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.