University of Mauritius, University of Johannesburg,, BCDP, Nigeria, CRIAA SA-DC, Centre for Herbal Medicine in Mali were PARTNERS to this action which was undertaken by the Phytomedicine Programme, University of Pretoria seat of Chairman of AAMPS.

4.1.4 the cost of the action

Approximately €61,000 excluding contributions in kind.

4.1.5 donors to the action (name, amount contributed)

- University of Pretoria €7,500 euros (in kind)
- Essential Nutrition €5,000 euros
- Parceval Pharmaceuticals €5,000 euros

Please note CDE and CTA funded the main partner
CDE funded the Phytomedicine Programme, University of Pretoria with a grant of c. €41,000 euros
CTA funded the Phytomedicine Programme, University of Pretoria with a grant of c. €20,000 euros

4.2. Resources

4.2.1 Annual income over the last three years, mentioning where applicable for each year, the names of the main financial backers and the proportion of annual income each has contributed

AAMPS
The first financial year not completed, so no accounts or audit can be presented. Main sources of income are CDE/CTA funding, funds sponsored by commercial members and membership fees.
Estimated annual income for 2005/2006 is Euros 109,500 including

- CDE grant 85,000 euros (to be released)
- Membership Fees paid and expected 4.500 Euros
- Industry Sponsorship 10,000 euros
- Consultancy fees expected 10,000 euros

4.2.2 Financial data. Please provide the following information on the basis of the profit and loss account and balance sheet of your organisation

AAMPS
See 4.2.1

CRIAA SA-DC

Financial Year (Jan.-Dec.)	Turnover or equivalent	Net earnings or equivalent	Total balance sheet or budget	Shareholders' equity or equivalent	Medium and long-term debt	Short-term debt (< 1 year)
2005	Euros 609,804	Euros 9,100	Euros 236,666	N/A	None	None
2004	Euros 619,100	Euros 8,138	Euros 204,218	N/A	None	None
2003	Euros 731,682	Euros 86,521	Euros 218,354	N/A	None	None

CRIAA SA-DC

Year	Main financial backers	% of annual income
2005:		
	OXFAMs in Namibia	12
	NASSP & NR International (8 ACP NAM 023)	11
	Namibian Agronomic Board –NAB (MAWF funds)	10
	Commercial partners through PhytoTrade Africa	18
2004:		
	NAB (MAWRD funds)	16
	PhytoTrade Africa	15
	OXFAMs in Namibia	8
	French Cooperation	6
2003:		
	NAB (MAWRD funds)	18
	PhytoTrade Africa	16
	OXFAMs in Namibia	14
	International Commercial buyer	5

MAWF: Ministry of Agriculture, Water & Forestry (Namibia), previously Ministry of Agriculture, Water & Rural Development (MAWRD)

Any guarantees granted by third parties: **None**

Any other factors demonstrating financial viability and any risks or uncertainties about implementation: **Nothing that is not in our audited financial statements**

Furthermore, where the grant requested exceeds EUR 300 000 please provide the references of the external audit report established by an approved auditor. This obligation does not apply to international organisations nor to public bodies:

Saunderson Theron & Partners, 30 Lister Street, PO Box 24305, Windhoek, Namibia
Tel. +264 61 228858, Fax: +264 61 246306, e-mail: saunderson@acsec.com.na

4.2.3 The number of full-time and part-time staff by category (e.g. number of project managers, accountants, etc), indicating their place of employment

AAMPS

Category	Full-time	Part-time	Place of employment
Secretary	1		Pretoria
Chairman		1	Pretoria
Treasurer		1	Berlin
Technical/Scientific Directors		4	Johannesburg, Addis Ababa, Accra, Mauritius
Commercial Directors		2	Wellington, Accra

CRIAA SA-DC

Category	Full-time	Part-time	Place of employment
Project managers	5	1	Windhoek
Accounting & administrative staff	3	1	Windhoek
Field officers	1	1	Omaheke region
Volunteers	2	-	Windhoek (1 at NBRI)
Technical staff	3	-	Katutura
Total :	**14**	**3**	

4.2.4 Equipment and offices

AAMPS

Various offices of board and association members
Data base of key medicinal plants
Voucher specimens and other research data
Lab/research facilities in RSA, Ethiopia, Mauritius, Ghana, Mali, Botswana, UK, etc.
Access to manufacturing facilities in RSA, Kenya, Ghana, UK

CRIAA SA-DC

Land & buildings: Euros 80,962 (N$ 756 k)
Plant & equipment: Euros 5,140 (N$ 48 k)
Furniture & fittings: Euros 7,924 (N$ 74 k)
Vehicles: Euros 88,362 (N$ 825 k)
Field machinery & equipment: Euros 8,247 (N$ 77 k)
Electronic equipment: Euros 40,057 (N$ 374 k)

4.2.5 Other relevant resources (e.g. volunteers, associated organisations, networks that might also contribute to implementation).

AAMPS

Members and their organisations (various universities in and outside Africa), commercial ventures in Africa and Europe, scientific organisations and government agencies in Africa, Europe and the US (e.g. PROTA. ESCOP, USHP).

CRIAA SA-DC

Member of PhytoTrade Africa (The Southern Africa Natural Products Trade Association): technical and financial support, R&D and market information, market development and market linkage services
Member of the Devil's Claw Range State Working Group (Botswana, Namibia and South Africa): regional co-operation.
Member of the Devil's Claw Working Group in Namibia: a technical, financial and policy committee affiliated to IPTT, chaired by MET
Member of the Indigenous Plant Task Team (IPTT) in Namibia: national stakeholders' co-ordination body, guidance and financial contributions to research and development
Member of the National Plant Genetic Resources Committee of Namibia (NPGRComm), under the auspices of the National Botanical Research Institute (NBRI) of Namibia: national and regional strategy and co-ordination
Member of the National Programme Steering Committee (NPSC) of the Southern Africa Biodiversity Support Programme (SABSP), hosted by the Directorate of Environmental Affairs of the Ministry of Environment & Tourism (MET): national and regional strategy and co-ordination
Member of the Namibian Plant Sector Development Forum in Namibia (Public-Private Partnership initiated by MAWF), representing the Indigenous Plant sector: national strategy & action plans, co-ordination and financial resources
Invited member to the Integrated Community-based Ecosystem Management (ICEMA) Project Steering Committee of the Ministry of Environment and Tourism (MET) : co-ordination, guidance and support to Conservancies

5. OTHER APPLICATIONS MADE TO EUROPEAN INSTITUTIONS, THE EUROPEAN DEVELOPMENT FUND (EDF) AND EU MEMBER STATES

5.1 Grants, contracts and loans obtained over the last three years from European Institutions, the EDF and EU Member States.

AAMPS

Country of intervention	EC budget line, EDF or EU Member States	Amount (EUR)	Year obtained
South Africa	CDE ACP/0313/01/CP	40075	2005
South Africa	CTA	19924	2005

CRIAA SA-DC

Country of intervention	EC budget line, EDF or EU Member States	Amount (EUR)	Year obtained
Namibia	CS2006-116 French Cooperation	19 845	2006
Namibia	FFEM (AFD) French Government	139 200	2005
Namibia	FFEM (AFD) through MET	58 000	2005
Namibia	NASSP (8th EDF) NRInt. SC	60 000	2005
Namibia	NASSP (8th EDF) NRInt. SC	6 000	2004
Namibia	CS2004-0142 French Cooperation	20 000	2004
Namibia	NASSP (8th EDF) NRInt. SC	47 400	2003
Namibia	EC-B7-6000 through Oxfams in Namibia	186 450	2003
Namibia	Government of Finland	11 000	2003

NASSP: National Agricultural Support Services Programme (8 ACP NAM 023)
NRInt. SC: NR International service contracts

5.2 Grant applications submitted (or about to be submitted) to European Institutions, the EDF and EU Member States in the current year.

AAMPS

Country of intervention	EC budget line, EDF or EU Member States	Amount requested (EUR)
Africa wide (based in Mauritius)	Centre for Development of Enterprise	75,000

III. PARTNERS OF THE APPLICANT PARTICIPATING IN THE ACTION

1. DESCRIPTION OF THE PARTNERS

	Partner 1
Full legal name (business name)	The Centre for Research, Information, Action in Africa : Southern Africa - Development and Consulting (CRIAA SA-DC)
Nationality	Namibian
Legal status	Incorporated Association Not-For-Gain (Registration No. 21/97/069)
Official address	22 Johann Albrecht Street, P.O. Box 23778, Windhoek, Namibia
Contact person	Michel Mallet (Executive Director) & Dave Cole (Programme Coordinator)
Telephone number	+ 264 (0) 61 220117
Fax number	+ 264 (0) 61 232293
E-mail address	criaawhk@iafrica.com.na
Number of employees	17
Other relevant resources	▪ Government of Namibia (research projects and consultancies): Ministry of Agriculture Water & Forestry, Ministry of Environment & Tourism ▪ Namibian Agronomic Board (training and consultancies) ▪ PhytoTrade Africa (the Southern Africa Natural Products Trade Association) (technical R&D and marketing support)
Experience of similar actions, in relation to role in the implementation of the proposed action	▪ Piloting and implementing the “Sustainably Harvested Devil’s Claw” (SHDC) project in Namibia (Omaheke region) since 1997 to date ▪ Co-implementing as a local partner organisation of Intermòn (Spain) the “Omaheke Livelihood Project” co-funded by EC (ref. ONG/PVD/2002/020-819/NA 751) ▪ Organisation of the first Regional Devil’s Claw Conference (February 2002) ▪ Service provider to the Namibian “Indigenous Plant Task Team” (IPTT), a Namibian national stakeholder institution co-ordinating and financing interventions for the socio-economic promotion of botanicals for poverty alleviation and value-addition.
History of cooperation with the applicant	Key partner in the establishment of AAMPS (2003)
Role and involvement in preparing the proposed action	Direct consultation in conceptualisation & reviewing proposed action
Role and involvement in implementing the proposed action	Key administrative supporting role, as well as principal role in organising the AAMPS international evaluation / consultative workshop

2. PARTNERSHIP STATEMENT

I have read and approved the contents of the proposal submitted to Pro€Invest. I undertake to comply with the principles of good partnership practice.

Name:	M. Mallet
Organisation:	CRIAA SA-DC
Position:	Executive Director
Signature:	
Date and place:	9th November 2006, Windhoek, Namibia

IV. ASSOCIATES OF THE APPLICANT PARTICIPATING IN THE ACTION

1. DESCRIPTION OF THE ASSOCIATES

	Associate 1
Full legal name (business name)	Dept. of Chemistry, University of Mauritius
Nationality	Mauritian
Legal status	University
Official address	Reduit, Mauritius
Contact person	Prof. Ameenah Gurib-Fakim Pro Vice Chancellor
Telephone number	Tel: (230) 454 10 41; 465 6888
Fax number	Fax: (23) 465 1337; 454 9642
E-mail address	Email: fakima@uom.ac.mu;fakima@intnet.mu
Number of employees	20
Other relevant resources	Research and dissemination network for Indian Ocean Islands and East Africa
Experience of similar actions, in relation to role in the implementation of the proposed action	The department has already successfully carried out contracts for CDE, IPGRI, CIDA and SIDA.
History of cooperation with the applicant	Founder member of AAMPS, past secretary/treasurer
Role and involvement in preparing the proposed action	
Role and involvement in implementing the proposed action	cross-checking, reviewing, editing and disseminating herbal profiles

	Associate 2
Full legal name (business name)	Parceval Pty Ltd
Nationality	South African
Legal status	Limited Company
Official address	P.O.Box 158, Wellington 7654
Contact person	Mr. Ulrich Feiter

Telephone number	**27-21-8733895
Fax number	**27-21-8735955
E-mail address	ulrich.feiter@parceval.co.za
Number of employees	143
Other relevant resources	2 certified organic farms, production facility for complimentary medicines
Experience of similar actions, in relation to role in the implementation of the proposed action	Peer reviewer, sub-editor for herbal monographs AAMPS phase I
History of cooperation with the applicant	Founder Member and alternative director
Role and involvement in preparing the proposed action	
Role and involvement in implementing the proposed action	cross-checking, reviewing, editing and disseminating herbal profiles

	Associate 3
Full legal name (business name)	PlantaPhile
Nationality	German
Legal status	Freelancer
Official address	Immanuelkirchstr. 32, 10405 Berlin, Germany
Contact person	Thomas Brendler
Telephone number	+49-(0)30-44341943
Fax number	+49-(0)30-44341944
E-mail address	info@plantaphile.com
Number of employees	Varies, not full time
Other relevant resources	5-10 freelance personnel (technical, scientific, translators etc.)
Experience of similar actions, in relation to role in the implementation of the proposed action	Taking part in the organization in the initial and various follow-up AAMPS meeting, board meetings and AGM, taking part in the compiling, editing and (electronic) publishing of the initial 21 herbal monographs (AAMPS phase 1), helping to organize AHAM 2006, Nairobi
History of cooperation with the applicant	Founder member, board member

Role and involvement in preparing the proposed action	Organizer
Role and involvement in implementing the proposed action	Organizer

	Associate 4
Full legal name (business name)	Vicdoris Pharmaceuticals Ltd.
Nationality	Ghanaean
Legal status	Ltd. Company
Official address	71 Ring Road Central, POBox 15088, Accra-North, Ghana
Contact person	Victor Attafua
Telephone number	+233-21-227091 / 235145
Fax number	+233-21-220198
E-mail address	vicwad@myzipnet.com
Number of employees	30
Other relevant resources	
Experience of similar actions, in relation to role in the implementation of the proposed action	Trader in raw materials and African herbal products, long standing experience with local growing and collecting practices, quality standards and processing practices
History of cooperation with the applicant	Founder member of AAMPS, alternate director
Role and involvement in preparing the proposed action	-
Role and involvement in implementing the proposed action	Recommend plant species of commercial value, review and edit plant profiles

	Associate 5
Full legal name (business name)	Phytomedicine Programme, University of Pretoria
Nationality	South African
Legal status	University
Official address	Private Bag X04, Onderstepoort, 0110 South Africa
Contact person	Prof. J N Eloff Programme Leader
Telephone number	Tel: (+27) 12-529-8244
Fax number	Fax: (+27) 12-529-8525
E-mail address	Email: kobus.eloff@up.ac.za
Number of employees/post grad students	c. 38
Other relevant resources	Research on Medicinal Plants see http://www.up.ac.za/ academic/veterinary/depts_paracl_phyto.htm
Experience of similar actions, in relation to role in the implementation of the proposed action	The group has already successfully carried out contracts for CDE CTA. Experience in preparing Strategic Plans for National Botanic Gardens of South Africa and Research Strategic Plan for National Botanical Institute.
History of cooperation with the applicant	Founder member of AAMPS, leader chairman of Board
Role and involvement in preparing the proposed action	Wrote concept note and finalized application after input from partner and associates
Role and involvement in implementing the proposed action	Evaluating Strategic Plan and profiles

V. CHECKLIST

PRO€INVEST

Grant Application Form

Call for Proposals 2006 Demand Driven activities - Intermediary Organisations

ADMINISTRATIVE DATA	
Name of the Applicant	Association for African Medicinal Plants Standards
Nationality	Mauritius
Legal Entity Sheet number[1]	Not applicable
Legal status[2]	Non profit making Trade Association
Date of establishment of the organization	2005
Partner 1	Name: CRIAA SA-DC Nationality: Namibian Legal status: Non Profit Making

[1] If the applicant has already signed a contract with the European Commission

[2] E.g. non profit making, governmental body, international organisation…

BEFORE SENDING YOUR PROPOSAL, PLEASE CHECK THAT EACH OF THE FOLLOWING COMPONENTS IS COMPLETE AND RESPECTS THE FOLLOWING CRITERIA :	To be filled in by the applicant		To be filled in by the Contracting Authority	
	Yes	**No**	**Yes**	**No**
1. The correct grant application form, published for this call for proposals, has been used	x			
2. The proposal is typed and is in English or French,	x			
3. One original is included	x			
4. A floppy disk or Cd-Rom is enclosed	x			
5. Each partner has completed and signed a partnership statement and the statements are included (if any). Please indicate "Not applicable" (NA) if you have no partner	x			
6. The budget is presented in the format requested, is expressed in € and is enclosed	x			
7. The logical framework has been completed and is enclosed		x		
8. The duration of the action is equal to or lower than 12 months (the maximum allowed)	x			
9. The requested contribution is equal to or lower than the maximum allowed (refer to Guidelines)	x			
10. The requested contribution is equal to or lower than 67 % of the total eligible costs (maximum percentage allowed)	x			
11. The Declaration by the applicant has been filled in and has been signed	x			

Title of the Proposal	Institutional Strengthening of the Association for African Medicinal Plant Standards

VI. DECLARATION BY THE APPLICANT

A. The applicant declares that:

- It has the sources of financing and professional competence and qualifications specified in section 2.3of the Guidelines for Applicants.
- It undertakes to comply with the principles of good partnership practice foreseen in section III.2 of the grant application form.
- It is directly responsible for the preparation and management of the action with its partners, and is not acting as an intermediary.
- It and its partners do not fall in any of the categories (a) to (f) listed in section 2.1.1(2) of the Guidelines for Applicants.
- If selected, it is in a position to deliver immediately, upon request, the supporting documents stipulated under point 2.4 of the Guidelines for Applicants

Furthermore, the applicant declares that :	**To be filled in by the applicant**		**To be filled in by the Contracting Authority**	
	Yes	**No**	**Yes**	**No**
1. It is eligible in accordance with the criteria set out under point 2.1.1 of the guidelines.)	x			
2. Partner 1 is eligible (in accordance with the criteria set out under point 2.1.2 of the guidelines.) (if any)[3]	x			
3. Partner 2 is eligible (in accordance with the criteria set out under point 2.1.2 of the guidelines.) (if any) [4]				
4. Partner … is eligible (in accordance with the criteria set out under point 2.1.2 of the guidelines.) (if any) [5] **NB:** add as many rows as partners				

B. SIGNATURE:

I, the undersigned and person responsible in the applicant organisation for the proposal, certify that the information given in this Declaration is correct.

Date: 11 November 2006

Name: Prof Jacobus Nicolaas Eloff Signature:

Position: Chairman of AAMPS Board of Directors

[3] Please indicate "Not Applicable" (NA) if you have no partner
[4] Please indicate "Not Applicable" (NA) if you have no partner
[5] Please indicate "Not Applicable" (NA) if you have no partner

VII. ASSESSMENT GRID

(FOR THE USE OF THE CONTRACTING AUTHORITY ONLY)

	YES	NO
1. The Deadline has been respected		
2. The Application form satisfies all the criteria mentioned in the Checklist (Section V of the Grant application form).		
The verification of the Checklist has been conducted by …… On the ……..		
DECISION 1: The Committee has decided to recommend the Concept Note for Evaluation after having passed the Administrative check. (If not, reasons must be encoded in the Administrative check Grid in CRIS and in the Administrative Check report in CRIS).		
DECISION 2: The Committee has approved the Concept Note and decided to proceed with the evaluation of the full proposal after having pre-selected the best Concept Notes. (If not, reasons must be encoded in the Concept Note Evaluation Grid in CRIS – this includes the evaluation sheet for assessors and delegations, in the Concept Note Evaluation report and in the letters sent out to applicants.)		

	YES	NO
DECISION 3: A. The Committee has recommended the proposal for Eligibility verification after having been provisionally selected within the top ranked scored proposals and within the available financial envelope. (If not, reasons must be encoded in the Evaluation Grid in CRIS – this includes the evaluation sheet for experts and delegations, in the Evaluation report and in the letters sent out to applicants.)		
B. The Committee has recommended the proposal for Eligibility verification after having been put in the reserve list should any provisionally selected proposal fail to fulfil the eligibility verification, according to the top ranked scored proposals and within the available financial envelope. (If not, reasons must be encoded in the l Evaluation Grid in CRIS – this includes the evaluation sheet for experts and delegations, in the Technical Evaluation report and in the letters sent out to applicants.)		
3. The supporting documents listed hereunder, submitted according to the Guidelines (Section 2.2.5), satisfy all the eligibility criteria of the applicant and its partner(s) (if any).		

a. The applicant's statutes.		
b. The statutes or articles of association of <u>all partners</u> .		
c. The applicant's external audit report. (where applicable)[6]		
d. The Legal Entity Sheet (see annex D) is duly completed and signed by the applicant and is accompanied by the justifying documents requested.[7]		
e. A financial identification form conforming to the model attached at Annex E.		
f. Copy of the applicant's latest accounts.		
The assessment of the eligibility has been conducted by …… …On the …….		
<u>DECISION 4:</u> The Committee has selected the proposal for funding after having verified its eligibility according to the criteria stipulated in the Guidelines. (If not, reasons must be encoded in the Eligibility Verification Grid in CRIS, in the Eligibility Verification Report in CRIS and in the letters sent out to applicants.)		

[6] Please indicate "Not Applicable" (NA) if your grant does not request an audit report. (Grant < 300 000€ - Operating grant < 75 000€)

[7] To be inserted only where the Contracting Authority is a body of the European Commission