2003

Pesticidal properties of Anacardium occide1ttale L. against Tribolium casta1teum (Hbst.)

Parveen, Shamima Akhter

University of Rajshahi

PESTICIDAL PROPERTIES OF *ANACARDIUM OCCIDENTALE* L. AGAINST *TRIBOLIUM CASTANEUM* (Hbst.)

A Thesis Submitted for the Degree of
Doctor of Philosophy
in the Department of Zoology, University of Rajshahi,
Bangladesh

Submitted by

Shamima Akhter Parveen

B.Sc. (Hons.), M.Sc.

Crop Protection and Toxicology Laboratory
Department of Zoology
University of Rajshahi
Rajshahi 6205, Bangladesh

December 2003

Dedicated
to
my beloved parents

Certificate

This is to certify that Mrs. Shamima Akhter Parveen has been working under my supervision. I am pleased to forward her thesis entitled, **"Pesticidal properties of *Anacardium occidentale* L. against *Tribolium castaneum* (Hbst.)"** which is the record of bonafide research carried out at the Crop Protection and Toxicology Laboratory of the Department of Zoology, Rajshahi University, Rajshahi. She has fulfilled all the requirements of the regulations relating to the nature and prescribed period of research for submission of thesis for the award of Ph. D. degree of Rajshahi University, Rajshahi.

30 DEC 2003

(Dr. M. Khalequzzaman)
Professor
Department of Zoology
Rajshahi University

Declaration

I hereby declare that the whole work now submitted as a thesis entitled **"Pesticidal properties of *Anacardium occidentale* L. against *Tribolium castaneum* (Hbst.)"** in the Department of Zoology, Rajshahi University for the degree of Doctor of Philosophy is the result of my own investigation. The thesis contains no material which has been accepted for the award of any other degree or diploma elsewhere, and, to the best of my knowledge, the thesis contains no material previously published or written by another person, except where due reference is made in the text.

Shamima Akhter Parveen.

December 30, 2003
Rajshahi University
Rajshahi

(Shamima Akhter Parveen)
Candidate

List of Abbreviations

cm^2	=	centimetre square.
%kill	=	Insects killed per cent.
Cr%	=	corrected mortality per cent.
$CHCl_3$	=	Chloroform.
Df	=	degree of freedom.
E. Pr	=	Empirical Probit.
Ex. Pr	=	Expected Probit.
et al.,	=	and others (author).
EtOAc	=	Ethyl acetate.
Fig.	=	Figure.
F. Pro	=	Final Probit.
h	=	hour (s).
i. e.	=	that is
Kg	=	kilogram (s).
KI	=	Number of insects killed.
l	=	litre (s).
LD_{50}	=	dose required to kill 50% of test organism.
Ldos	=	Log dose.
LC	=	Leaf extract in chloroform

LM	=	Leaf extract in methanol.
mg	=	milligram (s).
ml	=	millilitre.
MeOH	=	Methanol.
NSC	=	Nut-shell extract in chloroform.
NSM	=	Nut-shell extract in methanol.
Pet. Benzene	=	Petroleum Benzene.
R_f	=	Retention factor.
RBC	=	Root-bark extract in chloroform.
RBM	=	Root-bark extract in methanol.
SBC	=	Stem-bark extract in chloroform.
SBM	=	Stem-bark extract in methanol.
#U	=	Number of test insects used.
Wk pro	=	Working probit.
Weght	=	Weighting coefficient.
$\mu g/cm^2$	=	Microgram per centimetre square.
χ^2	=	Chi-squared.

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Abstract

Insecticidal properties of *Anacardium occidentale* L. were investigated. All $CHCl_3$ and MeOH extracts of leaves, nut-shells, root-bark and stem-bark extracts were tested against *Valsa ceratosperma* (Tode Ex Fries) and *Tribolium castaneum* (Hbst.). At a dose of 100 µg/spot on the chromatogram and the $CHCl_3$ extract of nut-shell, $CHCl_3$ and MeOH extracts of root-bark and $CHCl_3$ extract of the stem-bark showed highest activity against the test fungus, whereas $CHCl_3$ extract of leaf and MeOH extract of leaf and nut-shell showed no activity.

Both the $CHCl_3$ and MeOH extracts of nut-shells have been found strongly effective against the test insect *T. castaneum* while tested by topical application method. However, the MeOH extract of the root-bark was stronger compared to its chloroform extract. After the nut-shell and the root-bark extract stem-bark extracts were found active but the methanolic extract was less effective in this regard. In case of leaf, MeOH extract was stronger than the $CHCL_3$ extract showing a similarity like the root-bark extract. All the extracts (for both $CHCl_3$ and MeOH) of different parts of *A. occidentale*, viz. leaf, nut-shell, root-bark and stem-bark were subjected to surface film test against *T. castaneum* adults and no trace of activity for the within the given dose ranges was found.

The nut-shell and the root-bark were found promising since the topical application bioassay offered LD_{50} values 22.096 and 16.868 μg/insect (regression equations were Y= 2.287933 + 2.017423X and Y= 2.970249 + 1.654154X) for $CHCl_3$ and MeOH extracts with an exposure of 24 h which reached at 19.322 and 15.503 μg/insect (regression equations Y= 2.514534 + 1.932630X and Y= 3.140641 + 1.561937X) respectively after 48 hours of exposure. Nut-shell extracts offered LD_{50} values 18.892 and 19.777 μg/insect (Y= 2.577399 + 1.898174X and Y= 1.917841 + 2.377903X) which reached at 17.574 and 17.049 μg/insect (regression equations Y= 2.374147 + 2.109339X and Y= 2.254093 + 2.229367X) respectively for $CHCl_3$ and MeOH extracts. Stem-bark extracts extracted in the similar manner with the same solvents offered LD_{50} values 64.110 and 179.019μg/insect (Y= 0.8454337 + 2.299246X and Y= 3.27767 + 0.7644957X) which reached at 47.013 and 121.012μg/insect (regression equations Y= 1.080848 + 2.343688X and Y= 3.035686 + 0.9431001X) respectively. The leaf extracts of $CHCl_3$ and MeOH showed relatively weak activity compared to the root-bark, nut-shell and the stem-bark extracts while the LD_{50} values were 207.238 and 85.515 μg/insect (Y= 2.343019 + 1.146996X and Y= 2.669598 + 1.206187X) which reached at 248.284 and 56.535μg/insect (regression equations Y= 2.830691.+ 0.905786X and Y= 2.093695 + 1.658553X) respectively. In this case effect of exposure time affected a little, thus a mere change was traced so far in the LD_{50} values with no increase in their effect.

Repellency was found promisingly in the order of $CHCl_3$ extract of root-bark, MeOH extract of nut-shell, leaf root-bark and stem-bark extract and then $CHCl_3$ extracts of nut-shell, stem-bark and leaf respectively. The repellency was found for all the 8 extracts of the 4 different parts of the title plant and it could be arranged in the descending order as nut-shell (MeOH<root-bark ($CHCl_3$)<leaf (MeOH)<root-bark (MeOH)< nut-shell ($CHCl_3$) <stem-bark (MeOH) <leaf ($CHCl_3$) <stem-bark ($CHCl_3$)

while P-values were 4.69E-07 <7.35E-07 <6.33E-06 <3.63E-05 <0.0001 <0.0009 <0.02 <0.05 respectively in the above order.

Two compounds from the nut-shell extract were isolated and named NS-1 and NS-2. About 140mg of the NS-1 has been purified which was a light brownish oil and after Godin reagent spray it takes pink in color on the TLC. NS-2 was 48mg in amount and it was a dark brown oil. It gets violet in color after Godin reagent spray.

The first root-bark component RB-1 was 30mg and it was white crystal needles. After Godin it gets purple in color and the second one RB-2 was a reddish powder and only 5.7mg in amount, which gets violet in color after Godin reagent spray.

The stem-bark compounds isolated were SB-1 and SB-2 and were 12- and 39mg in amount respectively. SB-1 was a white crystal needles and similar to that of the white crystals form the root-bark as mentioned above as RB-1. After Godin reagent spray it was also purple in color like the RB-1 (and after taking NMR it was confirmed that both the compounds from two different sources were the same, but bioassay with them was not possible as they were applied separately and separate amounts were not sufficient to do bioassays with).

The LD_{50} values found in this investigation indicate potentiality in the order of NS-1>NS-2>RB-1>SB-2. Compound NS-1 offered LD_{50} value 8.918 µg/insect (regression equation Y= 3.146098 + 1.950926X) for 24 h of exposure which reached at 8.039 µg/insect (regression equation Y= 3.260723 + 1.921474X) after 48 hours of exposure. Compound NS-2 offered LD_{50} value 11.940 µg/insect (regression equation Y= 3.220169 + 1.652573X) for 24 h of exposure, which reached at 10.546 µg/insect (regression equation 3.299809 + 1.661833X) after 48 hours of exposure. Compound RB-1 offered LD_{50} value 13.727 µg/insect (regression equation Y= 3.45836 + 1.355214X) for 24 h of exposure, which reached at 11.291 µg/insect (regression equation Y= 3.692723 + 1.241786X) after 48 hours of exposure. Compound SB-2

offered LD_{50} value 14.248 µg/insect (regression equation Y= 3.158351 + 1.596216X) for 24 h of exposure, which reached at 12.487 µg/insect (regression equation Y= 3.414889 + 1.445666X) after 48 hours of exposure.

Among the NS-1, NS-2, RB-1 and SB-2 all except the last one were performed the highest average percent repellency on the beetle, and the differences were statistically significant for SB-2. The efficacy for repellency was found as in the order of NS-2>NS-1>RB-1>SB-2 ($P<0.05$) which supports that the name compound of this plant anacardic acid (NS-2) is showing repellency relatively mild to that of the compound NS-1 which was an unsaturated fatty acid (may be linoleic acid as tentatively assigned). RB-1 is the β-sitosterol is comparatively mild to that of the anacardic acid. P-values were in the descending order 2.51E-07 <7.27E-07 <6.48E-05 <0.702 for the NS-2, NS-1, RB-1 and SB-2 respectively.

ACKNOWLEDGEMENT

The author expresses her humble indebtedness and profound sense of gratitude to her respectable teacher and supervisor Dr. M. Khalequzzaman, Professor, Department of Zoology, University of Rajshahi, Bangladesh for his continued encouragement, indefatigable patience, proper guidance, criticism, stimulating discussion and valuable suggestions during the hole course of the present investigation. She declares the period of this work as of her proud as spent to work with a scientist like him.

Professor Dr. Md. Mahtab Ali, Chairman, Department of Zoology, University of Rajshahi deserves her thanks for providing required laboratory space and financial facilities to run the experimental works of this project.

She is very much thankful to all dignified persons abroad who came in touch with this work, especially to Dr. M. Suzuki, N. Nakazawa and H. Hasegawa, T. Kondo of Aomori Green BioCenter, Japan for their constructive suggestions; and to Prof. M. Hashimoto and T. Takashi (the then MS student) of Hirosaki University, Japan without whose help this work would not have been done.

She wishes to express her deep sense of gratitude and sincere devotion to her honorable teachers of the Department of Zoology, Rajshahi University for their kind

cooperation during the course of this work. She expresses thanks to Prof. Dr. Ekramul Haque of the Department of Pharmacy, University of Rajshahi, Bangladesh for his kind guidance in assigning the tentative structures of the purified compounds from the ^{1}H proton spectra. Dr. Qi Wang of Sinatur, China is cordially thanked for his kind assistance in providing information regarding the spectral difficulties. Dr. Moniruzzaman Sarker and Jinat Ferdousi are thanked for their encouragement throughout the course.

Student researchers, especially Dr. Abdul Awal (Ph.D. Fellow of the I.Bio. Sc., R.U.), Dr. Inun Nahar and Shahina Begum Rekha (M.Phil. researchers of the I.Bio.Sc., R.U.), Hedayetul Islam Roxy, Kanta Farhana, Rashed Zaman Shoag, Firoz Kabir, Shafiqullah Dipon, Apurba Kumar Sarkar, Sultan Mahmud, Afaz Uddin, Mehedi, and Mina Monadal (all M.Sc. students) of the Crop Protection and Toxicology Laboratory are also thanked for their kindness and cooperation during the course of this work.

Lastly, she owes her best thanks to her family members for their kind and patient sacrifice to carry out this project.

The author

Chapter 1

General Introduction

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GENERAL INTRODUCTION

1.1 Background to investigation

The earth is inhabited by about one million insect species, roughly half of which feed on plants (Schoonhoven, 1982), as well as many molluscs, annelids, nematodes, etc. belong to which groups there are pest species of animals. Among all the organisms only plant species, during millions of years have developed strong chemical defense, have survived the heavy selection pressure by those greedy early-created animals. Although each of the defense systems having own intrinsic merit, some of which have featured to render them as more generally applicable and, from a technical point of view, easier to handle than others. It seems that modern science has discovered one of these rare opportunities in the naturally occurring compounds in plants act as toxic or repellent to the pest. Historical references to insect pests are found in the Bible. Long before synthetic pesticides were invented, farmers around the world had their own home remedies against harmful insects even in the Neolithic time (7000 B.C). Typically they crushed the leaves of a poisonous plant, dissolved in water and then sprayed the solution on their crops (Ghosh, 2000). Documentation of the ancient use of chemical controls for the insects appear in Homer's writing before 1000 B.C. where sulfur was identified as an insecticide. Pliny (79 A.D.) recommended the use of arsenic as an insecticide (Cremlyn, 1978).

Until now only a small part of the plant kingdom (estimated at 2,50,000-5,00,000 species around the globe) has been investigated phytochemically and the fraction subjected to biological and pharmacological screening is even lower. Since plants may contain hundreds or even thousands of metabolites, there is currently a resurgence of interest in the vegetable kingdom as a possible source of new lead compounds for introduction into the therapeutical screening programs (Hostettmann *et al.*, 1995). Moreover, botanicals, because of their low-mammalian toxicity, have received much attention as control agents against pests. According to Feinstein (1952), over 2000 species of plants representing 170 odd families are said to have some insecticidal values.

Use of pesticides is often considered to be the most potent control technology for pests. But continuous or heavy use of some pesticides has created serious problems arising from factors, such as, direct toxicity to parasites, predators, pollinators, fish and man (Munakata, 1977; Pimental, 1981), pesticide resistance (Brown, 1968; Georghiou and Taylor, 1977; Schmutterer, 1981; Waiss *et, al.* 1981), susceptibility of crop plants to insect pests (Pimental, 1977), and increased environmental and social costs (Pimental *et al.*, 1980). Resistance to one or more pesticides has been reported in at least 477 species of insects and mites (Georghiou and Mellon, 1983), cross and multi-resistant strains in many important insect species have also been reported (Dyte, 1970; Pasalu and Bhatia, 1983; Dyte and Halliday, 1985; Irshad and Gillani, 1990; Zettler and Cuperus, 1990; Zettler, 1991).

The increasing serious problems of pest resistance to pesticides, and of contamination of the biosphere associated with the large-scale use of broad spectrum synthetic pesticides have dictated the need for effective biodegradable less hazardous safe pesticides with greater selectivity (Saxena, 1983). The awareness has created a worldwide interest in the re-evaluation and use of age-old, traditional botanical pest control agents (Heyde *et al.*, 1983). Further synthetic insecticides are not only a threat to the farmer's major resources but also used beyond the permissible limits (Lepigre,

1951). Safe and inexpensive insecticides coupled with simple application methods are needed at the rural level (Periera and Wohlgemuth, 1982). In many areas of the world locally available materials are widely used to protect stored products (Golob and Webley, 1980).

Natural products derived from plants, as an alternative to conventional insecticides for insect control is now a days very popular among the IPM practitioners. Plant-derived pesticides are more readily biodegradable. Therefore they are less like to contaminate the environment and may be less toxic to mammals (Freedman *et al.*, 1979). The botanical pesticides break down readily in soil and are not stored in plant or animal tissues. Often their effects are not as long lasting as those of synthetic pesticides. Environmentally they are less harmful than synthetic pesticides and acting in many insects in different ways (Schmutterer, 1990, 1992; Luik, 1994; Kuusik *et al.*, 1995).

The research for naturally occurring antifeedants against pests of field crops and storage has been intensified (Islam, 1983). A number of investigators isolated, identified and screened promising biologically active compounds from different parts of many botanical families for insect feeding deterrence and growth inhibitor (Jacobson *et al.*, 1975; Bernays and Chapman, 1977; Doskotch *et al.*, 1977; Jacobson, 1977; Carpentier *et al.*, 1979; Warthen, 1979; Jurd and Mannérs, 1980; Menn, 1980). Insecticides of plant origin are considered as alternatives to the synthetic chemical ingredients for being pest specific and biodegradable in nature (Periera and Wohlgemuth, 1982). So, derivatives of some plants have had temporary to restricted use in pest control or have been considered items of regional interest (Saxena, 1983).

The chemistry and biological activity of some of these plants have recently been studied. But even after a long history of pest control potential, these plants have not been fully utilized for pest control. This paradox can be attributed to the earlier prediction to research workers for chemicals that will rather than those that subtly

alter the pest's behavior and physiology. Only recently the potential of behavioral and physiological aberrations in pest management has been recognized. In some situations such aberrations may be highly desirable to minimize the risk of exposing the pest's natural enemies to poisoned food or starvation. The wide scale commercial use of plant extracts as an insecticide began in the 1850s with the introduction of nicotine from *Nicotina tobacum*, rotenone from *Lonchocarpus* sp., derris dust from *Derris elliptca* and pyrethrum from the flower heads of *Chrysanthemum cinerariaefolium.* Now a days, a number of traditionally used plant preparations have found in local commercial markets, for example *Ryania speciosa* (Ryania) (Flacourtiaceae), which contain an insecticidal alkoloid. *Haplophyton* spp. (Apocynaceae) has been used in the West Indies and Mexico for crop protection. Nicotine, an alkaloid from some *Nicotiana* species (Solanaceae) and the related compound anabasine from *Anabasis* species continue to be used against various insect pests (Pinniger *et al.*, 1996; Whitehead, 1997).

Constituents of many aromatic plants are used for flavouring or medicinal purpose has been found to possess insecticidal properties. Surveys of desert and semi desert plants have revealed a range of sesquiterpenes, benzopyrans, chromenes and prenylated quinines that are cytotoxic (Bell *et al.*, 1990). Some plant families may accumulate a restricted number of anti-insect chemicals, so-called secondary metabolites, whilst other possesses a wide variety of different structural compounds.

Farmers have been using plant extracts in pest control for centuries. This method of pest control provides an ideal source of low cost, safe and effective pesticides. Extracts of plant material rely on the solubility of the active components and it may cause repellent to insects (Sighamony *et al.*, 1984), antifeedant or other type of bioactivities against insects (Jayasinghe and Fujimoto, 1990; Morallo-Rejesus *et al.*, 1990; Adalla *et al.*, 1993; Facknath and Kawol, 1993; Morallo-Rejesus *et al.*, 1993; Kim *et al.*, 1994; Naumann *et al.*, 1994; Niber, 1994; Rajuraman and Saxena,

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1994; Braverman and Chizov-Ginzburg, 1997; Ho *et al.*, 1997; El-Lakwah and Abdel-Latif, 1998; Hermawan *et al.* 1998; Ndungu *et al.*, 1999; Jannet *et al.*, 2000).

Trials of plant materials admixed with a commodity, usually directly assess contact toxicity to adult insects, number of egg lying, number of eggs which hatch or the percentage of first filial (F_1) adult emergence (Ali *et al.*, 1983; Bowry *et al.*, 1984; Salas, 1985; Salas and Hernandez, 1985; Ahmed and Koppel, 1986; Das, 1986; Mueke and Apouli, 1986; Dakshinamurthy, 1988; Khalique *et al.*, 1988; Pal *et al.*, 1988; Babu *et al.*, 1989; Prakash and Jagadiswari, 1989; Choudhary, 1990; Doharey *et al.*, 1990; Morallo-Rejesus *et al.*, 1990; Prakash *et al.*, 1990; Tiwari, 1994).

Essential oil and their constituents have also been shown to be a potent source of botanical pesticide (Doharey *et al.*, 1990; Giga and Munetsi, 1990; Hassanali *et al.*, 1990; Koul *et al.*, 1990; Singal and Singh, 1990; Coats *et al.*, 1991; Kumar and Okonronkwo, 1991; Cockfield, 1992; Ahmed *et al.*, 1993; Rice and Coats, 1994; Schmidt and Streloke, 1994; Ho *et al.*, 1996; Shaaya, *et al.*, 1997; Liu and Ho, 1999; Huang and Ho, 1998; Huang *et al.*, 1999a, Kéïta, *et al.*, 2000, 2001, Bouda *et al.*, 2001; Tripathi *et al.*, 2002). Admixture of oil and cereals may cause repellence, antifeedant and mortality to insects (Qi and Burkholder, 1981; Messina and Renwick, 1983; Pereira, 1983; Salas, 1985; Salas and Hernandez, 1985; Ivbijaro and Agbaje, 1986; Ahmed *et al.*, 1988; Don-Pedro, 1989; Doharey *et al.*, 1990; Morallo-Rejesus *et al.*, 1990; Singal and Singh, 1990; Sube *et al.*, 1991; Khalequzzaman and Chowdhury, 2003). Spices and extract of spices have various effects on insect pests including stored product insects (Grainge and Ahmed, 1988; Jacobson, 1989, 1990; Shaaya *et al.*, 1991; Ho *et al.*, 1996; Huang *et al.*, 1997). Many workers have given their attention to isolate and identify active compounds from plant extracts and plant materials (Perry, 1980; Ayensu, 1981; Messina and Renwick, 1983; Duke, 1985, 1992; Oliver-Bever, 1986; Grainge and Ahmed, 1988; Southon and Buckingham, 1988; Rheenen *et al.*, 1989; Jouhar and Poucher, 1991).

Secondary metabolites from plants include alkaloids, terpenoids, phenolics, flavonoids, chromenes and other minor chemicals can affect insects life in several ways: they may disrupt major metabolic pathways and cause rapid death, act as attractants, deterrents, phagostimulants, antifeedants or an agent to modify oviposition. Essential oils and especially their main compounds monoterpenoids offer promising alternatives to classical fumigants (Papachristos and Stamopoulos, 2003). These compounds may act as fumigants against stored product insects (Liu and Ho, 1999; Huang *et al.*, 2000a; Kim and Ahn, 2001; Papachristos and Stamopoulos, 2002a), contact insecticides (Huang *et al.*, 1997, 1999b, 2000b; Huang and Ho, 1998, Chun *et al.*, 2000; Tripathi *et al.*, 2000; Tunc *et al.*, 2000, Papachristos and Stamopoulos, 2002a), antifeedant or repellent effects (Chiam *et al.*, 1999, Kim *et al.*, 2003a,b; Park *et al.*, 2003a,b) and may also affect some biological parameters, such as growth rate, life span and reproduction (Regnault-Roger and Hamraoui, 1995; Gurusubramanian and Krishna, 1996; Pascual-Villalobos, 1996).

They may also retard or accelerate development or interfere with the lifecycle of the insect in other ways (Harborne, 1988; Bell *et al.*, 1990; Houghton, 1996). Good secondary metabolites having a high degree of structural complexity as Azadirachtin, are normally obtained from plant materials by stem distillation or by extraction with organic or aqueous solvents (Balandrin *et al.*, 1985).

Products from several floral species have been demonstrated to act as repellents and antifeedants against a number of Coleoptera that attack stored products and the toxicity of a large number of plant extracts and their constituents have been evaluated against a number of stored-product insects (Raja *et al.*, 2001; Papachristos and Stamopoulos, 2002b; Tapondjou *et al.*, 2002). Quite a good number of plants have been identified and utilized for insecticidal and medicinal purpose till to date. But it is true that quite a large number of plants have still been untouched or less touched from which significant results can be obtained to control the pest and disease problems of human beings. *A. occidentale* L. is one of such plants under the family

Anacardiaceae that has been studied too much on medicinal context but a very little attempts have been made in details on biological activity of this plant till to date. Plant extracts and plant materials exhibit toxicity to the stored product beetles, *T. castaneum* and *Sitpophilus zeamais* Motsch. (Deb-Kritaniya *et al.*, 1980; Su, 1984; Sighamony *et al.*, 1986; Rauf and Harahap, 1991; Bekele, 1994; Ho *et al.*, 1994, 1995; Jembere *et al.*, 1995).

1.2 Description of the test plant

1.2.1 The Anacardiaceae plant family

The Anacardiaceae includes 76 genera with over 600 species. There are four tribes of poisonous Anacardiaceae. The poisonous genera *Anacardium*, *Gluta*, *Mangifera*, and *Switonia* are members of the tribe Anacardeae. *Comocladia*, *Metopium*, *Toxicodendron*, are in the tribe Rhoeae. *Semecarpus*, *Holigarna*, and *Melanochyla* are in the tribe Semecarpeae (Mitchell, 1990). Obviously, Anacardiaceae are evergreen trees and are found around the tropical regions of the globe and millions of people and animals are acquainted with them, chiefly because of the irritant effects of their chemicals rather than their botanical interest. While for the members of the tribe Anacardeae medicinal use is well known by the common people.

Table 1. Anacardiaceae family data

Family	Subfamily	Tribe	Genus
Anacardiaceae	Anacardioideae	Anacardeae	*Anacardium*
			Gluta
			Mangifera
			Switonia
	Spondioideae	Spondieae	*Spondias*
		Rhoeae	*Achinus*
			Comocladia
			Cotinus
			Metopium,
			Rhus
			Schinopsis
			Toxicodendron
		Semecarpeae	*Holigarna*
			Melanochyla
			Semecarpus
	Julianioideae		
	Pistacioideae		*Pistacea*

1.2.2 Morphological attributes and systematic position of the title plant

A. occidentale L. (synonyms=*Acajuba occidentalis, Anacardium microcarpum, Cassuvium pomiverum*) is known in French: anacardier, acajou; Spanish: anacardo, maranon; Italian: anacardo; German: kaschunuss and Bangla: kazu badam and other common names in various other languages are: cajueiro, cashew, cashu, casho, acajuiba, caju, acaju, acajaiba, alcayoiba, anacarde, anacardier, cacajuil, cajou, gajus, jocote maranon, merey, noix d'acajou, pomme cajou, pomme, jambu, jambu golok, jambu mete, jambu monyet, jambu terong.

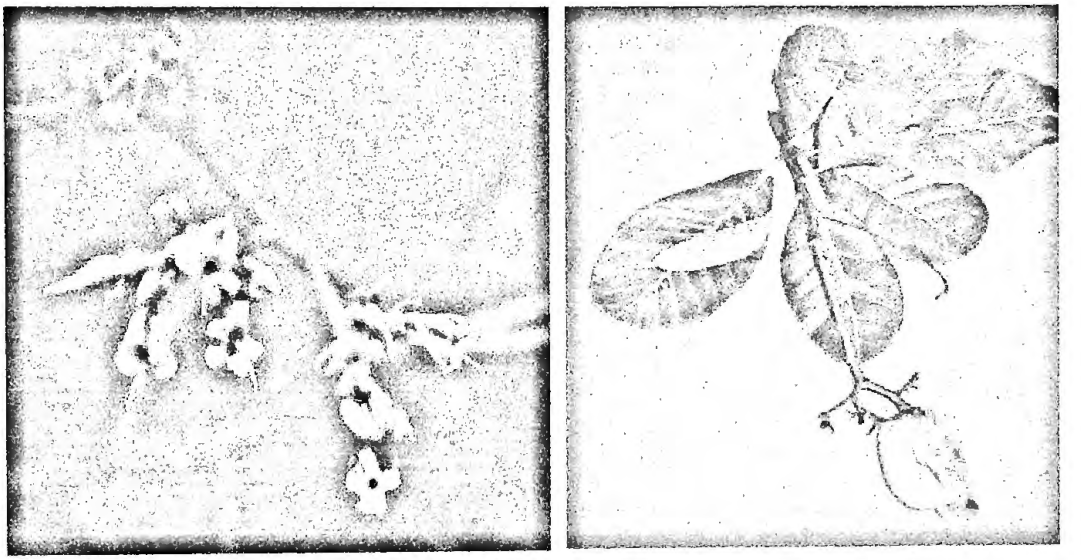


Plate 1. *Anacardium occidentale* L. flowering top and a fruit.

Its a spreading evergreen perennial tree to 12 m tall; leaves simple, alternate, obovate, glabrous, penninerved, to 20 cm long, 15 cm wide, apically rounded or notched, entire, short petiolate; flowers numerous in terminal panicles, 10–20 cm long, male or female, green and reddish, radially symmetrical nearly; sepals 5; petals 5; stamens 10; ovary one-locular, one-ovulate, style simple; fruit a reniform achene, about 3 cm long, 2.5 cm wide, attached to the distal end of an enlarged pedicel and hypocarp, called the cashew-apple; this shiny, red or yellowish, pear-shaped, soft,

juicy, 10–20 cm long, 4–8 cm broad; fruit reniform, edible, with two large white cotyledons and a small embryo (Jøker, 2000) surrounded by a hard pericarp which is cellular and oily, oil is poisonous causing allergenic reactions in some humans (Standley and Steyermark, 1949). Chromosome numbers for common varieties as mentioned in *Species Plantarum* 1: 383 (1753) $2n$ =24, 40, 42. The systematic position of the title plant is given below (Molina, 1975):

Kingdom	Plante– Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae –
Order	Sapindales –
Family	Anacardiaceae – Sumac family
Genus	*Anacardium* L. – anacardium
Species	*Anacardium occidentale* L. –cashew

1.2.3 Distribution and ecology of *A. occidentale*

The cashew tree, *A. occidentale*, is a botanical species native of eastern Brazil and was introduced into other tropical countries such as India, Africa, Indonesia and South East Asia in the 16th century (Mitchell and Mori, 1987). The cashew (*A. occidentale*, Anacardiaceae) is a tropical evergreen tree from North-east Brazil. They are pantropical, especially in coastal areas and native to tropical America, from Mexico and West Indies to Brazil and Peru. It ranks third in world production of edible tree-nuts with a current world production of about 7,00,000 MT of nut-in-shell (NIS).

Ranging from warm temperate moist to tropical very dry to wet forest life zones, cashew is reported to tolerate annual precipitation of 7 to 42 dm (mean of 32 cases = 19.6), annual temperature of 21 to 28°C (mean of 31 cases 25.2), and pH of 4.3 to 8.7 (mean of 21 cases = 64). Grows on sterile, very shallow and impervious savanna soils, on which few other trees or crops will grow, but is less tolerant of saline soil than most coastal plants. Does not tolerate any frost. In Brazil, Johnson (1973) summarizes "optimal ecological conditions;" annual rainfall 7–20 dm, minimum temperature 17°C, maximum temperature 38°C; average annual temperature 24–28°C, relative humidity 65–80%; insulation 1,500 to 2,000 hours per year, wind velocity 2.25 km/hr, and dry season 2–5 months long. It is recommended that cultivation be limited to nearly level areas of red-yellow podzols, quartziferous sands, and red-yellow latosols.

1.2.4 Uses of the title plant

Anacardiaceae are found, now a days, around the tropical and subtropical regions of the world and millions of people and animals are acquainted with them, chiefly because of the irritant effects of their chemicals rather than their botanical interest. Many parts of the cashew plant are used. The cashew "apple," the enlarged fully ripe, fruit may be eaten raw, or preserved as jam or sweetmeat. The juice is made into a beverage (Brazil cajuado) or fermented into a wine. Fruits or seeds of the cashew are consumed whole, roasted, shelled and salted, in Madeira wine, or mixed in chocolates. Shelling the roasted fruits yields the cashew nut of commerce. Seeds yield about 45% of pale-yellow, bland, edible oil, resembling almond oil. From the shells or hulls is extracted a black, acrid, powerful vesicant oil, used as a preservative and water-proofing agent in insulating varnishes, in manufacture of typewriter rolls, in oil- and acid-proof cements and tiles, in brake-linings, as an excellent lubricant in magneto armatures in airplanes, and for termite proofing timbers. Timber is used in furniture making, boat building, packing cases and in the production of charcoal. Bark used in tanning. Stems exude a clear gum, Cashew gum, used in pharmaceuticals and

as a substitute for Gum arabic (Bose and Biswas, 1970, de Silveira *et al.*, 2002). Juice turns black on exposure to air and provides an indelible ink. Along the coast of Orissa in India, shelter-belts and wind breaks, planted to stabilize sand dunes and protect the adjacent fertile agricultural land from drifting sand, have yielded economic cashew crops 5 years after planting (Patro and Behera, 1979).

The principle function of the secondary chemicals in the Anacardiaceae is probably to serve as a defense against vertebrate and insect herbivores. Contact with the poisonous members of the Anacardiaceae usually causes a cell-mediated dermatitis. Faupel and Kurki (2002) enlisted cashew plant as a source of bio-diesel and mentioned its output as 148 kg/hectare in general.

1.2.5 Chemistry and biomedical properties of the title plant

Per 100 g, the mature seed is reported to contain 561 calories, 5.2 g H_2O, 17.2 g protein, 45.7 g fat, 29.3 g total carbohydrate, 1.4 g fiber, 2.6 g ash, 38 mg Ca, 373 mg P, 3.8 mg Fe, 15 mg Na, 464 mg K, 60 mg *(*-carotene equivalent, 0.43 mg thiamine, 0.25 mg riboflavin, and 1.8 mg niacin. The apple contains 87.9% water, 0.2% protein, 0.1% fat, 11.6% carbohydrate, 0.2% ash, 0.01% Ca, 0.01% P, .002% Fe, 0.26% vitamin C, and 0.09% carotene (Duke, 1985). The fruit-shell contains *(*-catechin, *(*-sitosterol, and *l*-epicatechin; also proanthocyanadine leucocyanadine, and leucopelargodonidine. The dark color of the nut is due to an iron-polyphenol complex. The shell oil contains about 90% anacardic acid ($C_{22}H_{32}O_3$ and 10% cardol ($C_{32}H_{27}O_4$) mentioned by Deszcz and Kozubek (2000). It yields glycerides, linoleic, palmitic, stearic, and lignoceric acids, and sitosterol. Examining 24 different cashews, Murthy and Yadava (1972) reported that the oil content of the shell ranged from 16.6 to 32.9%, of the kernel from 34.5 to 46.8%. Reducing sugars ranged from 0.9 to 3.2%, non-reducing sugars, 1.3 to 5.8%, total sugars from 2.4 to 8.7%, starch from 4.7 to 11.2%. Gum exudates contain arabinose, galactose, rhamnose, and xylose.

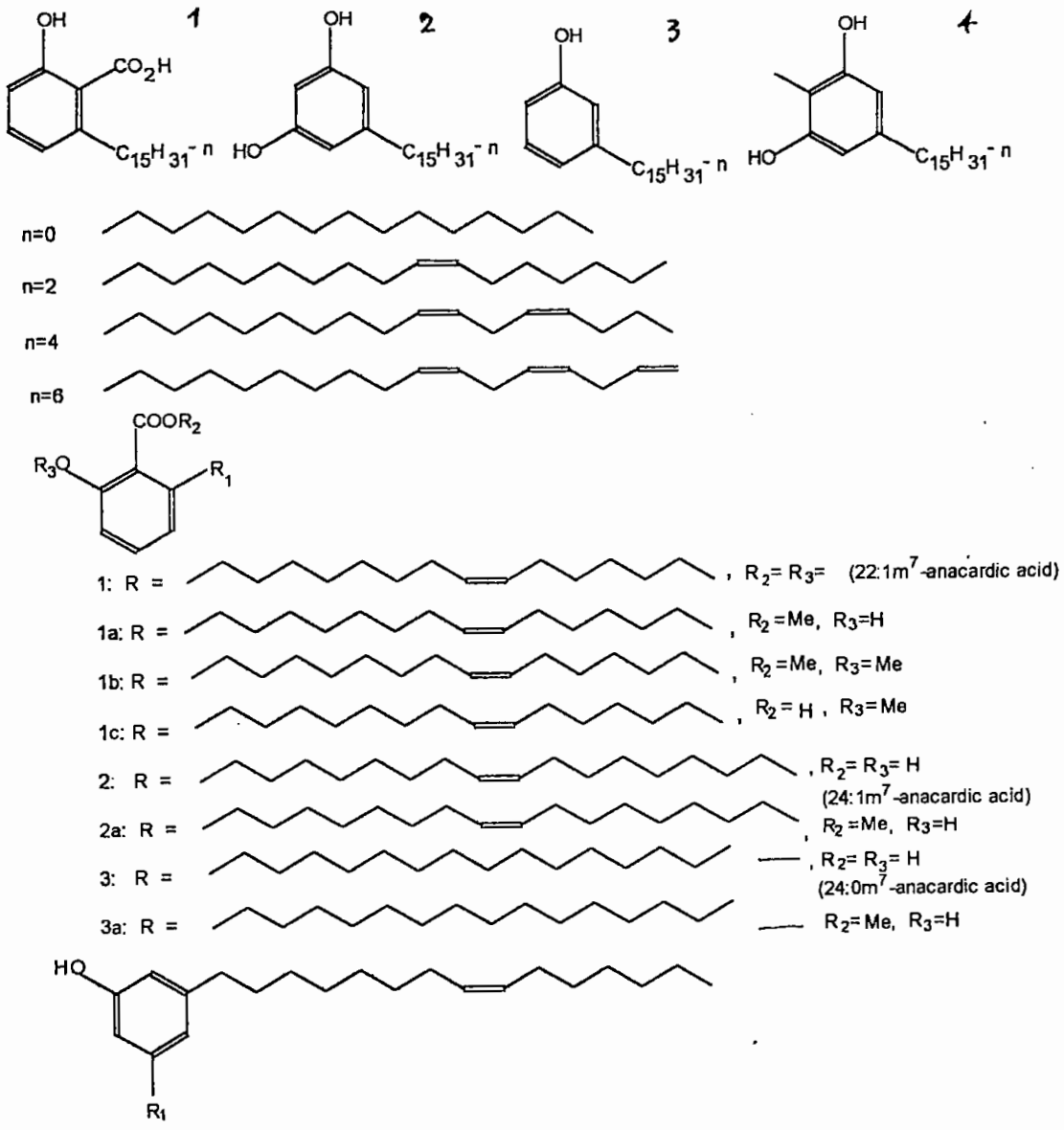


Fig. 1. Naturally occurring non-isoprenoid phenolic lipids from *A. occidentale*

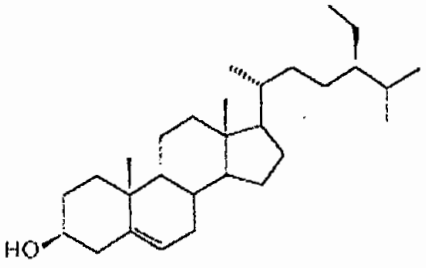
The true fruit of cashew is the nut, a kidney shaped structure of approximately 2-3 cm in length which is attached to the end of a fleshy bulb, generally called the cashew apple. Crude cashew nut shell liquid represents one of the major and cheapest sources of naturally occurring non-isoprenoid phenolic lipids, such as anacardic acids, cardols, cardanols, methylcardols (Fig. 1) and polymeric materials (Santos and Magalhães, 1999). They also mentioned the occurrence of alkylresorcinols, polyketide compounds that in the same homologous series as cardol isolated from *Anacardium occidentale* (cashew) or bilobol from *Ginkgo biloba* which are derivatives of 1,3-dihydroxy-5-alk (en)ylbenzene, have been demonstrated in developing rye (*Secale cereale* L.) kernels.

The fruit-shell juice and the nut oil are both said to be folk remedies for calluses, corns, and warts, cancerous ulcers, and even elephantiasis. Lans (2003) mentioned that stem-bark of *A. occidentale* is used to control diarrhea in ruminants and other pet animals. Anacardol and anacardic acid have shown some activity against Walker carcinosarcoma 256. Decoction of the astringent bark is given for severe diarrhea and thrush. Old leaves are applied to skin afflictions and burns (tannin applied to burns is liepatocarcinogenic) (George and Kuttan, 1997). Oily substance from pericarp used for cracks on the feet. Cuna Indians used the bark in herb teas for asthma, colds and congestion (Ippen, 1983; Fernandes and Mesquita, 1995, Menezes *et al.*, 2002; Teuber *et al.*,2002; Wang *et al.*,2003). The seed oil is believed to be alexeritic and amebicidal; used to treat gingivitis, malaria, and syphilitic ulcers (Mota *et al.*, 1985).

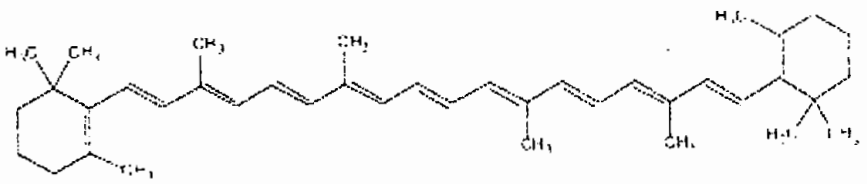
Bicalho and Rezende (2000) reported the volatile compounds of a largely consumed Brazilian cashew apple variety (*A. occidentale* L. var. *nanum*) were recovered by headspace extraction or simultaneous distillation-extraction and several compounds including esters, terpenes, hydrocarbons, carboxylic acids, aldehydes, alcohols, ketones, lactones and norisoprenoids were characterized and quantified by gas chromatography and mass spectrometry analyses (Begum *et al* 2002). However,

Beare-rogers *et al.*, (2001) reported presence of β-sitosterol and β-carotine in the title plant as very common constituents.

The most know sources of resorcinolic lipids in plants are the members of families *Anacardiaceae* (Cashew, Mango), *Ginkgoaceae* (*Ginkgo biloba*) and *Granineae* (cereals). Kozubek *et al.* (2001) advocated resorcinolic lipids as amphiphilic phenols in the study plant, as well as demonstrated their biological activity. It has also been introduced as a promising source of biodiesel (Faupel and Kurki, 2002) with its potentiality in absence of fossil fuels.



β-Sitosterol



β-Carotene

Fig. 2. Beta-sitosterol and beta-carotin from *A. occidentale*

1.2.6 Ethnobotany of the title plant

People from round the world found *A. occidentale* as a multipurpose tree of the Amazon and considered as of their own. They have made various use of it including food, shelter, remedies, etc. but ethnobotanical use of it is very interesting (www.rain-tree.com/cajuero/htm).

Ethnobotany: Worldwide Uses	
Africa	Intoxicant, malaria, tattoo
Brazil	Analgesic, aphrodisiac, asthenia, asthma, bronchitis, callosity, corn, cough, diabetes, diuretic, dyspepsia, eczema, fever, gargle, genital, impotence, intestinal colic, leishmaniasis, mouthwash, muscular debility, psoriasis, scrofula, stimulant, syphilis, throat, tonsillitis, ulcers (mouth), urinary, venereal disease, vesicant, wart, wounds
Haiti	Caries, diabetes, stomatitis, toothache, wart
Malaysia	Catarrh, constipation, dermatosis, diarrhea, nausea, thrush
Mexico	Caustic, diabetes, diarrhea, freckle, leprosy, liqueur, poison, skin, swelling, syphilis, ulcer, wart
Panama	Asthma, cold, congestion, diabetes, diarrhea, hypertension, inflammation
Peru	Antiseptic, diarrhea, douche, flu, infection, skin infections
Trinidad	Asthma, cough, diarrhea, dysentery, dyspepsia, stomachache
Turkey	Diarrhea, fever, poison, wart
Venezuela	Dysentery, gargle, leprosy, sore throat
Elsewhere	Asthma, astringent, cold, colic, congestion, corn, cough, debility, diabetes, diuretic, dysentery, liqueur, piscicide, poison, purgative, scurvy, skin, tumor, vesicant, wart

1.2.7 Aim of the work

Quite a good number of plants have been identified and utilized for insecticidal and medicinal purpose till to date. But it is true that quite a large number of plants have still been untouched or less touched from which significant results can be obtained to control the pest of crops and disease problems of human beings.

A. occidentale is one of such plants that has been studied a lot phycochemically and only a few studies have been done only with its medicinal properties, but in details a very few works have been done till to date on its use for the control of crop pests. Accordingly, a research topic entitled "Pesticidal properties of *Anacardium occidentale* L. against *Tribolium castaneum* (Herbst)" was undertaken for study.

Chapter 2

General Materials and Methods

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Selection of plant materials

In this investigation different parts of *A. occidentale*, viz. leaves, nut-shell, root-bark and stem-bark have been collected for screening to trace the presence of toxic, as well as, bio-active constituents since the plant is well known as a medicinal plant and also considered to contain toxic constituents.

2.1.1 Collection of plant materials

For the extraction fresh materials of *A. occidentale* were collected from the botanical garden of Rajshahi University Campus, where there is a single fruiting cashew tree planted some 10 years ago. The plant was taxonomically identified and a voucher specimen is deposited in the Herbarium of the Department of Botany, Rajshahi University, Bangladesh [Collection No. 140]. Disease-free healthy mature leaves and nut-shells of mature fruits, root-bark and stem-bark were collected for this investigation. Cashew fruits (nut with apple attached) were collected and the attached apples were separated before cutting the nuts (seed kernel) out. Leaves were collected green and fresh avoiding ripened or dried ones carefully. Stem-bark and root-bark scratched out carefully and soil was removed from the root-bark strips with a brush. The materials were dried in a well-ventilated room under shade for 6 days and were

then powdered in a hand grinder slowly to avoid heat and were stored in an air-tight container in the laboratory (Plate 2).

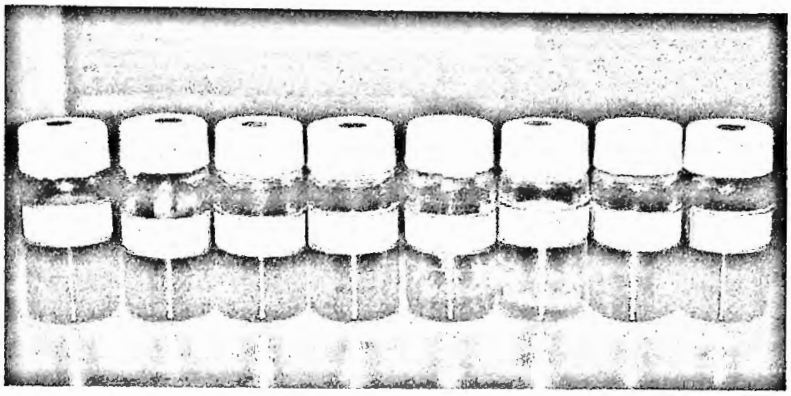


Plate 2. Collected extracts of *A. occidentale*

2.1.2 Preparation of materials for extraction

Fresh leaves, nut-shells, root-bark and stem-bark of *A. occidentale* were prepared for extraction in the following way:

Leaves- After collection of leaves of *A. occidentale* were spread out to dry without heaping the material together. It was done in the shade of the sun.

Nut-shell- Fruits were collected fresh and the nut-shells were picked up and were cut into small pieces and spread out to dry under shade.

Stem-bark- Stem-bark was collected by striping from the stem and cutting into small pieces as thin as possible. After collection the pieces of bark were dry thoroughly in a well-ventilated place.

Root- Roots were collected by digging up without damaging them and, shake and brush away excess soil without washing with water. Then the roots were cut into thin slivers and spread out to dry under the shade.

After drying well the plant materials were powdered in a hand grinder slowly to avoid excess heat. Powdered materials were then packed and sealed.

2.1.3 Extraction of test materials

There are basically two methods for extracting compounds from plant materials. Which one to choose, depends on whether the aim is to extract the more polar compounds (especially glycosides) which are present in the cell vacuole, or to obtain the less polar aglycones present on the surface of the plant, in aerial parts, heartwood or roots. In the present study two solvents, chloroform and methanol were selected to extract four different parts of *A. occidentale* separately.

For extraction, powdered materials i.e., leaf, nut-shell, root-bark and stem-bark were extracted separately by chloroform and methanol successively. The powdered materials were weighed and placed in separate conical flasks to add chloroform (500g × 1500ml × 3 filtration by Whatman filter paper at 24 h interval in the same collection flask) to yield the first extracts [for leaves, nut-shell, root-bark and stem-bark] separately (Plate 3). After filtration extraction by $CHCl_3$ (Merck, Germany) to collect aglycones (or the components from the inter-cellular space) was completed and the same amount of MeOH (Merck, Germany) was added in the same way to extract glycosides (or the components mostly from the cell vacuoles) to yield the other ones [for leaves, root-bark and stem-bark] and thus extraction of all possible components has done. The extracts were then fitted one after another with a round bottom flask to a vacuum rotary evaporator. The output extracts were removed to glass vials and preserved in a refrigerator at 4°C with proper labeling. For each of the items two solvents have been used separately for extraction successively, and the amount of materials were recorded deducing the amount of the extract afforded in the previous extraction for some conveniences in preparing doses for the insecticidal tests. Process of extraction and collection of extracts from the plant materials have been shown in Fig. 3.

Plate 3. Extraction by $CHCl_3$ and MeOH

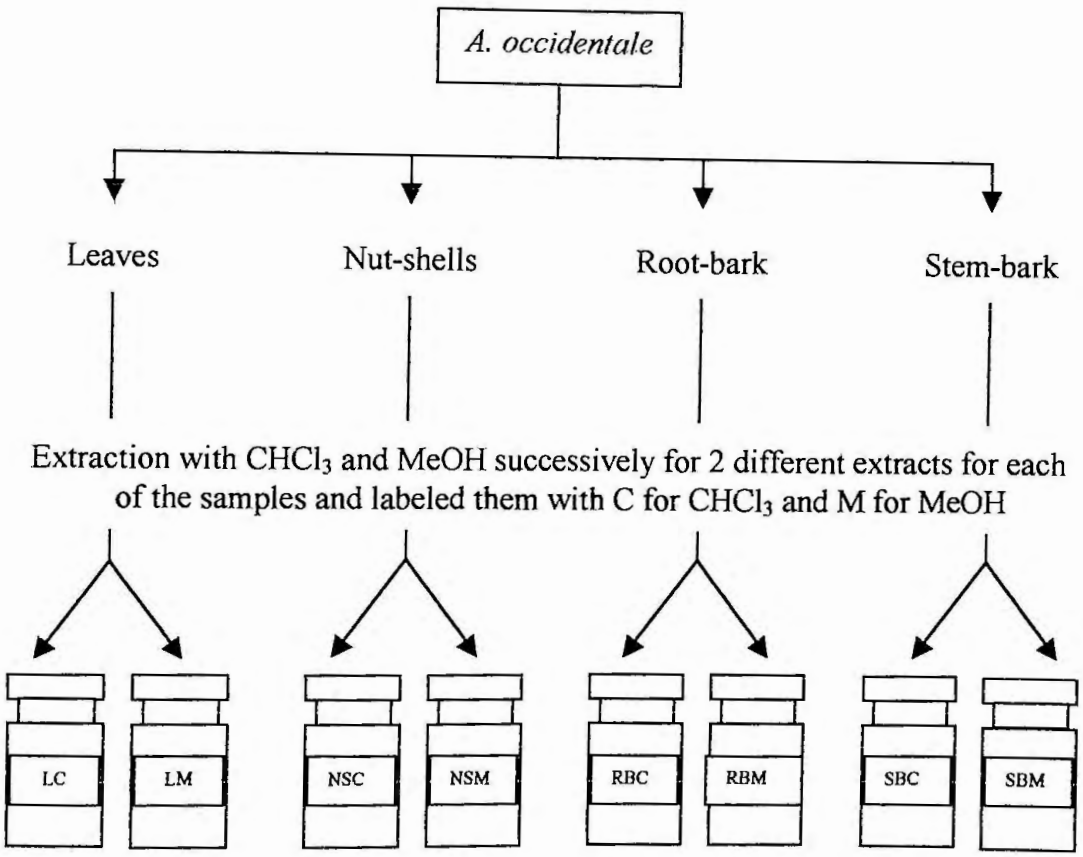


Fig. 3. Collection of extracts in chloroform from the plant materials.

2.2 Selection of test organisms

To carry on screening for insecticidal properties of the extracts of different parts of the title species *A. occidentale*, a test organism *T. castaneum* was selected, because it is an easy cultivable and noble laboratory animal. It is an important stored grain pest in a wide variety of cereal products. The life history has made this insect a popular choice as a test insect for biological studies. They are also easy to culture in large numbers and require no sophisticated equipment for maintenance.

2.2.1 Test insect

Source of test insects- The *Tribolium* species, *T. castaneum* used in the present experiment was originally received from the Crop Protection Department of the University of Newcastle upon Tyne, U.K. and were reared in the Crop Protection and Toxicology Laboratory, Department of Zoology, University of Rajshahi, Bangladesh.

2.2.1.1 Culture of test insects

Mass cultures were maintained in plastic containers (1200ml) and sub-cultures in beakers (1000ml) with the food medium. The beakers were kept in an incubator at 30°C ± 0.5°C without light and humidity control (Plate 4). Each container and beaker contained 250g and 150g of food respectively. About 200 adults in each container and 100 adults in each beaker were introduced. The cultures were checked in regular intervals and eggs and larvae were separated to grow properly. A crumpled filter paper was placed in side each container and beaker for easy movement of the beetles. The containers and beakers were covered with pieces of muslin cloth tightly fixed with the help of rubber bands to avoid possible escape of the beetles.

2.2.1.2 Preparation of food medium

The whole-wheat flour was used as the food medium for the insect species. The flour was sterilized at 60°C for 24 hours in an oven. A standard mixture of whole-wheat flour with powdered dry yeast in a ratio of 19:1 (Park and Frank, 1948; Park,

1962; Zyromska-Rudzka, 1966; Khalequzzaman *et al.*, 1994) was used as food medium throughout the experimental period. Both the flour and the powdered dry yeast were sterilized at 60°C for six hours in an oven. Food was not used until at least 15 days after sterilization to allow its moisture content to equilibrate with the environment.

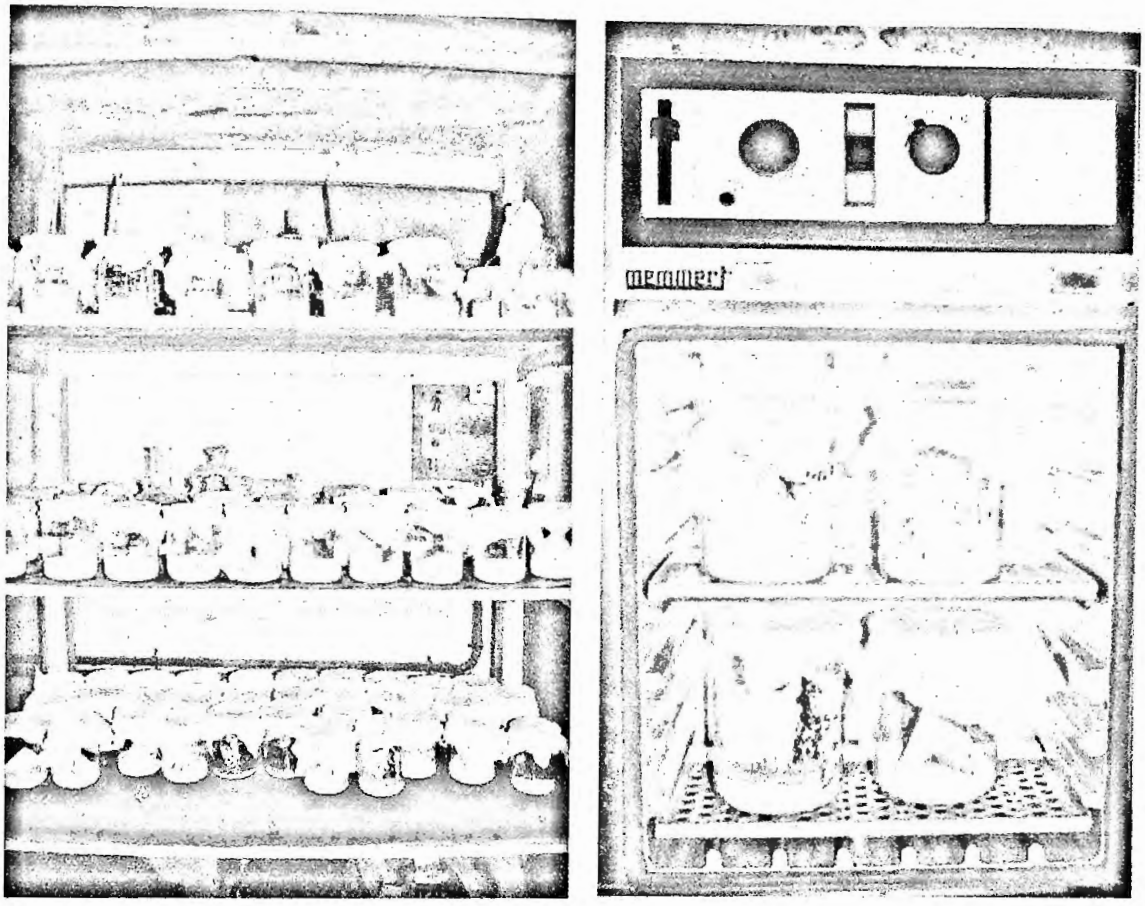


Plate 4. Culture of *T. castaneum* at room temperature (left) and in an incubator (right).

2.2.1.3 Collection of eggs

About 500 beetles were placed in a 500ml beaker containing food medium. The beaker was covered with a piece of cloth and kept in an incubator at 30°C ± 0.5°C. In regular intervals the eggs were collected by sieving the food medium by two sieves of 500- and 250 μ aperture separating the adults and eggs respectively

following the methods of Khan and Selman (1981). Eggs were then transferred to petri dish (90mm in diameter) with food and incubated at the same temperature.

2.2.1.4 Collection of newly hatched larvae

After 3-5 days, larvae hatched out in described conditions. Newly hatched larvae were then collected with a fine pointed camel hairbrush and then shifted to the fresh food medium for culture. The larvae are yellowish white in color and long cylindrical in shape. It appears 1 mm in length after hatching and become 6-7mm at maturation.

2.2.1.5 Collection of mature larvae

Most larvae had six instars as reported by Good (1936). The larval instars were determined by counting the number of exuvae (larval skin) deposited in the food medium according to Good (1936). Two days-old larvae were considered as first instar larvae while second, third, fourth, and fifth instar larvae were considered on fourth, seventh, tenth and thirteenth day from hatching respectively. Depending on these days according to larval instars sixteen days old larvae have been considered as a mature larvae. Larval cultures were maintained in an incubator in the same procedure at 30°C ± 0.5°C without light and humidity control. The food medium was replaced by three days interval to a fresh one to avoid conditioning by the larvae.

2.2.1.6 Collection of adults

A huge number of flour beetles were thus reared to get a regular supply of the newly formed adults. When sufficient adults produced in the sub-cultures, they were collected form the food medium. For this purpose some pieces of filter papers were kept inside the beaker on the food. Adults crawled on the paper were taken out with the help of forceps and the beetles were collected in a small beaker (100 ml) with the help of a camel hairbrush. Adults were 2.3 to 4.4 mm in length with sexes externally alike. Body is flattened with a pronotum that is broader and wide.

2.2.2 Test Fungus

As per of the present search for pesticidal properties from *A. occidentale* 8 crude extracts of different parts, viz. leaves, nut-shell, root-bark and stem-bark extracted in $CHCl_3$ and MeOH have been screened for antifungal activity test against a plant pathogenic fungus, *Valsa ceratosperma* (Tode ex Fries). Initially it was cultured in the Plant Protection Division of the Aomori Green BioCenter (AGBC), Aomori, Japan to collect spores and brought to the Crop Protection Laboratory of the Department of Zoology, Rajshahi University. This fungus was a suitable indicator organism for screening purposes (Islam *et al.*, 2003) and bioautographic assays on TLC with this was successful since most of the anti-*Valsa* compounds found active against the test insect *T. castaneum*.

2.3 Statistical analysis

The percent mortality was subjected to statistical analysis according to Finney (1947) and Busvine (1971). The dose-mortality relationship was expressed as a median lethal dose (LD_{50}).

During probit mortality calculation percent mortality of the adult beetles were corrected by using Abbott's (1925) formula:

$$P_r = \frac{P_o - P_c}{100 - P_c} \times 100$$

Where,

P_r = Corrected mortality (%)

P_o = Observed mortality (%)

P_c = Mortality (%) at control

2.4 Detailed Materials and Methods

The detailed Materials and Methods are described in the respective chapters.

Chapter 3

Crude Extract Bioasasay

CHAPTER 3

CRUDE EXTRACT BIOASSAY AND REPELLENCY

3.1 Introduction

Biological activity test is an essential tool to evaluate activities of any compound that could be used to develop pesticides and medicines. Now a days, plant derived compounds are being subjected to produce insecticides, insect repellents or insect antifeedants for safer protection of field crops and stored grains with eco-friendly means for sustainable development. According to the item crops biological assays could be of different types. For field crops infesting agents normally different from that of stored products, thus selection of test agents might not be the same. However, there are some test-organisms, uses of them in bioassays have wide range of comparable phenomenon with that controlling attempt should be taken. But it depends on the type of action of the testing materials.

Recently, the search for naturally occurring antifeedants against pests of field crops and storage has been intensified. A number of investigators isolated, identified and screened chemical compounds from leaves and seeds of many botanical families for insect feeding deterrence and growth inhibition and toxicant (Jacobson *et al.*, 1975; Bernays and Chapman, 1977; Doskotch *et al.*, 1977; Carpenter *et al.*, 1979; Warthen 1979; Jurd and Manners, 1980; Menn, 1980; Ho *et al.* 1995).

A large number of scientific papers and reports cover the subject of naturally occurring insect antifeedants, feeding deterrents and insect toxicants in plants (Munakata, 1970; Jacobson *et al.*, 1975; Bernays and Chapman, 1977; Doskotch *et al.*, 1977; Sudhakar *et al.*, 1978; Warthen, 1979; Deb-Kritaniya *et al.*, 1980; Menn, 1980; Huang and Ho, 1998). From the academic point of view, plants represent a vast storehouse of potentially useful natural products, and indeed, many laboratories worldwide have screened thousands of species of higher plants not only in search of pharmaceuticals, but also for pest control products (Van Beek and Breteler, 1993; Gonzáles-Coloma *et al.*, 1994ab; Addor, 1995; Cornelius *et al.*, 1995; Assabgui *et al.*, 1997; Bläske and Hertel, 2001). These studies have pointed to numerous plant species possessing potential pest-controlling properties under laboratory conditions, but the step from the laboratory to the field eliminates many contenders, even when judged only on their efficacy against pests under realistic field conditions. Unfortunately, efficacy against pests is only one of a number of important criteria that need to be met for a plant extract or derivative to move successfully toward commercialization and use (Isman, 1995).

Further, testing of plant materials for insecticidal properties may help in the development of new antifeedant, repellents or synthetic insecticides. Since the whole experiment was based on the activity guided fractionation antifungal activity was the preliminary test and that has been done successfully. After that the crude extracts of *A. occidentale* were subjected to insecticidal tests against red flour beetle *T. castaneum* by topical and surface film methods of application. Besides these repellency tests were also carried out.

3.2 Materials and Methods

Extracts collected from different parts of *A. occidentale* in different solvents were weighed and dissolved in the respective solvents according to the proportion of dry weight in different parts. This was considered as stock solution. Then the stock solution was diluted as desired concentration with the same solvent.

3.2.1 Bioassay with *V. ceratosperma*

Direct bioautography with *V. ceratosperma* was performed on Al backed TLC sheets according to Homans and Fuchs (1970) and Gottstein *et al.* (1984). After developing the TLC plates they were dried well to remove the solvent and UV active spots were detected at 254 and 366 nm. A suspension of the spores of the fungus *V. ceratosperma* in potato dextrose agar (liquid at 45 °C that allows the spores to survive) sprayed over the plates and kept them at a room temperature for two days in polythene boxes provided with water to give a humid condition (Islam *et al.*, 2003). The grayish color created by the grown mycelia on the plates flushed the inhibition zones clear. To kill the fungus on TLC plates EtOH solution is sprayed.

Direct bioautography for antimicrobial test is very sensitive and it gives accurate localization of active compounds and therefore permits a target-directed isolation of the active constituents (Rahalison *et al.*, 1991). For simple comparison with the inhibiting compounds present in the sample, and sample and compounds can be used on the same plate as a reference (Rahalison, 1994).

Pycnidiospores of *V. ceratosperma* produced in the culture plates within 70-80 days (after inoculation) showed yellow patches, which oozed out from matured ascospores. Spores were collected with a syringe and preserved in 0.5 ml of autoclaved distilled water in 1.5 ml snap-cap tubes. They were then centrifuged for 5

minutes at 10,000 rpm and most of the water was discarded before use in the bioautographic assays.

Dehydrated Potato Dextrose Agar (PDA, DIFCO Laboratories, Detroit MI 48232-7058 USA) was used to prepare an immersion of spores to spray on TLC plates. PDA (39 g) was suspended in 1 liter of distilled water, heated (without boiling) for complete dissolution and sterilized at 120°C for 15 minutes. The same PDA medium used for culture of the test fungus was also used for the inoculum. About 15 ml of PDA solution was allowed to cool down. As soon as the temperature reached 48°C, spores (which after removal of water from the snap-cap tube weighed about 0.55g) were added to the PDA to give a concentration of approx. 1×10^6 spores/ml for a 20×20 cm plate (calculated with a haemocytometer). Spraying of the plate was performed rapidly to avoid immediate solidification.

Lyophilized extracts were measured 10 mg in a 1.5 ml vial to add 1 ml of solvent to give a concentration of 10 mg/ml or 10 µg/µl. All samples were prepared prior to the bioassay. For crude extracts activity at 100 µg or less were subsequently investigated against the same fungus in classical dilution experiments (Barry, 1980).

Silica-gel G60 F_{254} coated Al sheets (Merck) were used. The volume spotted on the TLC plate was 10 µl, corresponding to 100 µg of extract. For active extracts, the assays were repeated in duplicate at a lower sample amount (50 µg). The following mobile phases were used for the separation: Pet. ether-EtOAc (1:1) for $CHCl_3$ extract and $CHCl_3$ –MeOH-H_2O (65:35:05) for MeOH extracts. Thin layer chromatograms were run in duplicate of which one plate was used for the bioassay, whereas the second plate serving as reference chromatogram, which was stained with Godin reagent (Godin, 1954). The color bands of the compounds for the reaction with Godin were also recorded as physical remarks of the compounds, and this was also served for the record of the retention factor (Rf).

Inoculum was sprayed onto TLC plates with a 30 ml sprayer and atomizer bulb. After solidification of the medium, the test plates were put in polythene boxes lined with moist filter paper. The whole system was wrapped in a polythene bag to avoid drying and was kept in an incubator at 25°C for 60-72 hours. Clear zones were observed against a dark background that had been produced by the fungus itself. The bioassay results indicated promising biological activity in the chloroform extracts of the nut-shell, root-bark and the stem-bark.

3.2.2 Topical bioassay

A general concentration for each of the extracts was selected as 10mg/ml to afford 10µg/µl as the stock dose for topical application to make other successive doses by serial dilution to give a series of doses 100-, 50-, 25-, 12.5-, 6.25-, 3.125 and 1.56 or 30-, 15-, 7.5- and 3.75 or 10-, 5-, 2.5- and 1.25 µg/µl concentrations. Applicable doses have been taken by observing the mortality at the pilot experiments that killing below 100% at the highest and at least positively at the lowest dose.

Prepared insects were taken in a petri dish and 1µl of each of the doses from the choose series were applied on to the pronotum of each of the insects by a micro-syringe and at least 10 insects were used for each of the doses. Three replications were made for each of the doses as well, while a control (application of only solvent) was also set at the same time under the same condition. Placing the upper lids marked with dose levels, time and dates the petri dishes were kept at the room temperature. One µl of each of the doses was applied on the pronotum of each of the insects so carefully that the total amount was dropped onto its body. All 30 treated insects were released (for each of the dose) with 3 replications in three 90mm petri dishes and covered with the upper lid during the whole exposure period of 48 hours. For the control batch the same was also done and all sets kept on a secured place. The mortality of the treated insects was recorded after 24- and 48 hours of exposure.

3.2.3 Residual-film bioassay

All extracts were diluted with the solvents in which they were extracted and the actual amount of extracted matter in a dose was recorded. The application of dose was carried out by residual film method (Busvine, 1971). For each dose one ml of mixture was dropped on a petri dish (90 mm) in such a way that it made a uniform film over the petri dish. Then the petri dishes were air-dried leaving the extraction on it. The actual extract present in one ml mixture was calculated and dividing the value by the area of the petri dish the dose per square centimeter was calculated. After drying 10 red flour beetles (3-5 day old) were released in each petri dish with 3 replications. A control batch was also maintained with the same number of insects after preparing the petri dish by applying and evaporating the solvent only. The treated beetles were placed in the incubator at the same temperature as reared in stock cultures and the mortality of the beetles was counted after 24- and 48 hours of treatment.

3.2.4 Repellency test

The repellency test used was adopted from the method (No. 3) of McDonald *et al.*, (1970) with some modifications by Talukder and Howse (1993, 1994). Half filter paper discs (Whatman No. 40, diameter 9 cm) were prepared and 100-, 80-, 60-, 40- and 20 µL of all the $CHCl_3$ and MeOH extracts separately applied onto each of the half-disc and allowed to dry out as exposed in the air for 10 minutes. Each treated half-disc was then attached lengthwise, edge-to-edge, to a control half-disc with adhesive tape and placed in a petridish (diameter 9 cm), the inner surface of which was smeared with fluon to prevent insects escaping. The orientation of the same was changed, in the replica to avoid the effects of any external directional stimulus affecting the distribution of the test insects. Twenty adult insects were released in the middle of each filter-paper circle (Plate 5). Each concentration was tested five times. Insects that settled on each half of the filter paper disc were counted after 1 h and then

at hourly intervals for 5 h. No significant difference was detected between the repellency of only solvent impregnated and untreated filter papers in tests designed to check for any possible influence of $CHCl_3$ and MeOH. The average of the counts was converted to percentage repellency (*PR*) using the formula of Talukder and Howse (1993, 1995):

$$PR = 2(C - 50),$$

where, C is the percentage of insects on the untreated half of the disc. Positive values expressed repellency and negative values for attractant activity.

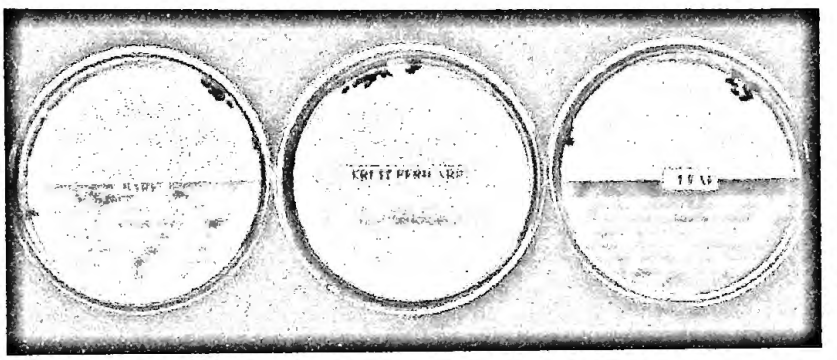


Plate 5. Petri-dishes used in repellency test

3.3 Results

3.3.1 Effect of crude extracts on *V. ceratosperma*

All $CHCl_3$ and MeOH extracts of the leaves, nut-shells, root-bark and stem-bark extracts of *A. occidentale* were tested against *V. ceratosperma* and at a dose of 100 µg/spot on the chromatogram. The results have been presented in Table 2. It was observed that the $CHCl_3$ extract of nut-shell, $CHCl_3$ and MeOH extract of root-bark and $CHCl_3$ extract of the stem-bark showed highest activity against the test fungus, whereas $CHCl_3$ extract of leaf and MeOH extract of leaf and nut-shell showed no activity.

Table 2. Activity of crude extracts against *V. ceratosperma.*

Plant organ	Extract	Amount (g)	Activity on *V. ceratosperma*
Leaf	$CHCl_3$	1.0	-
	MeOH	0.5	-
Nut-shell	$CHCl_3$	1.0	++
	MeOH	4.0	-
Root-bark	$CHCl_3$	2.5	++
	MeOH	1.0	++
Stem-bark	$CHCl_3$	15.0	++
	MeOH	5.0	+

3.3.2 Topical bioassay on *T. castaneum* adults

Both the chloroform and MeOH extract of nut-shells have been found strongly effective against the test insect *T. castaneum*, however the MeOH extract of the root-bark was stronger compared to its chloroform extract (Table 3). After the nut-shell and the root-bark extract stem-bark extracts were found active and methanolic extract was less effective in this regard. In case of leaf MeOH extract was stronger than the $CHCL_3$ extract showing a similarity like the root-bark extract.

The crude extract dose mortality results against *T. castaneum* were found promising. Both the chloroform and methenolic extract of nut-shells have been found strongly effective LD_{50} 17.574- and 17.049 µg/insect, except the methenolic extract that shows LD_{50} 15.503 µg/insect, while the chloroform extract offered LD_{50} 19.322 µg/insect. For chloroform and methenolic extracts of stem-bark LD_{50} values were 47.012- and 121.012 µg/insect respectively, but in case of leaf it is just adverse to that of stem-bark extract. In that case methenolic extract showed stronger activity than chloroform extract, and the above mentioned LD_{50} values were for 48 h of treatment. For 24 h of exposure chloroform and methenolic extracts of leaves, nut-shell, root-bark and stem-bark were 207.238-, 85.514-, 18.892-, 19.777-, 22.096-, 16.868-, 64.110- and 179.019 µg/insect respectively (Table 4; Appendix Tables I-XVI).

Regression equations for chloroform and methenolic extracts for leaf, nut-shell, root-bark and stem-bark are shown in Table 4 and the lines are presented in Figs. 1-4. The insignificant χ^2 values indicated the good fit of the lines.

Table 3. Dose mortality data of *T. castaneum* treated with *A. occidentale* extracts with 24 and 48 hours of exposure.

Plant organ	Solvent used	Dose used µg/insect	Mortality %	
			24 hours	48 hours
Leaf	$CHCl_3$	100.00	33.333	33.333
		50.00	30.000	33.333
		25.00	23.333	26.667
		12.50	6.667	10.000
	MeOH	100.00	56.667	73.333
		50.00	33.333	36.667
		25.00	26.667	26.667
		12.50	16.667	16.667
Nut-shell	$CHCl_3$	50.00	83.333	86.667
		25.00	56.667	60.000
		12.50	36.667	36.667
		6.25	23.333	23.333
	MeOH	50.00	86.667	90.000
		25.00	53.333	56.667
		12.50	33.333	36.667
		6.25	13.333	20.000
Root-bark	$CHCl_3$	50.00	76.667	80.000
		25.00	53.333	56.667
		12.50	33.333	36.667
		6.25	13.333	16.667
	MeOH	50.00	80.000	83.333
		25.00	60.000	60.000
		12.50	36.667	40.000
		6.25	26.667	30.000
Stem-bark	$CHCl_3$	100.00	73.333	43.333
		50.00	36.667	30.000
		25.00	20.000	30.000
		12.50	10.000	16.667
	MeOH	100.00	80.000	50.000
		50.00	53.333	30.000
		25.00	26.667	30.000
		12.50	13.333	16.667

Table 4. LD_{50}, 95% confidence limits, regression equation and χ^2 value of dose mortality experiments of *A. occidentale* crude extracts against adult *T. castaneum* with 24 and 48 hours of exposure.

Plant organ	Exp. (Hour)	Solvent	LD_{50} value (µg/insect)	95% confidence limits		Regression equations	χ^2 value (df)
				Upper	Lower		
Leaf	24	$CHCl_3$	207.238	718.395	59.783	Y= 2.343019 + 1.146996X	2.748 (2)
		McOH	85.515	164.868	44.355	Y= 2.669598 + 1.206187X	0.693 (2)
	48	$CHCl_3$	248.284	1356.347	45.449	Y= 2.830691 + 0.905786X	2.211 (2)
		McOH	56.535	82.134	38.914	Y= 2.093695 + 1.658553X	2.019 (2)
Nut-shell	24	$CHCl_3$	18.892	25.373	14.066	Y= 2.577399 + 1.898174X	0.561 (2)
		McOH	19.777	25.271	15.478	Y= 1.917841 + 2.377903X	0.659 (2)
	48	$CHCl_3$	17.574	22.953	13.456	Y= 2.374147 + 2.109339X	0.985 (2)
		McOH	17.049	22.057	13.178	Y= 2.254093 + 2.229367X	1.694 (2)
Root-bark	24	$CHCl_3$	22.096	29.400	16.608	Y= 2.287933 + 2.017423X	0.078 (2)
		McOH	16.868	23.490	12.113	Y= 2.970249 + 1.654154X	0.472 (2)
	48	$CHCl_3$	19.322	25.828	14.455	Y= 2.514534 + 1.932630X	0.018 (2)
		McOH	15.503	22.083	10.884	Y= 3.140641 + 1.561937X	.0652 (2)
Stem-bark	24	$CHCl_3$	64.110	85.960	47.814	Y= 0.845433 + 2.299246X	0.999 (2)
		McOH	179.019	908.266	35.284	Y= 3.27767 + 0.7644957X	0.636 (2)
	48	$CHCl_3$	47.013	60.592	36.477	Y= 1.080848 + 2.343688X	0.129 (2)
		McOH	121.012	346.842	42.220	Y= 3.035686 + 0.943101X	0.906 (2)

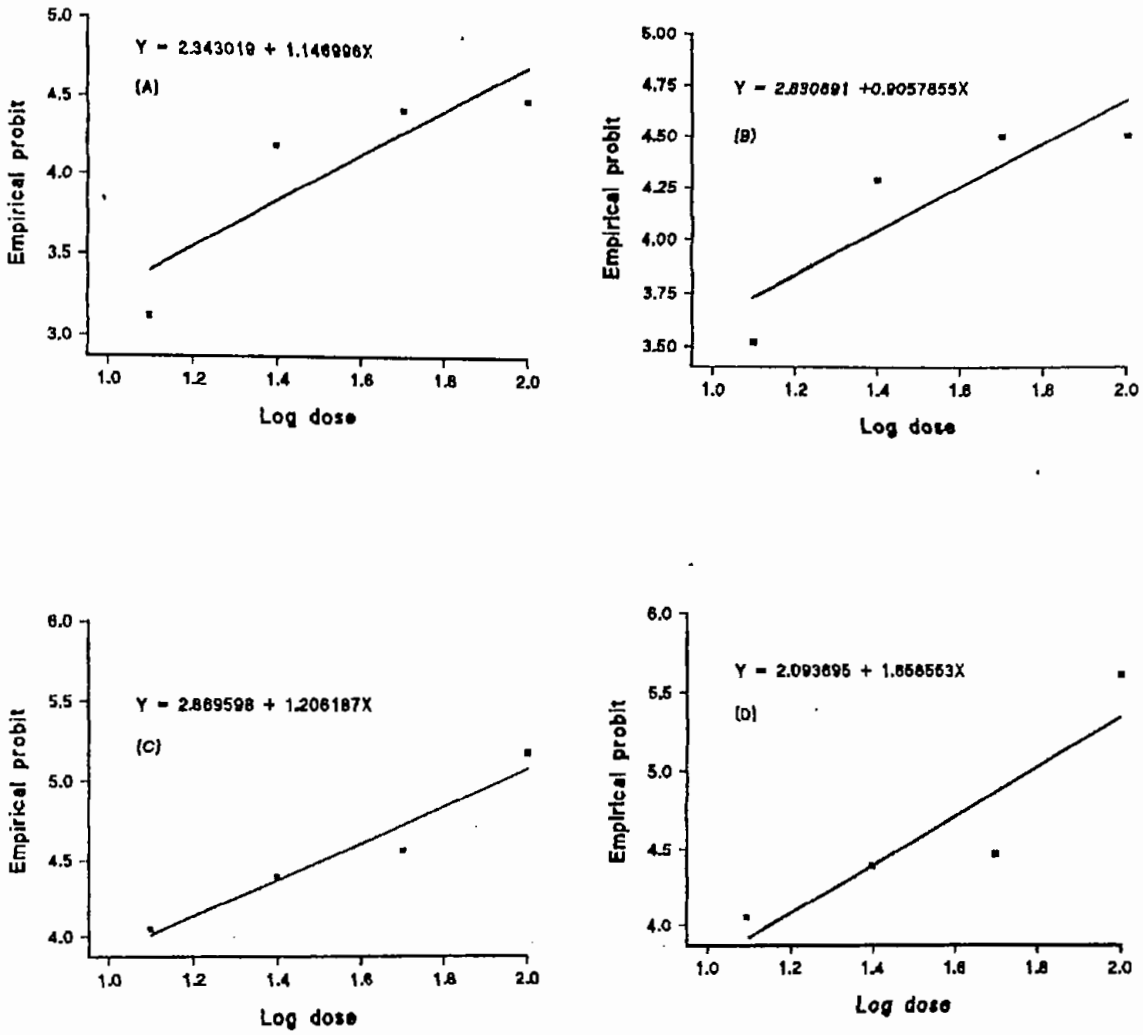


Fig. 4. Regression lines of log-dose of leaf extract of *A. occidentale* in chloroform [A] and [B]; and methanol [C] and [D] after 24 and 48 hours of exposure respectively.

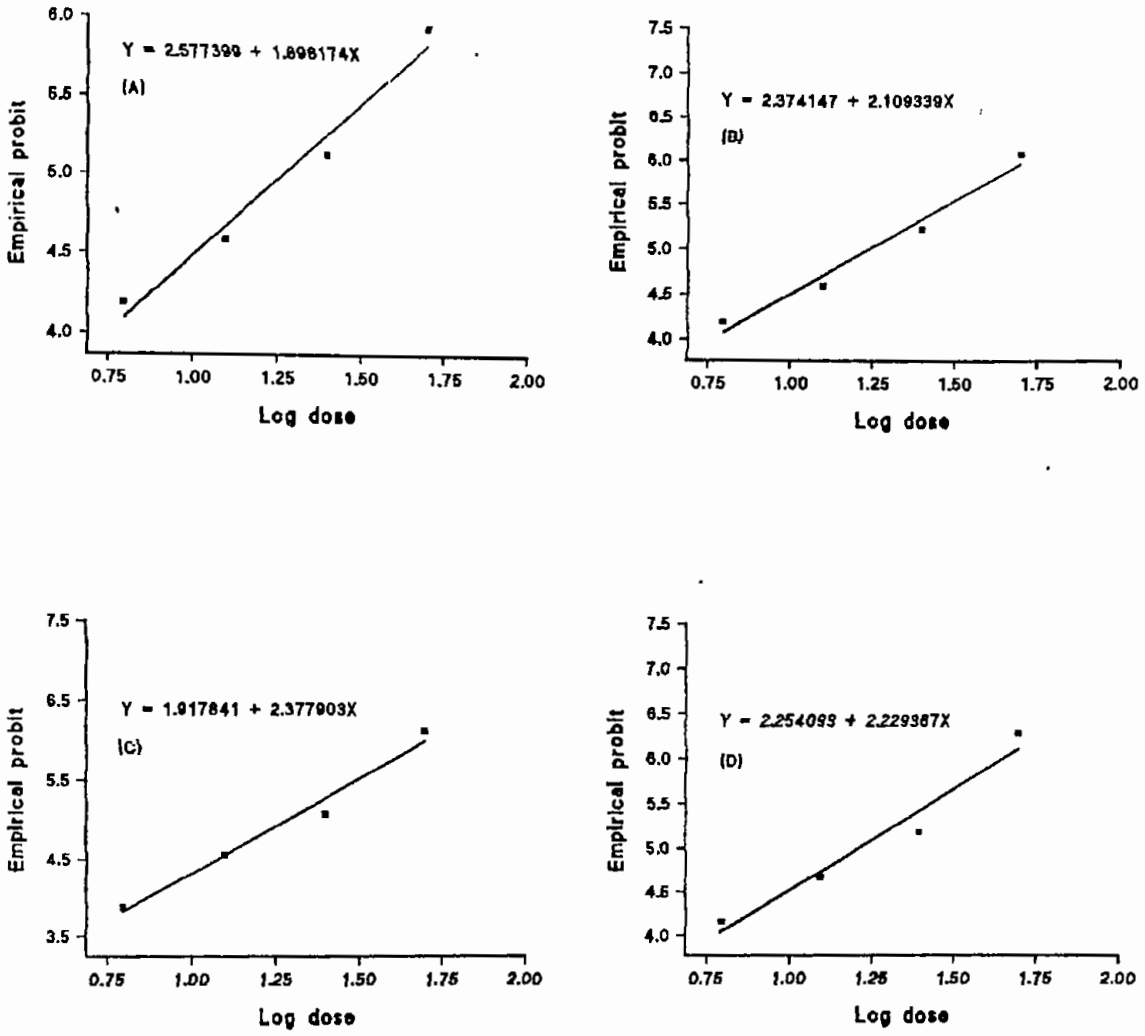


Fig. 5. Regression lines of log-dose of nut-shell extract of *A. occidentale* in chloroform [A] and [B]; and methanol [C] and [D] after 24 and 48 hours of exposure respectively.

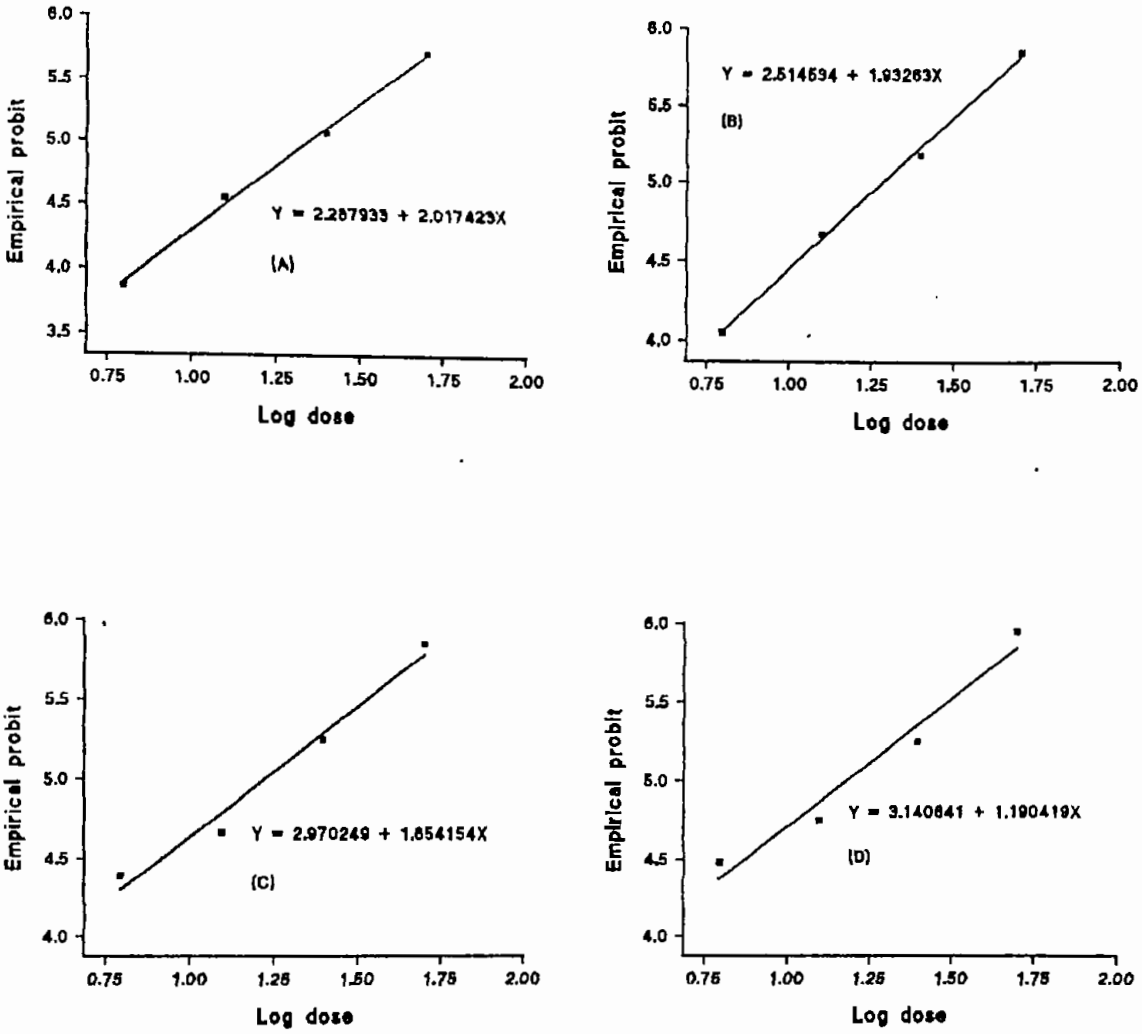


Fig. 6. Regression lines of log-dose of root-bark extract of *A. occidentale* in chloroform [A] and [B]; and methanol [C] and [D] after 24 and 48 hours of exposure respectively

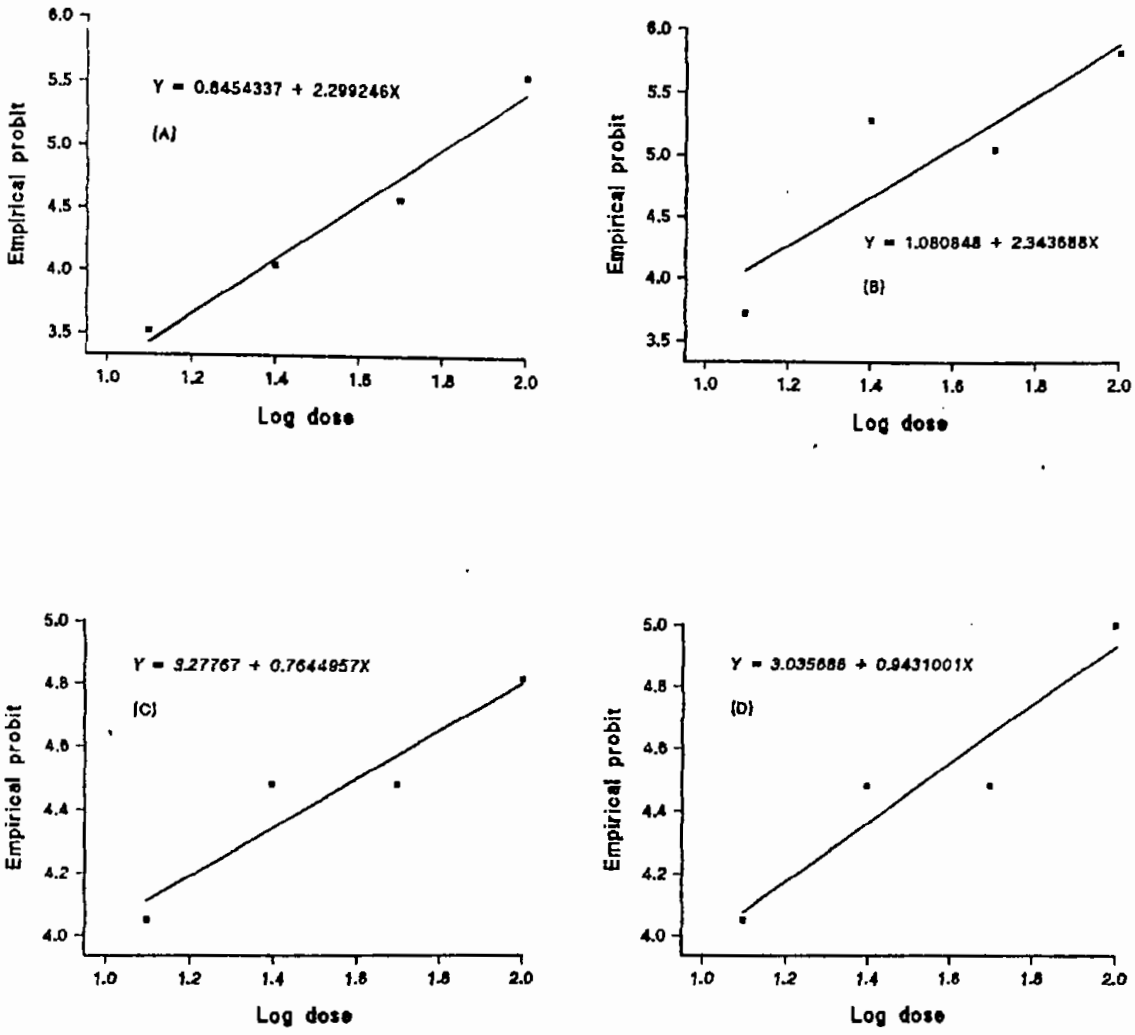


Fig. 7. Regression lines of log-dose of stem-bark extract of *A. occidentale* in chloroform [A] and [B]; and methanol [C] and [D] after 24 and 48 hours of exposure respectively

3.3.3 Surface-film bioassay on *T. castaneum* adults

All the extracts (for both $CHCl_3$ and MeOH) of different parts of *A. occidentale*, viz. leaf, nut-shell, root-bark and stem-bark were subjected to surface film test against *T. castaneum* adults and no trace of activity for them within the given dose ranges was found.

3.3.4 Repellency effect test

All *A. occidentale* extractives showed repellant activity against adult beetles of *T. castaneum* even for a concentration from 10 mg/ml to as less as 1.25mg/ml (10-, 5-, 2.5 and 1.25 mg/ml). The data was read with 1 h interval for up to 5 hours of exposure and was subjected to ANOVA after transforming them into arcsin percentage value and the result is given in Table 5 and 6.

Table 5. Repellency of *T. castaneum* adults by *A. occidentale* $CHCL_3$ extracts.

Plant part	Solvent	Dose (mg/ml)	Repellency percentage (arcsin) at intervals				
			1st hour	2nd hour	3rd hour	4th hour	5th hour
Leaf	$CHCl_3$	10	93.2 (74.88)	86.6 (68.53)	86.6 (68.53)	93.2 (74.88)	86.6 (68.53)
		5	46.6 (43.05)	80 (63.43)	73.2 (58.82)	66.6 (54.70)	-13.4 (-21.30)
		2.5	-13.4 (-21.30)	26.6 (31.05)	53.2 (46.83)	53.2 (46.83)	73.2 (58.82)
		1.25	13.2 (21.30)	13.2 (21.30)	6.6 (14.89)	26.6 (31.05)	-26.8 (-31.05)
Nut-shell	$CHCl_3$	10	93.2 (74.88)	93.2 (74.88)	93.2 (74.88)	93.2 (74.88)	86.6 (68.53)
		5	93.2 (74.88)	53.2 (46.83)	66.6 (54.70)	73.2 (58.82)	. 80 (63.43)
		2.5	26.6 (31.05)	66.6 (54.70)	66.6 (54.70)	53.2 (46.83)	66.6 (54.70)
		1.25	26.6 (31.05)	-20 (-26.57)	40 (39.23)	20 (26.57)	13.2 (21.30)
Root-bark	$CHCl_3$	10	93.2 (74.88)	93.2 (74.88)	93.2 (74.88)	86.6 (68.53)	93.2 (74.88)
		5	93.2 (74.88)	86.6 (68.53)	93.2 (74.88)	86.6 (68.53)	86.6 (68.53)
		2.5	73.2 (58.82)	66.6 (54.70)	66.6 (54.70)	73.2 (58.82)	80 (63.43)
		1.25	40 (39.23)	20 (26.57)	13.2 (21.30)	20 (26.57)	26.6 (31.05)
Stem-bark	$CHCl_3$	10	86.6 (68.53)	93.2 (74.88)	86.6 (68.53)	93.2 (74.88)	93.2 (74.88)
		5	13.2 (26.57)	66.6 (43.05)	80 (39.23)	60 (47.88)	53.2 (86.53)
		2.5	20 (39.23)	46.6 (58.82)	40 (63.43)	93.2 (58.82)	86.6 (50.77)
		1.25	40 (21.30)	73.2 (54.70)	80 (63.43)	73.2 (50.77)	60 (46.83)

Table 6. Repellency test of *T. castaneum* adults by *A. occidental* MeOH extracts against.

Plant part,	Solvent	Dose (mg/ml)	Repellency percentage (arcsin) at intervals				
			1st hour	2nd hour	3rd hour	4th hour	5th hour
Leaf	MeOH	10	86.6 (68.53)	80 (63.43)	86.6 (68.53)	93.2 (74.88)	86.6 (68.53)
		5	66.6 (54.70)	73.2 (58.82)	66.6 (54.70)	73.2 (58.82)	73.2 (58.82)
		2.5	60 (50.77)	46.6 (43.05)	13.2 (21.30)	46.5 (43.05)	46.6 (43.05)
		1.25	53.2 (46.83)	53.2 (46.83)	40 (39.23)	40 (39.23)	46.6 (43.05)
Nut-shell	MeOH	10	93.2 (74.88)	93.2 (74.88)	86.6 (68.53)	93.2 (74.88)	93.2 (74.88)
		5	53.2 (46.83)	66.6 (54.70)	73.2 (58.82)	60 (50.77)	73.2 (58.82)
		2.5	46.6 (43.05)	73.2 (58.82)	66.6 (54.70)	66.6 (54.70)	73.2 (58.82)
		1.25	-33.4 (-35.30)	-33.4 (-35.30)	-33.4 (-35.30)	-33.4 (-35.30)	-33.4 (-35.30)
Root-bark	MeOH	10	73.2 (58.82)	93.2 (74.88)	66.6 (54.70)	66.6 (54.70)	80 (63.43)
		5	66.6 (54.70)	86.6 (68.53)	86.6 (68.53)	66.6 (54.70)	66.6 (54.70)
		2.5	53.2 (46.83)	53.2 (46.83)	46.6 (43.05)	13.2 (21.30)	26.6 (31.05)
		1.25	40 (39.23)	20 (26.57)	20 (26.57)	20 (26.57)	33.2 (35.24)
Stem-bark	MeOH	10	80 (63.43)	93.2 (74.88)	86.6 (67.53)	86.6 (68.53)	86.6 (68.53)
		5	66.6 (54.70)	73.2 (58.82)	80 (63.43)	66.6 (54.70)	73.2 (58.82)
		2.5	46.6 (43.05)	46.6 (43.05)	73.2 (58.82)	66.6 (54.70)	66.6 (54.70)
		1.25	0 (0)	26.6 (31.05)	53.2 (46.83)	53.2 (46.83)	46.6 (43.05)

Table 7. ANOVA results of repellency by *A. occidental* extracts against *T. castaneum.*

Material	Extract	Source of Variation	SS	df	MS	F	P-value
Leaf	$CHCl_3$	Between groups	9139.796	3	3046.599	4.302	0.02
		Within groups	11330.53	16	708.158		
		Total	20470.33	19			
	MeOH	Between groups	2632.662	3	877.554	22.026	6.33E-06
		Within groups	637.442	16	39.840		
		Total	3270.104	19			
Nut-shell	$CHCl_3$	Between groups	8292.960	3	2764.320	12.347	0.0001
		Within groups	3581.902	16	223.868		
		Total	11874.86	19			
	MeOH	Between groups	26323.72	3	8774.575	32.680	4.69E-07
		Within groups	4295.887	16	268.492		
		Total	30619.61	19			
Root-bark	$CHCl_3$	Between groups	5487.855	3	1829.285	30.585	7.35E-07
		Within groups	956.950	16	59.810		
		Total	6444.805	19			
	MeOH	Between groups	3621.235	3	1207.078	16.582	3.63E-05
		Within groups	1164.653	16	72.790		
		Total	4785.888	19			
Stem-bark	$CHCl_3$	Between groups	1873.900	3	624.633	3.234	0.05
		Within groups	3089.890	16	193.118		
		Total	4963.790	19			
	MeOH	Between groups	3288.092	3	1096.030	9.190	0.0009
		Within groups	1908.250	16	119.265		
		Total	5196.341	19			

3.4 Discussion

Consoli *et al.*, (1988) studied effect of anacardic acid against *Aedes fluviatilis* with much success. In their work anacardic acid was found effective at 10 ppm, which is promising in the field of crop protection and pesticide technology. Molluscicidal activity was found in the nut-shell liquid of *A. occidentale* (Laurens *et al.*, 1987; Mendes *et al.*, 1990). The molluscicide activity of hexanic extract from *A. occidentale* L. (cashew) nut shell, of copper complex, of lead complex and anacardic acid has been compared in the laboratory in an attempt to obtain better stability than anacardic acid. Ten products of the mixture obtained were tested on adults of *Biomphalaria glabrata*, the schistosomiasis transmitting snails at 1 to 10 ppm. The most active products were copper complex, obtained by cupric sulfate plus sodium hydroxide, and anacardic acid (sodium hydroxide), which presented activity at 4 ppm. The anacardic acid's lead content was above the limits accepted by the United States standards (de Souza *et al.*, 1992).

Plant stimuli (visual, mechanical, and chemical) can influence animal behaviour positively by attracting them from a distance or by stimulating oviposition and feeding on contact. Negative reactions are triggered by repellents acting at some distance or elicited by antifeedants on contact (Städler, 2000). A means of saving plants from pests would thus be disguising their natural odour with an extraction from a repellent plant, i.e. the odour of another plant having repellent effect on insects. Since plants contain very different compounds simultaneously, they exert not only repellent but also antifeedant, morphogenetic and toxic effects on insects (Dethier *et al.*, 1960; Dethier, 1970; Schoonhoven, 1982; Schmutterer, 1992; Kuusik, *et al.*, 1995). The consequences of these effects can be determined in laboratory conditions.

Deterrents prevent the insect from further feeding by affecting the peripheral nervous system. Toxicants work once digested by the insect by disrupting cellular,

biochemical, and physiological processes (Mendel *et al.*, 1991a,b). Most other insecticidal compounds act by affecting the insect's central nervous system (Gonzales *et al.*, 1995). Most frequently available allelochemical compounds to cause antifeedancy are alkaloids flavonoids, terpene, lactones and phenoles (Smith, 1989).

The surface film method of application of crude extract doses, as well as the fumigant activity test gave no positive results, while topical application of the same extracts gave interesting results by posing toxic effect to the test beetles. Bicalho and Rezende (2001) reported presence of volatiles also in the fruit of *A. occidentale*, but no fumigant activity was found in the crude extracts of the above mentioned materials of the title plant. However, repellency was found promisingly in the order of $CHCl_3$ extract of root-bark, MeOH extract of nut-shell, leaf root-bark and stem-bark extract and then $CHCl_3$ extracts of nut-shell, stem-bark and leaf respectively

Chapter 4

Isolation of Compounds

CHAPTER 4

ISOLATION AND PURIFICATION OF COMPOUNDS

4.1 Introduction

Although people knew the therapeutic uses of many plants, people could not isolate the physiologically active substances from plants before 1803. Narcotine was firstly isolated from syrupy extract of *Opium.* This beginning opened up a new era of successful isolation of a large number of other active substances from the plants. Plants are the important source of a diverse range of bioactive principles, so plants are referred, 'as a chemical factory'. Plants can synthesize carbohydrates, fats, proteins, essential oils, resins, tannins and bitter principles.

The principle function of secondary chemicals in Anacardieaceae family is presumably as a defense against herbivores. Murthy *et al.*, (1968) describes the separation and identification of cardanol and cardol fractions of cashew nut shell liquid using paper, thin-layer, and column chromatographic methods. Separation of cardanol according to the degree of unsaturation was achieved by chromatography on a silica gel-silver-nitrate column. However, phenolic, resorcinolic and catecholic lipids have also been illustrated (Murthy and Yadava, 1972; Przeworska *et al.*, 2001). Cardanol, the main constituent of technical cardanol, was successfully separated into four components, viz. saturated, mono-, di-, and tri-olefins. The presence of a vinyl double bond in the tri-olefins was also confirmed. Anacardic acids, 2-methylcardols,

and cardols isolated from various parts of the cashew (*A. occidentale*) fruit have been found to exhibit tyrosinase inhibitory activity. The Anacardiaceae is an exceptional plant family. Secondary chemicals found within the Anacards have a wide-spread influence on human life (Hartley, 2003). Kubo *et al.* (1994) completed studies with the two principle active compounds 6-[8(Z), 11(Z),14-pentadecatrienyl salicylic acid and 5-[8(Z),11(Z),14-pentadecatienyl] resorcinol. The results indicated that both of these phenolic compounds exhibit characteristic competitive inhibition of the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA). Tyrosinase is one of the most important enzymes in the molting process and this research could results in the development of an alternative insect control agent.

For the separation of pure substances the availability and choice of chromatographic techniques are essential for the successful program involving the investigation of biologically active plant constituents. The aim is to have maximum yield with minimum effort (to reduce the time and cost of the separation procedure). Preparative separation techniques can be tedious and time consuming, especially when complex mixtures, such as crude plant extracts have to be resolved. Over the passed decade or so, several new techniques have been introduced, leading to acceleration and simplification of different separation problems (Hostettmann *et al.*, 1986; Marston and Hostettmann, 1991; Hostettmann *et al.*, 1991). However, there is no universal technique capable of solving every isolation problem. All methods have advantages and limitations so much, so that the best results are often obtained by a combination of two or more of these.

Among the most important preparative separation techniques employed in the isolation and purification of plant constituents thin layer chromatography and open column chromatography have been used simultaneously in this investigation since we do not have other equipment available in our laboratory. Thin layer chromatography

was used to select the slurry or the solvent system for the successful run of the open column chromatography.

4.2. Materials and Methods

4.2.1 Chromatography on TLC plates

To select the solvent system for the run of the open column separation was made on the preparative thin layer chromatographic plates. For the normal phase chromatography silica gel G60 F_{254} on Al sheets (Merck) were used. Ten mg/ml of the sample in the solvent extraction offered 100 µg/spot by spotting 10 µl for each of the sample extracts. The chromatograms then developed within a conventional camber with the following solvent systems: Pet. ether-EtOAc (1:1) for $CHCl_3$ extract and $CHCl_3$ –MeOH-H_2O (65:35:05) for MeOH extracts. All chromatograms were observed under UV at 254 and 366 nm and marked with a pencil.

4.2.1.1 Revelation of compound spots by reagent spray

A mixture of the equal volume of 1% ethanolic solution of vanillin and 3% aqueous solution of perchloric acid was sprayed and soon after that 10% ethanolic solution of H_2SO_4 was also applied in the same way before drying the plate at 100°C to reveal the compound spots (Godin, 1954) (Plate 6).

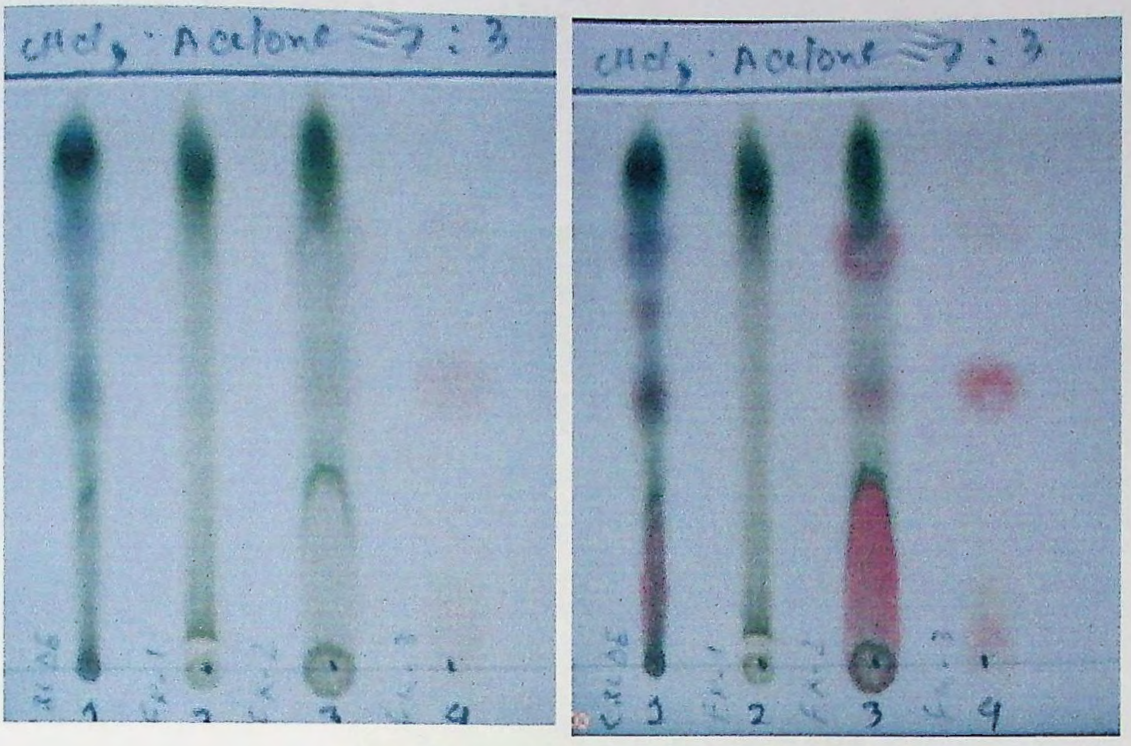


Plate 6. Revelation of compound spots by Godin reagent spray

4.2.2 Open column chromatography

Of the methods in the solid phase category, column chromatography is very popular and used extensively. It can include non-exchange resins, polymeric columns, gel-filtration and chromatography over silica gel or chemically modified silica gel. Open column chromatography has a high load capacity but the separation time is long and the resolution is respectively low.

The stationary phase for the open column chromatography was silica gel Si60 (70-230 mesh and 230-400 mesh) (Merck) and glass column of different size (2.5×28 cm, 2.5×32 cm, 3×35 cm, 3.25 × 48 cm, etc.) were used. Cotton pads washed with acetone, chloroform and MeOH was used at the base of the gel column. A similar cotton cloud was used at the top of the column (after application of the sample and the solvent) to protect destruction of the sample layer (Plate 7). Selected solvent systems

were used as eluents and the elution rate was 1 ml/min. Fractions were collected carefully.

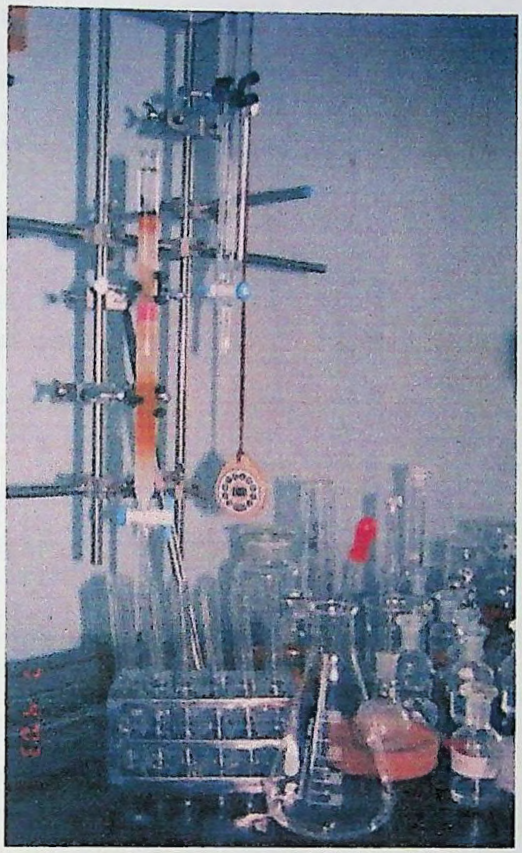


Plate 7. Open column used in the experiment.

4.2.2.1 Gel filtration

Open columns were used to apply sephadex LH-20 (Pharmacia) for the chromatography of exclusion. For methanol soluble samples MeOH (100%) and for $CHCl_3$ soluble samples $CHCl_3$–MeOH (1:1) system were used. The eluent allowed about 0.5 ml/min.

4.2.3 Isolation of compounds

Chromatography is an analytical technique for separating compounds on the basis of differences in affinity for a stationary and mobile phase. The separation of pure constituents from plant materials chromatography is a popular technique. The aim of choice any technique for separation is to have maximum yield with minimum effort to reduce the time and cost of the separation procedure. Preparative separation techniques can be tedious and time consuming, especially when complex mixtures, such as, crude plant extracts have to be resolved. In the present study for isolation of the pure compounds from different parts, viz. leaves, nut-shell, root-bark and stem-bark of *A. occidentale* were done mainly by open column chromatography, while Thin Layer Chromatography (TLC) was used as a supporting tool.

4.2.3.1 Selection of extracts for fractionation

For fractionation of the extraction with a view to isolate biologically active compounds all the extracts were subjected to biological assay. Repetition of the same assay is required until the purification of the target compound, and thus a suitable bioassay technique was selected. A plant pathogenic fungus, *V. ceratosperma* was taken in this regard as a test organism, however pure products were again tested with the title test insect *T. castaneum.*

Test samples of 10 mg/ml of solvent were diluted to give a concentration through which it was possible to diversified doses from 10 µg/ml. This was spotted on a TLC to make a chromatogram to culture the test fungus on to it to see clear zones on it as a phenomenon of bioactive compound's existence in it.

Inoculum of spores in PDA medium at below 45°C was sprayed onto TLC plates with a 30 ml sprayer and atomizer bulb. After solidification of the medium, the test plates were put in polythene boxes lined with moist filter paper. The whole system was wrapped in a polythene bag to avoid drying and was kept in an incubator

at 25°C for 60-72 hours. Clear zones were observed against a dark background that had been produced by the fungus itself. The bioassay results indicated promising biological activity in the chloroform extracts of the nut-shell, root-bark and the stem-bark.

4.2.3.2 Selection of slurry (solvent system) for respective extracts

Thin layer chromatography is a very convenient technique for isolation and purification of different types of secondary metabolites. The mixtures of the compounds were well separated from each other and resolved by preparative thin layer chromatographic technique. This tool is considered to be one of the most helpful methods for the detection of organic compounds, which involves an adsorbent (using silica gel) as stationary phase and a solvent system as the mobile phase. Due to the differential rate of adsorption on the adsorbent, the components in a mixture migrate differentially along with the TLC plate. In other words due to the difference in mobility of the components often depend on their polarity and on that of the solvents used.

Aluminium backed precoated preparative thin layer chromatographic (TLC) plates (20 × 20 cm) with silica gel GF_{254} with 0.5 mm thickness and active in the usual manner (Merck, Germany) were used. The sample was applied on the activate plates with the help of a gradient micropipettes as a narrow band at 1 cm above the lower edge of the plate to make sure that the sample was not washed away when the plates were placed inside the TLC chamber with the solvent system. The plates were then developed in the usual manner.

After development, the chromatograms were air dried and observed visually under UV light (254 nm) and sprayed with Godin reagent (Godin, 1954). The distinct bands were expected and by changing the solvent system with increase of either the polar or the apolar one. After having a better separation the selected solvent system was applied on the open column for isolation of the compounds by fractionation.

Thin layer chromatography was used to select the solvent systems for the selected chloroform extracts. Small pieces of aluminium backed TLC plate was taken to spot the target extracts and run with a mixture of a relatively polar and a relatively apolar solvent (1:1). For the better separation on the TLC with a known stationary phase the amount of both solvents were increased or decreased and applied accordingly. The combination given a better separation was selected for that extract for fractionation on the open column. However, no choice was for the LH_{20} if it was cloroform soluble property then $CHCl_3$-MeOH (1:1) was applied. If any fraction found soluble only in MeOH then 100% MeOH was the eluent for that fraction.

4.2.3.3 Isolation of compounds by open column chromatography

Open column chromatography is a very convenient technique for fraction and separation of different types of secondary metabolites. The mixture of components in column chromatography is separated into several fractions according to the relative affinity of the component between the stationary phase and the mobile phase. For the development of the column gradient elution technique is preferred. The component having lower affinity to the adsorbed component eluted gradually. The chromatographic column was prepared as follows.

Cylindrical columns made of glass; drown at one end to from narrow tube. The lower constricted end of the column was fitted with a stop cork for controlling the flow of the effluent. The column was made by pouring down the slurry of the silica gel (70-230 mesh and 230-400 mesh) in the suitable solvent and allowing the silica gel to settle down. The pouring of slurry that was selected earlier by thin layer chromatography was continued until the column of desire height was obtained. The solvent layer should always be kept above the absorbent bed to avoid cracking of the column. At the end of preparation of the column a little amount of the slurry kept on the upper surface of the gel matrix for the convenience in application of the extract in dissolved state.

4.2.4 Detection of the compound on TLC by Godin revelation

The properly developed plates were dried and viewed visually under UV light and Godin reagent (Godin, 1954) spray were used and number of compounds separated.

Visual detection: The development chromatogram was examined visually to detect the presence of colored compound.

I) UV light (254 and 366 nm): After development and drying the chromatogram was examined under UV light to detect fluorescent compound and the glowing spots, which were then marked.

II) Godin reagent spray: Equal volume of 1% ethanolic solution of vanillin and 3% aqueous solution of perchloric acid was mixed and sprayed on to the prepared chromatogram and 10% ethanolic solution of H_2SO_4 was also sprayed afterwards and allowed the plate to dry out at 100°C by using a hair dryer. Revelation was observed in different colors for different compounds (Godin, 1954).

III) Measurement of R_f values: The R_f values of the separated compounds were calculated on a developed chromatogram using the pre-established solvent system. The R_f values were calculated by the following formula.

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

4.2.5 Isolation of nut-shell compounds

For the first fractionation LH_{20} (Pharmacia) was used as the stationary phase and $CHCl_3$-MeOH (1:1) was the eluent on a glass column of 3.25 × 60 cm for 2g of the nut-shell extract. Elution time was adjusted to yield 1 ml/min. It gave 26 tubes, which were then spotted on TLC to run and reveal the compounds by reagent spray.

Two fractions were made for tubes 1-21 (T/1-21) and tubes 22 to 26 (T/22-26). Biological assay with *V. ceratosperma* indicated T/22-26 for the presence of bioactive components there in and that was then subjected to fractionation. Selecting a solvent system by TLC, equal volume of chloroform and acetone ($CHCl_3$-acetone 1:1) was applied on a glass column of 3.25 × 48 cm was packed with silica gel (70-230 mesh) (Sigma). The elution was kept similar to that of the previous one. This fractionation yielded 28 tubes and TLC was made for all of them to get 4 fractions as T/1-4, T/5-8, T/9-16 and T/17-28. Biological assay with *V. ceratosperma* indicated T/9-16 for the presence of bioactive components and that was then subjected to fractionation.

Similar to above solvent system was found $CHCl_3$-acetone 9:1 to use on the same type of silica gel (70-230 mesh) and again this column gave 28 tubes to have 3 fractions, T/1-3, T/4-11 and T/12-28. Biological assay then indicated T/12-28 for the presence of bioactive components and this was subjected to fractionation. Selecting a solvent system by TLC, cent percent MeOH was applied on LH_{20} on a glass column of 2.5 × 32 cm to give 10 tubes which were then spotted on TLC to run and reveal the compounds by reagent spray. Two fractions were made for tubes 1-6 (T/1-6) and tubes 7 to 10 (T/7-10). Biological assay with *V. ceratosperma* indicated T/7-10 for the presence of bioactive components and that was then subjected to fractionation. Selection of a solvent system by TLC, equal volume of chloroform and ethyl acetate ($CHCl_3$-EtOAc 1:1) was applied on a glass column of 2.5 × 28 cm was packed with silica gel (230-400 mesh) to give 15 tubes. Four fractions were made for tubes 1-5 (T/1-5), 6 to 9 (T/6-9), tubes 10 & 11 (T/10-11) and tubes 12 to 15 (T/12-15). Fractions T/6-9 and T/12-15 were pure compounds and biological assay indicated that both of them biologically active.

4.2.6 Isolation of root-bark compounds

For the first fractionation of the chloroform extract of the root-bark LH_{20} (Pharmacia) was used as the stationary phase and $CHCl_3$-MeOH (1:1) was the eluent

on a glass column of 3.25 × 48 cm for 1g of the extract. Elution time was adjusted to yield 1 ml/min. It gives 28 tubes, which were then spotted on TLC to run and reveal the compounds by reagent spray. Five fractions were made for tubes 1-6 (T/1-6), 7-11 (T/7-11), 12-15 (T/12-15), 16-22 (T/16-22) and tubes 23 to 28 (T/23-28). Biological assay with *V. ceratosperma* indicated T/12-15 for the presence of bioactive components there in and that was then subjected to fractionation. Selecting a solvent system by TLC, Pet. Benzene and EtOAc (Pet. Benzene-EtOAc 9:1) was applied on a glass column of 2.5 × 32 cm was packed with silica gel (70-230 mesh) (Sigma). The elution was kept similar to that of the previous one. This fractionation yielded 42 tubes and TLC was made for all of them to get 4 fractions as T/1-9, T/10-17, T/18-31, and T/32-42. The third fraction gave white crystal needles as a pure compound which was tested on TLC under UV and Godin reagent spray to give purple colour. Just washing the 2nd fraction (T/10-17) with only MeOH a pinkish powder was appeared as compound RB-2.

4.2.7 Isolation of Stem-bark compounds

Similar to that of the above two fractionation in the first fractionation for the stem-bark extract LH_{20} (Pharmacia) was used as the stationary phase and $CHCl_3$-MeOH (1:1) was the eluent on a glass column of 3.25 × 48 cm for 750 mg of the extract. Elution time was adjusted to yield 1 ml/min. This column gave 45 tubes, which were then spotted on TLC to run and reveal the compounds by reagent spray. Three fractions were made for tubes 1-15 (T/1-15), 16 to 23 (T/16-23) and tubes 24 to 45 (T/24-45). Biological assay with *V. ceratosperma* indicated T/16-23 and T/24-45 for the presence of bioactive components there in and those were then subjected to fractionation.

Selecting a solvent system by TLC, Pet. Benzene and EtOAc (Pet. Benzene-EtOAc, 9:1) was applied on a glass column of 2.5 × 28 cm was packed with silica gel (70-230 mesh) (Sigma). The elution was kept similar to that of the previous one. This

fractionation yielded 31 tubes and TLC was made for all of them to get 4 fractions as T/1-6, T/7-17, T/18-20 and T/21-31. The second fraction was the white crystal (needles) and a pure compound confirmed by TLC with visual observation under UV and Godin reagent spray. Pet. Benzene-$CHCl_3$ (1:1) was the solvent system on silica gel (230-400 mesh) column for the fraction T/24-45. With the same rate of elution 58 tubes were collected while biological assay indicated the second fraction (T/12-24) among the six fractions T/1-11, T/12-24, T/25-37, T/38-42, T/43-49 and T/50-58 for the presence of bioactive component, which was also a crystal.

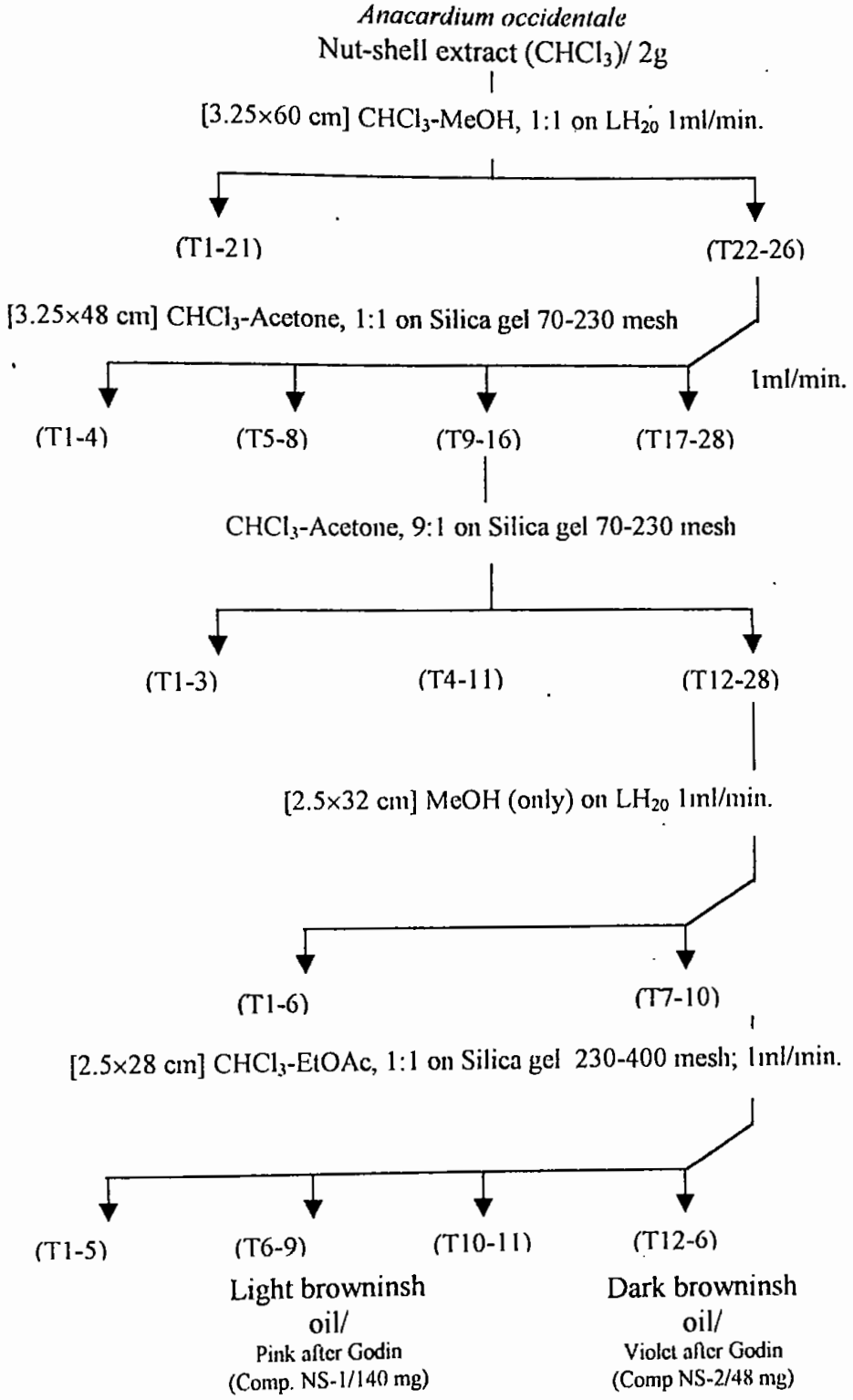


Fig. 8. Isolation pathway of nut-shell compounds

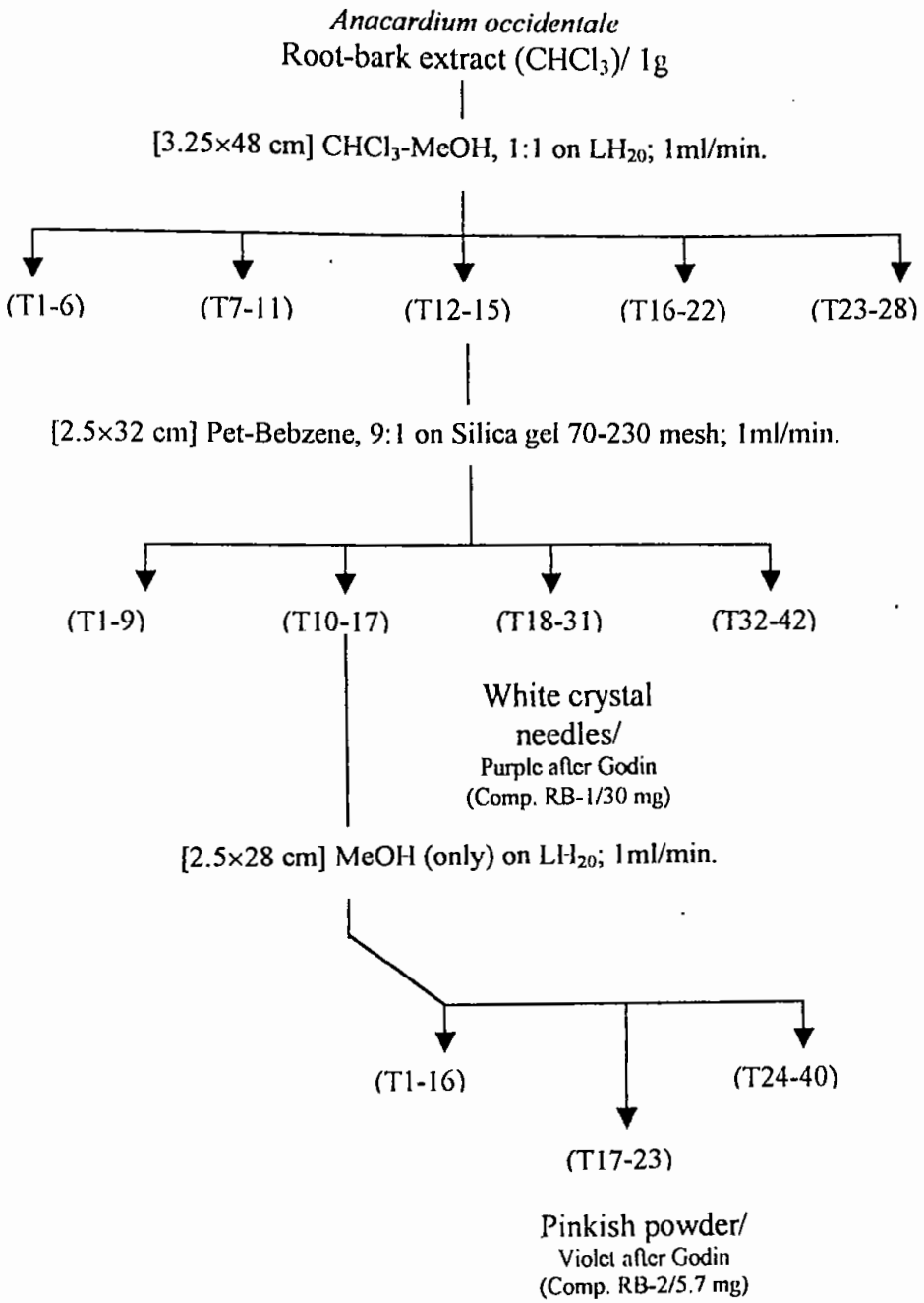


Fig. 9. Isolation pathway of root-bark compounds

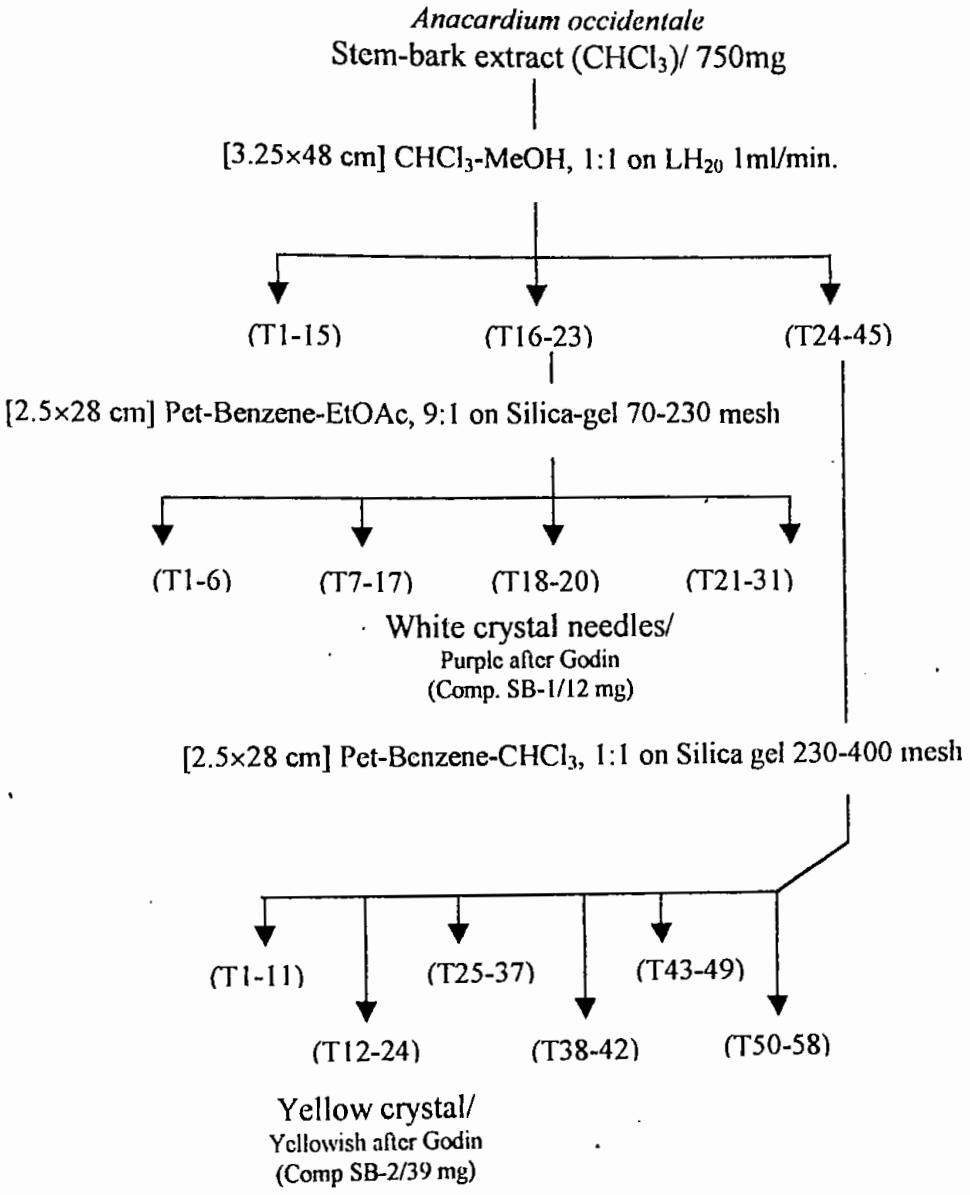


Fig. 10. Isolation pathway of stem-bark compounds

4.3 Results

4.3.1 Physical remarks of the pure compounds

The isolated compounds and there physical stature have been presented in Table 8. Two compounds from the nut-shell extract were isolated and named NS-1 and NS-2. About 140 mg of the NS-1 has been purified which was a light brownish oil and after Godin reagent spray it takes pink in color on the TLC. NS-2 was 48mg in amount and it was a dark brown oil. It gets violet in color after Godin reagent spray.

The first root-bark component RB-1 was 30 mg and it was white crystal needles. After Godin it gets purple in color and the second one RB-2 was a pinkish powder and only 5.7 mg in amount, which gets violet in color after Godin reagent spray.

The stem-bark compounds isolated were SB-1 and SB-2 and were 12- and 39 mg in amount respectively. SB-1 was a white crystal needles and similar to that of the white crystals form the root-bark as mentioned above as RB-1. After Godin reagent spray it was also purple in color like the RB-1 (and after taking NMR it was confirmed that both the compounds from two different sources were the same, but bioassay with them was not possible as they were applied separately and separate amounts were not sufficient to do bioassays).

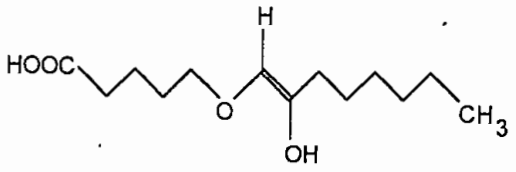
Table 8. Pure compounds (with their physical remarks) isolated from *A. occidentale*.

Plant part	Extract	Comp.	Amount mg	Physical identity of the compounds/after Godin	Bioassay done
Nut-shell	$CHCl_3$	NS-1	140.0	Light brownish oil/Pink	Y
		NS-2	48.0	Dark brownish oil/Violet	Y
Root-bark	$CHCl_3$	RB-1	30.0	White crystal needles /Purple	Y
		RB-2	5.7	Pinkish powder/Violet	-
Stem-bark	$CHCl_3$	SB-1	12.0	White crystal needles /Purple	Y
		SB-2	39.0	Yellow crystals/Yellow	Y

4.3.2 Structures of the compounds

Compound NS-1 (Fig. 11)

The spectrum shows characteristics of an unsaturated fatty acid. Compared with the spectrum of NS-2, one significant difference is the lack of aromatic signals, which suggests it represents a free unsaturated acid. Possibly linoleic acid.

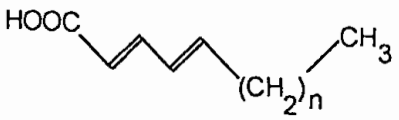


Compound NS-1

Compound NS-2 (Fig. 12)

Apart from the same solvent residual and TMS, the rest of the signals of the spectrum can be divided into four distinctive groups: a, below 1 ppm, only one signal at 0.85 ppm. The signal is a triplet, which means a methyl group attached to a methylene chain (CH_3-CH_2-...); b, from 1ppm to 3 ppm, all the methylene signals (-CH_2-) in the molecule, which either appears as distinctive signals, or superposed strong singals at 1.30 ppm, according to the different chemical environment of the individual protons); c, from 4.8 ppm to 6 ppm, signals in this region indicate olenific protons); d, 6.5 ppm to 7.5 ppm, where aromatic protons appear. It can be suggested from these spectral characters that the compound has two structure parts. One is an aromatic ring with certain substitution pattern; another is an aliphatic side chain with certain degree of unsaturation. The aromatic region shows the signal of three protons, the singal at 7.28 ppm is a triplet (split by two protons), while the signals at 6.82 ppm and 6.74 ppm are both doublets (split by one adjacent proton), indicating the aromatic ring is a tri-substituted ring, with three free aromatic protons adjacent to one another. This can translate into a 2-hydroxybenzoic acid unit with a side chain substitution at position 6, which agrees with the aromatic part of the structure of anacardic acid.

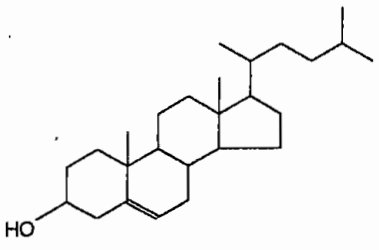
Examination of the rest of the signals also reveals that they match those of anacardic acid. Therefore, compound NS-2 was identified as anacardic acid.



Compound NS-2

Compound RB-1 (Fig. 13)

Compound RB-1 and SB-1 are the same in their physical remarks as white needle crystals and has no UV absorption. After Godin reagents gave bright purple spot after the development in TLC plate, which suggests it to be a terpenoid or steroid. From the 1H NMR spectrum, firstly the solvent residue peak at ca. 7.27 ppm (from the residual H of $CDCl_3$) and reference peak at 0 ppm from TMS were identified. The true signals gave out the following information: It has the general characteristics of a sterol. Aliphatic methylene and methine proton signals were second to and partially superposed with six methyl proton signals. Two of the methyl signals were singlets, which means no vicinal carbon has a proton attached (or quaternary carbon), at 1.01 ppm (Me-19) and 0.68 ppm (Me-18). The left four methyl signals were doublets, which means they are coupled with and split by one proton carried on the vicinal carbons. The signals of one olenific proton at 5.36 ppm and one proton attached to an oxygenated carbon at 3.52 ppm were also observed. These characteristic data were in perfect match with reported values of beta-sitosterol by Yu *et al.* (1989).

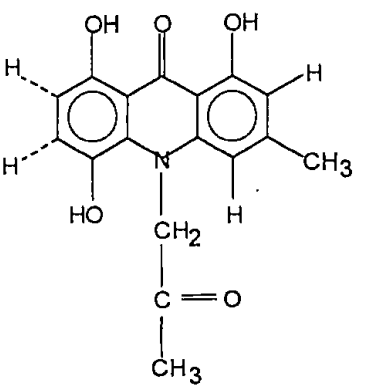


Compound RB-1

Compound SB-2 (Fig. 14)

Compound SB-2 shows the following ^{1}H-NMR spectral data: ^{1}H-NMR (400MHz, $CDCl_3$) δ: 7.65 (1H, bs, ArH), 7.35 (1H, d, J=4.0 Hz, ArH), 7.07 (1H, bs, ArH), 6.67 (1H, d, J=4.0 Hz, ArH), 3.95 (3H, S, OAc), 3.48 (2H, S, N-CH2), 2.45 (3H, S, $ArCH_3$)

From the above ^{1}H-NMR spectral data of the compound SB-2 two aromatic signals appeared at δ 7.35 and 6.67 with a J value of 4.0 Hz are assigned to be vicinal aromatic protons. Moreover, broad singlets or ortho, para aromatic protons appeared at δ 7.07 and 7.65 of another aromatic ring. Methyl protons of an acetyl group appeared at δ 3.95 equivalent to three protons. Another three proton singlet appeared at δ 2.45 and can be assigned as aromatic methyl group protons. A methylene proton appeared at δ 3.48 as singlet can be assigned as N-CH_2 proton with a neighbouring OAc group. Lack of other spectral data the structure of the compound SB-2 isolated from the chloroform extract of the stem-bark of *A. occidentale* can tentatively be assigned as Fig. on the basis of ^{1}H-NMR spectral data only.



Compound SB-2

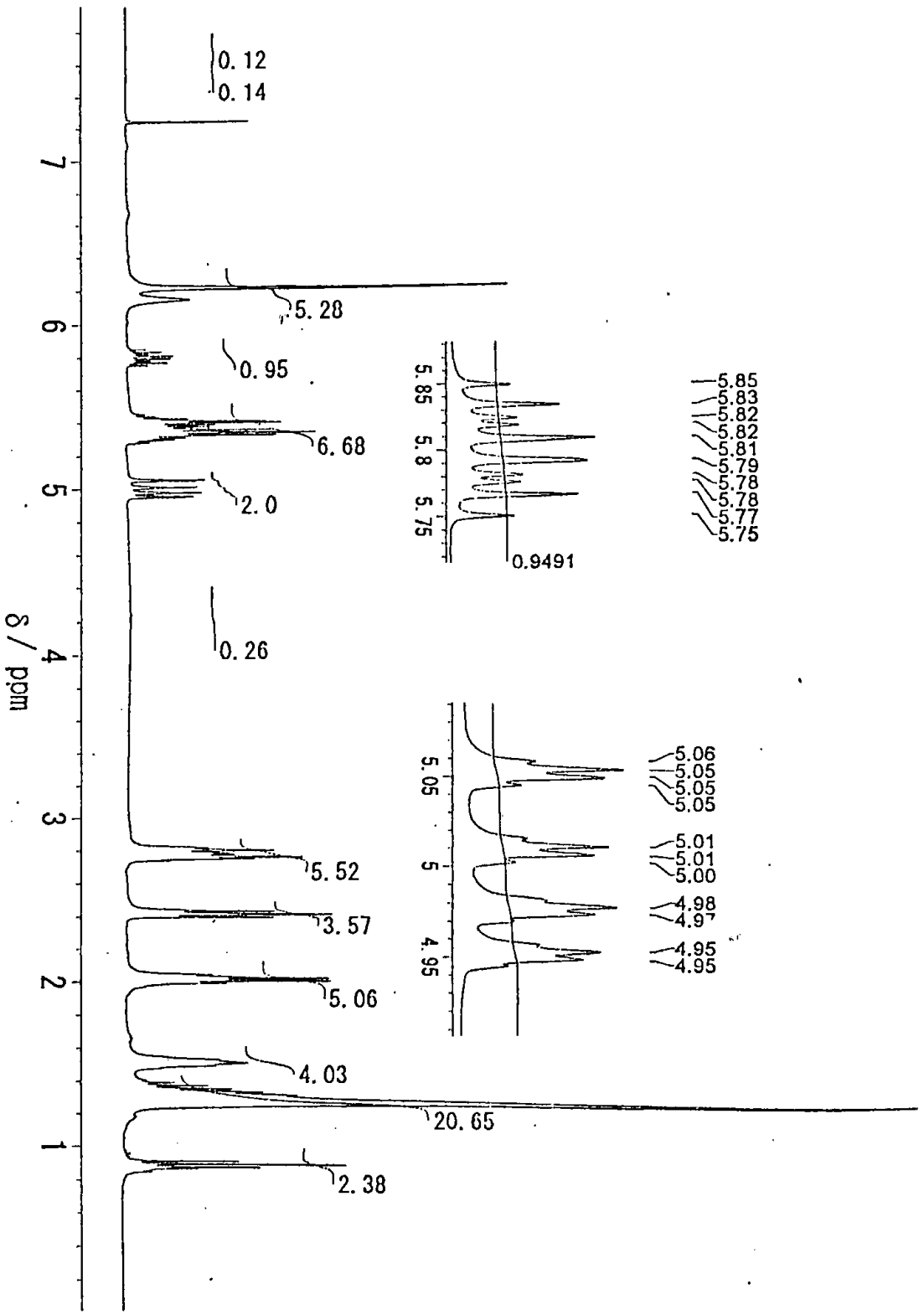


Fig. 11. ^{1}H-NMR proton spectra of nut-shell compound NS-1

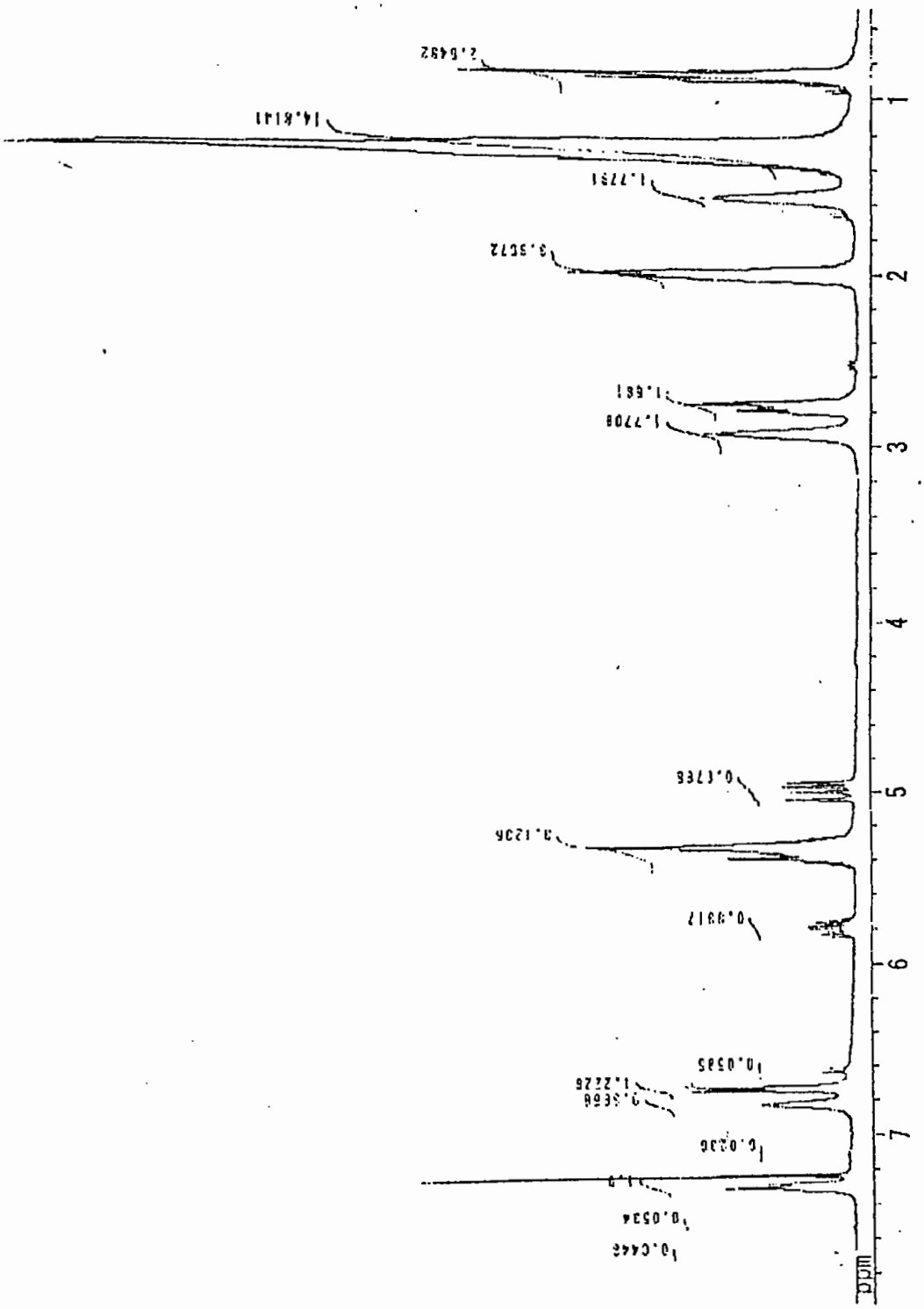


Fig. 12. ^{1}H-NMR proton spectra of nut-shell compound NS-2

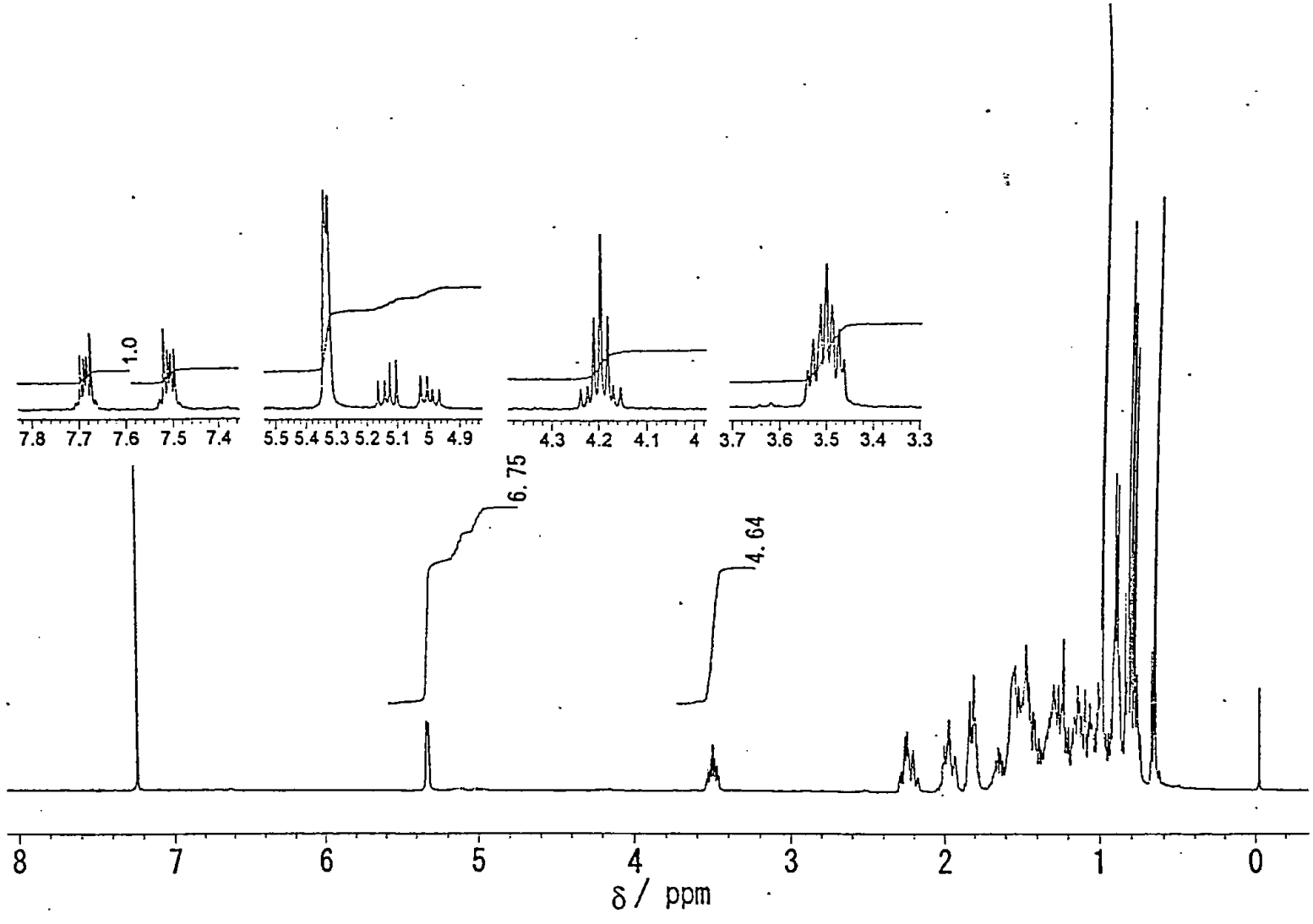


Fig. 13. ^{1}H-NMR proton spectra of root-bark compound RB-1

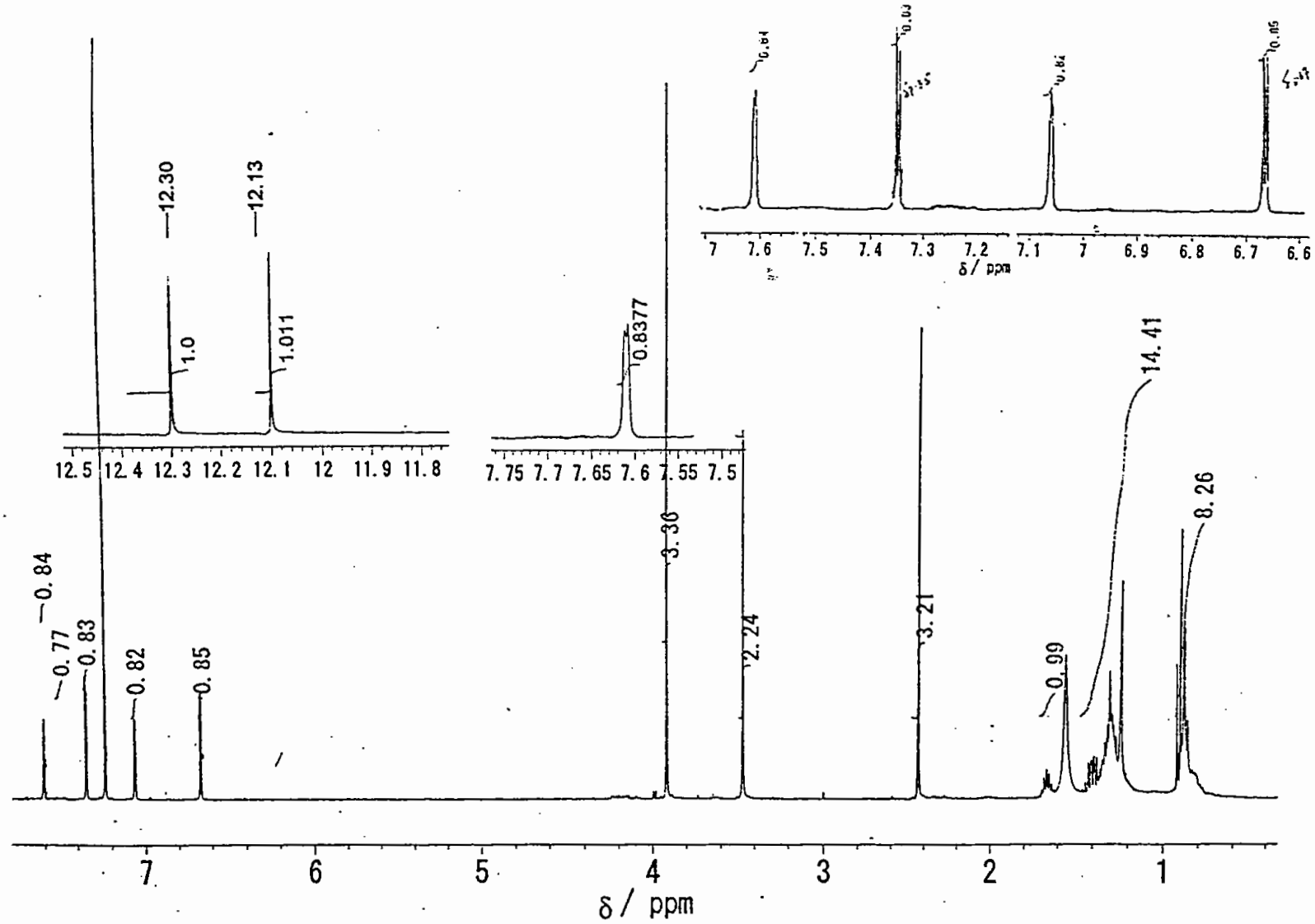


Fig. 14. ^{1}H-NMR proton spectra of stem-bark compound SB-2

4.4 Discussion

Bicalho and Rezende, (2000) reported the volatile compounds of a largely consumed Brazilian cashew apple variety (*A. occidentale* L. var. *nanum*) were recovered by headspace extraction or simultaneous distillation-extraction and several compounds including esters, terpenes, hydrocarbons, carboxylic acids, aldehydes, alcohols, ketones, lactones and norisoprenoids were characterized and quantified by gas chromatography and mass spectrometry analyses. However, Beare-rogers *et al.*, (2001) reported presence of β-Sitosterol and β-Caritene in the title plant as very common constituents.

The nutshell oil of the test plant afforded mixtures of anacardic acids and cardols in varying degrees of unsaturation. This work was attempted and performed by Consolacion, *et al.*, (2002) to elucidate their structures by extensive 1D and 2D NMR and high resolution mass spectrometry. The anacardic acids were identified as 1a (50%), 1b (17%) and 1c (33%), while the cardols were identified as 2a (80%) and 2b (20%). Small amounts (<1%) of mono-, di- and tri-unsaturated seventeen-carbon side-chain analogues of sample 1, and bilobol, the monounsaturated analog of sample 2, were also observed by mass spectrometry.

The fruit-shell contains β-catechin, β-sitosterol, and *l*-epicatechin; also proanthocyanadine leucocyanadine, and leucopelargodonidine. The dark color of the nut is due to an iron-polyphenol complex. The shell oil contains about 90% anacardic acid ($C_{22}H_{32}O_3$ and 10% cardol ($C_{32}H_{27}O_4$) mentioned by Deszcz and Kozubek (1999). It yields glycerides, linoleic, palmitic, stearic, and lignoceric acids, and sitosterol. Examining 24 different cashews, Murthy and Yadava (1972) reported that the oil content of the shell ranged from 16.6 to 32.9%, of the kernel from 34.5 to 46.8%. Reducing sugars ranged from 0.9 to 3.2%, non-reducing sugars, 1.3 to 5.8%, total sugars from 2.4 to 8.7%, starch from 4.7 to 11.2%. Gum exudates contain arabinose, galactose, rhamnose, and xylose.

Chapter 5

Bioassay with Purified Compounds

CHAPTER 5

BIOASSAY WITH PURIFIED COMPOUNDS

5.1 Introduction

Bioassay is the basic technique for the evaluation of substances for their efficacy against any living organism. However, the mode of action of different compounds may not always be the same but it could indicate the interaction it makes with the living cells or disturbs in the pathway of metabolic processes or it disrupts the chemical coordination among the principal units of the body. Bioassay with the natural products, i.e. plant secondary metabolites is important in the search for pharmacological or even pesticidal properties. So, it is a tool of the general screening procedure (Champagne *et al.*, 1989).

Considering of plant secondary products being involved in plant pest interactions, the strategy of randomly isolating, identifying, and bioassaying these compound may also an effective methods of pesticide discovery. Biologically active compounds from plants will often have activity against organisms with which the producing plant does not have to cope (Bernays and Chapman, 1977). Many secondary compounds described in the natural product, pharmacological and chemical ecology literature have not been screened for pesticidal activity (Pascual-Villalobos and Robledo, 1999). This is due, in part to the very small amounts of these compounds have been available for screening.

The discovery process for natural pesticides is more complicated than that for synthetic pesticides. Traditionally, new pesticides have been discovered by synthesis,

bioassay and evaluation. If the compound is sufficiently promising, quantitative structure activity relationship based synthesis of analogues is used to optimize desirable pesticidal properties. The discovery process with natural compounds is complicated by several factors.

First, the amount of purification initially conducted is a variable for which there is general rule. Furthermore, secondary compounds are generally isolated in relatively small amounts compared to the amounts of synthesized chemicals available for screening for pesticide activity. Therefore bioassay requiring very small amounts of material will be helpful in screening natural products from plants (Champagne *et al.*, 1989).

Thus phytochemical investigation or screening is an evaluatory process for the detection of plant constituents through chemical analysis. In the present investigation the crude extracts of *A. occidentale* was purified, the active compounds were separated and bioassays were done with them to test the insecticidal effect.

5.2 Materials and methods

Topical application and repellency tests have been done in case of the pure compounds isolated from the nut-shells, root-bark and the stem-bark of the title plant. Since, the crude extracts showed no effect on the mortality of *T. castaneum* in the surface film method as well in case of fumigant activity tests, those experiments were not conducted.

The whole work was based on activity-guided fraction for the isolation and purification of biologically active plant secondary metabolites, bioassay with the pure component was very much essential for a complete project and that was done accordingly. Four compounds NS-1, NS-2, RB-1 and SB-2 were applied against the test

insect, *T. castaneum* by topical application method as described earlier. Repellency effect test was also done in the similar manners.

5.3. Results

5.3.1 Insecticidal effect by topical application test

The LD_{50} values found in this investigation indicate potentiality in the order of NS-1>NS-2>RB-1>SB-2. Compound NS-1 offered LD_{50} value 8.918 µg/insect (regression equation Y= 3.146098 + 1.950926X) for 24 h of exposure which reached at 8.039 µg/insect (regression equation Y= 3.260723 + 1.921474X) after 48 hours of exposure. Compound NS-2 offered LD_{50} value 11.940 µg/insect (regression equation Y= 3.220169 + 1.652573X) for 24 h of exposure, which reached at 10.546 µg/insect (regression equation 3.299809 + 1.661833X) after 48 hours of exposure. Compound RB-1 offered LD_{50} value 13.727 µg/insect (regression equation Y= 3.45836 + 1.355214X) for 24 h of exposure, which reached at 11.291 µg/insect (regression equation Y= 3.692723 + 1.241786X) after 48 hours of exposure. Compound SB-2 offered LD_{50} value 14.248 µg/insect (regression equation Y= 3.158351 + 1.596216X) for 24 h of exposure, which reached at 12.487 µg/insect (regression equation Y= 3.414889 + 1.445666X) after 48 hours of exposure (Tables 9-10, Appendix Tables XVII-.XXIV).

The regression lines are presented in Figs. 13-14, which shows the good fit of the lines corroborate with the insignificant χ^2 values.

Table 9. Dose mortality data of *T. castaneum* treated with *A. occidentale* extracts with 24 and 48 hours of exposure.

Plant part	Compound	Dose used µg/insect	Mortality %	
			24 hours	48 hours
Leaf	NS-1	25.000	83.333	86.667
		12.500	60.000	60.000
		6.250	40.000	40.000
		3.120	13.333	16.667
		1.560	10.000	13.333
	NS-2	25.000	73.333	76.667
		12.500	50.000	50.000
		6.250	33.333	36.667
		3.120	13.333	16.667
		1.560	10.000	10.000
Root-bark	RB-1	50.000	80.000	83.333
		25.000	60.000	60.000
		12.500	50.000	56.667
		6.250	33.333	36.667
Stem-bark	SB-2	30.000	73.333	76.667
		15.000	53.333	53.333
		7.500	30.000	33.333
		3.750	23.333	30.000

Table 10. Dose-mortality effect of pure nut-shell compounds of *A. occidentale* against *T. castaneum* adults.

Plant organ	Exp. (Hour)	LD_{50} (µg/insect^2)	95% conf. limits		Regression equations	χ^2 value (df)
			Upper	Lower		
NS-1	24	8.918	11.723	6.784	Y= 3.146098 + 1.950926X	1.061 (3)
	48	8.039	10.562	6.118	Y= 3.260723 + 1.921474X	2.035 (3)
NS-2	24	11.940	16.905	8.433	Y= 3.220169 + 1.652573X	0.783 (3)
	48	10.546	14.681	7.576	Y= 3.299809 + 1.661833X	0.719 (3)
RB-1	24	13.727	20.750	9.080	Y= 3.45836 + 1.355214X	0.351 (2)
	48	11.291	18.439	6.914	Y= 3.692723 + 1.241786X	1.076 (2)
SB-2	24	14.248	20.421	9.941	Y= 3.158351 + 1.596216X	0.640 (2)
	48	12.487	18.246	8.546	Y= 3.414889 + 1.445666X	1.426 (2)

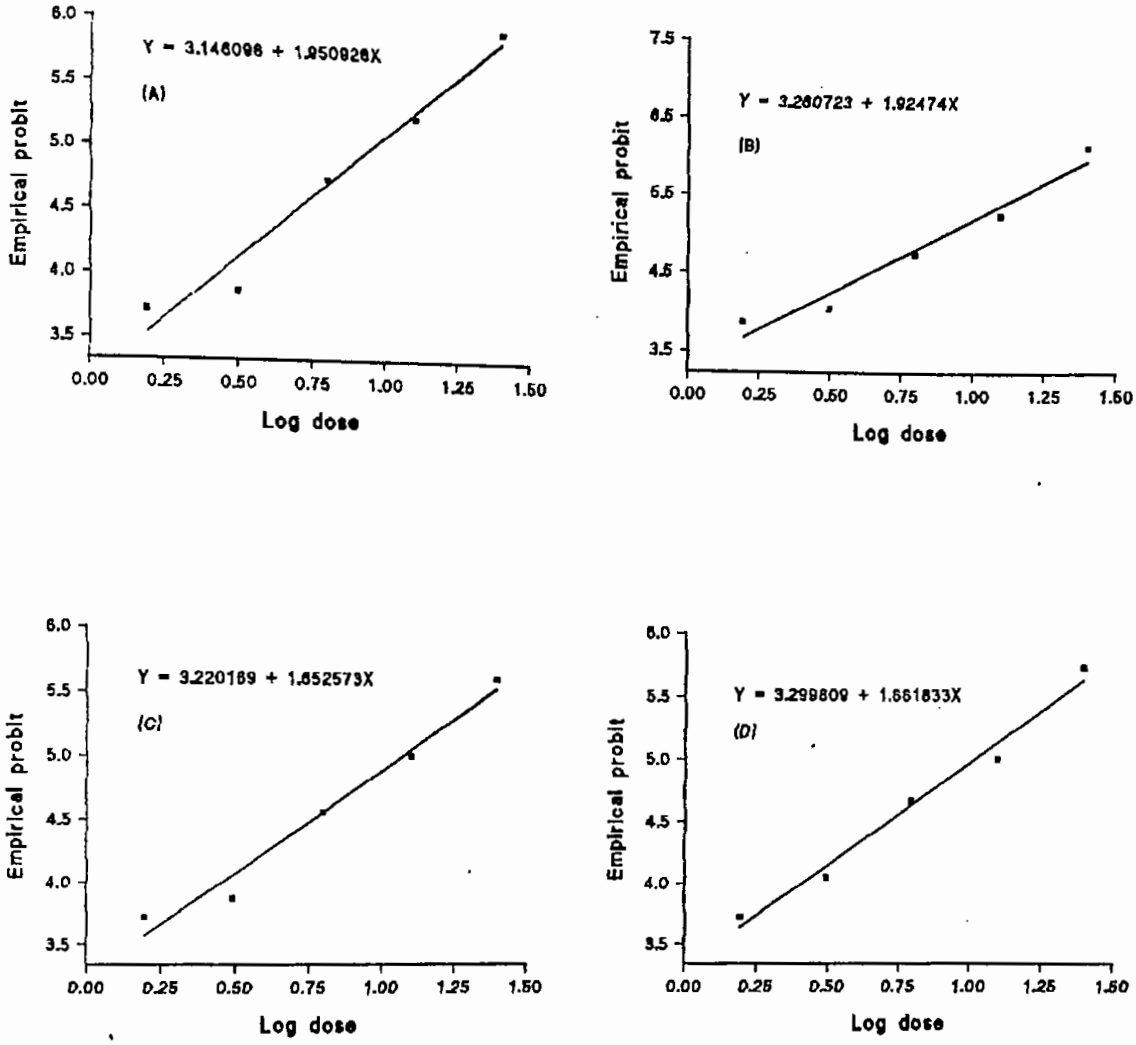


Fig. 15. Regression lines of log-dose of nut-shell compounds NS-1 [A] and [B] and NS-2 [C] and [D] after 24 and 48 hours of exposure respectively.

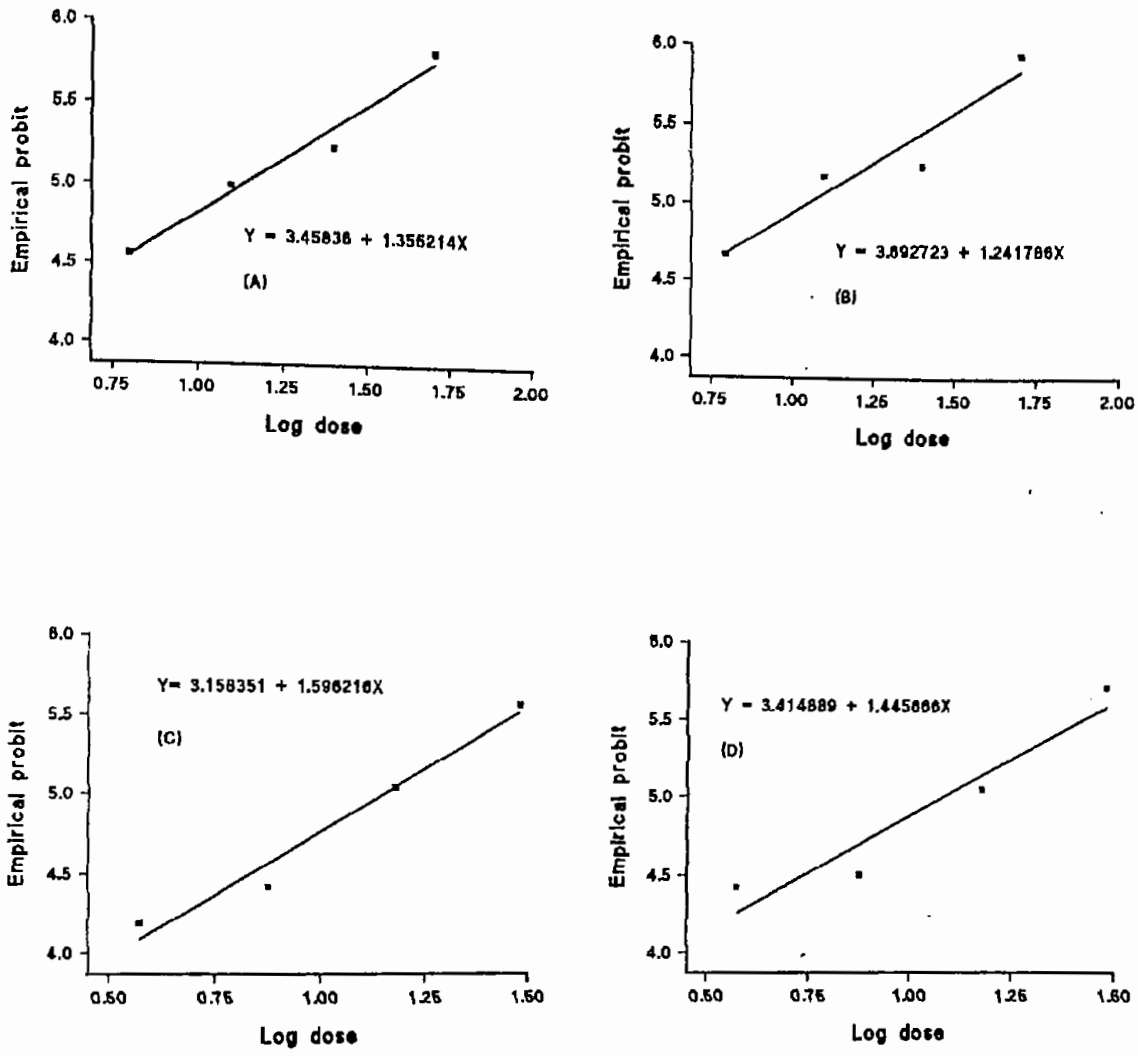


Fig. 16. Regression lines of log-dose of nut-shell compounds RB-1 [A] and [B] and SB-2 [C] and [D] after 24 and 48 hours of exposure respectively.

5.3.2 Repellency effects of pure compounds

Among the 4 pure compounds 3 showed repellency effect against adult beetles of *T. castaneum* even for a concentration from 5mg/ml to as less as 0.75mg/ml (5-, 2.5-, 1.25 and 0.75mg/ml). The data was read with 1 hour interval for up to 5 hours of exposure and was subjected to ANOVA after transforming them into arcsin percentage value and the result is given in Tables 11-12. Observation of data was conducted with 1 hour interval. Data were analyzed by analysis of variance (ANOVA) after transforming them into arcsin percentage value.

Among the NS-1, NS-2, RB-1 and SB-2 all except the last one were performed the highest average percent repellency on the beetle, and the differences were statistically significant for SB-2. The efficacy for repellency was found as in the order of NS-2>NS-1>RB-1>SB-2 ($P<0.05$) which supports that the name compound of this plant anacardic acid (NS-2) is showing repellency relatively mild to that of the compound NS-1 which was an unsaturated fatty acid (may be linoleic acid as tentatively assigned). RB-1 is the β-sitosterol is comparatively mild to that of the anacardic acid. P-values were in the descending order 2.51E-07 <7.27E-07 <6.48E-05 <0.702 for the NS-2, NS-1, RB-1 and SB-2 respectively.

Table 11. Repellency effect of pure compounds against *T. castaneum* adults.

Organ	Comp.	Dose (mg)	Repellency (%) at intervals				
			1st hour	2nd hour	3rd hour	4th hour	5th hour
Nut-shell	NS-1	5	93.2 (74.88)	80 (63.43)	66.6 (54.70)	80 (63.43)	86.6 (68.53)
		2.5	66.6 (55.70)	60 (50.77)	86.6 (68.53)	80 (63.43)	80 (63.43)
		1.25	46.6 (43.05)	80 (63.43)	53.2 (46.83)	66.6 (54.70)	66.6 (54.70)
		0.75	33.2 (35.18)	13.2 (21.30)	13.2 21.30)	20 (26.57)	13.2 (21.30)
Nut-shell	NS-2	5	80 (63.43)	93.2 (74.88)	86.6 (68.53)	86.6 (68.53)	93.2 (74.88)
		2.5	66.6 (54.70)	60 (50.77)	73.2 (58.82)	53.2 (46.83)	53.2 (46.83)
		1.25	60 (50.77)	66.6 (54.70)	53.2 (46.83)	60 (50.77)	60 (50.77)
		0.75	53.2 (46.83)	46.6 (43.05)	46.6 (43.05)	46.6 (43.05)	33.2 (35.18)
Root-bark	RB-1	5	80 (63.43)	73.2 (58.82)	60 (50.77)	60 (50.77)	53.2 (46.83)
		2.5	46.6 (43.05)	46.6 (43.05)	46.6 (43.05)	40 (39.23)	40 (39.23)
		1.25	60 (50.77)	40 (39.23)	26.6 (31.05)	33.2 (35.18)	26.6 (31.05)
		0.75	33.2 (35.18)	26.6 (31.05)	26.6 (31.05)	26.6 (31.05)	20 (26.57)
Stem-bark	SB-2	5	-6.8 (-15.12)	-6.8 (-15.12)	-6.8 (-15.12)	6.6 (14.89)	6.6 (14.89)
		2.5	13.2 (21.30)	-20 (-26.57)	-13.4 (-21.47)	-26.8 (-31.18)	-40 (-39.23)
		1.25	-6.8 (-15.12)	6.6 (14.89)	0 (0)	-20 (-26.57)	-20 (-26.57)
		0.75	40 (39.23)	-13.4 (-21.47)	-26.8 (-31.18)	-6.8 (-15.12)	-6.8 (-15.12)

Table 12. ANOVA results of repellency by pure compounds against *T. castaneum* .

Source Material	Pure comp.	Source of Variation	SS	df	MS	F	P-value
Nut-shell	NS-1	Between groups	4756.274	3	1585.425	30.629	7.28E-07
		Within groups	828.191	16	51.762		
		Total	5584.464	19			
	NS-2	Between groups	2059.399	3	686.466	35.790	2.51E-07
		Within groups	306.884	16	19.180		
		Total	2366.283	19			
Root-bark	RB-1	Between groups	1427.353	3	475.784	15.026	6.48E-05
		Within groups	506.632	16	31.665		
		Total	1933.985	19			
Stem-bark	SB-2	Between groups	687.119	3	229.040	0.478	0.702
		Within groups	7665.618	16	479.101		
		Total	8352.737	19			

5.4 Discussion

Bioassays using hexanolic extracts of cashew nut shells against snails showed promising effect by previous workers. The lethal concentration, LC_{90}, of sample I was from 2.0 to 2.2 ppm for adult snails (Mendes *et al.*, 1990). *A. occidentale* has a good antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa* which are gram-negative bacteria (Kudi *et al.*, 1999). The nutshell oil of the test plant afforded mixtures of anacardic acids and cardols in varying degrees of unsaturation. Sixty percent methanolic extract of stem-bark exhibited antimicrobial activity against 13 out of 15 bacterial isolates at a concentration of 20 mg/ml.

Anacardic acid was found effective at 10 ppm (Consoli *et al.*, 1988), which is promising in the field of crop protection and pesticide technology. Mendes *et al.* (1990) and Laurens *et al.* (1987) showed molluscicidal effect of it found in the nut-shell liquid of *A. occidentale*.

In the present investigation it was observed that all pure compounds obtained were more toxic than the respective crude extracts. The result suggests that the pure compounds may be well utilized in controlling the stored grain pests.

Summary

Insecticidal properties of *Anacardium occidentale* L. were investigated. All $CHCl_3$ and MeOH extracts of leaves, nut-shells, root-bark and stem-bark extracts were tested against *Valsa ceratosperma* (Tode Ex Fries) and *Tribolium castaneum* (Hbst.). Crude chloroform and methanol extracts of leaves, nut-shell, root-bark and stem-bark were tested against *V. ceratosperma* and found positive and promising activity, and it was considered as the test tool agent for the activity guided fractionation of biologically active compounds from the nut-shell, root-bark and the stem-bark of the test plant. This tool agent helped to isolate 6 bioactive compounds NS-1, NS-2, RB-1, RB-2, SB-1 and SB-2 from nut shell, root bark and the stem-bark of *A. occidentale* respectively, of which RB-1 was the same as SB-1, even though they were from different sources.

The surface film method of application of crude extract doses, as well as the fumigant activity test gave no positive results, while topical application of the same extracts gave interesting results by posing toxic effect to the test beetles.

The crude extract dose mortality results against *T. castaneum* were found promising. Both the chloroform and MeOH extract of nut-shells have been found strongly effective against the test insect, while the lowest LD_{50} values were17.574- and 17.049 µg/insect for the $CHCL_3$ extract of nut-shell after 24 and 48 hours of

exposures, except the MeOH extract of root-bark that shows LD_{50} 15.503 µg/insect, while the extract offered LD_{50} 19.322 µg/insect after 48 hours of exposure.

For the pure compounds NS-1, NS-2, RB-1 and SB-2 the four compounds from the $CHCl_3$ extract of the nut-shells, root-bark and stem-bark of the test plant were tested and the LD_{50} values were 8.918-, 11.940-, 13.727 and 13.318 µg/insect respectively for 24 h of treatment, and 8.038-, 10.545-, 11.291 and 11.513 µg/insect for 48 h of exposure.

The LD_{50} values found in this investigation indicate potentiality in the order of NS-1>NS-2>RB-1>SB-2. Among the NS-1, NS-2, RB-1 and SB-2 all, except the last one, were performed the highest average percent repellency on the beetles and the differences were statistically significant for SB-2. The efficacy for repellency was found as in the order of NS-2>NS-1>RB-1>SB-2 ($P<0.05$) which supports that the name compound of this plant anacardic acid (NS-2) is showing repellency relatively mild to that of the compound NS-1 was an unsaturated fatty acid (may be linoleic acid as tentatively assigned). RB-1 is the β-sitosterol is comparatively mild to that of the anacardic acid.

Chapter 6

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Appendix Table I. Probit mortality of *T. castaneum* by $CHCl_3$ extract of leaves of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.00	2.000	30	10	33.333	31	4.50	4.640	4.497	18.03	4.637
50.00	1.699	30	9	30.000	28	4.42	4.296	4.422	15.09	4.292
25.00	1.398	30	7	23.333	21	4.19	3.953	4.246	12.15	3.946
12.50	1.097	30	2	6.667	3	3.12	3.609	3.261	9.06	3.601

Results:

Y = 2.343019 + 1.146996 X
Chi-squared is 2.748073 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 2.316469
LD_{50} is 207.2379 µg/insect
95% confidence limits are 59.78266 to 718.3948 µg/insect

Appendix Table II. Probit mortality of *T. castaneum* by $CHCl_3$ extract of leaves of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.00	2.000	30	10	33.333	31	4.50	4.675	4.497	18.03	4.642
50.00	1.699	30	10	33.333	31	4.50	4.360	4.522	15.96	4.370
25.00	1.398	30	8	26.667	24	4.29	4.045	4.324	13.17	3.097
12.50	1.097	30	3	10.000	7	3.52	3.730	3.546	10.08	3.824

Results

Y = 2.830691 + 0.9057855 X
Chi-squared is 2.210655 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 2.394949
LD_{50} is 248.2838 µg/insect
95% confidence limits are 45.44919 to 1356.347 µg/insect

Appendix Table III. Probit mortality of *T. castaneum* by $CHCl_3$ extract of nut shells of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.00	1.699	30	25	83.33	83	5.95	5.838	5.902	15.09	5.802
25.00	1.398	30	17	56.667	55	5.13	5.256	5.150	18.81	5.231
12.50	1.097	30	11	36.667	34	4.59	4.674	4.578	18.03	4.660
6.25	0.796	30	7	23.333	21	4.19	4.092	4.201	13.17	4.088

Results:

Y = 2.577399 + 1.898174 X
Chi-squared is 0.5605736 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.27628
LD_{50} is 18.89208 µg/insect
95% confidence limits are 14.06643 to 25.37324 µg/insect

Appendix Table IV. Probit mortality of *T. castaneum* by $CHCl_3$ extract of nut shells of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.00	1.699	30	26	86.667	86	6.08	5.969	6.098	14.13	5.958
25.00	1.398	30	18	60.000	59	5.23	5.338	5.214	18.48	5.323
12.50	1.097	30	11	36.667	34	4.59	4.707	4.584	18.48	4.688
6.25	0.796	30	7	23.333	21	4.19	4.076	4.201	13.17	4.053

Results:

Y = 2.374147 + 2.109339 X
Chi-squared is 0.9847603 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.24487
LD_{50} is 17.57397 µg/insect
95% confidence limits are 13.45565 to 22.95277 µg/insect

Appendix Table V. Probit mortality of *T. castaneum* by $CHCl_3$ extract of root-bark of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.00	1.699	30	23	76.667	77	5.74	5.732	5.734	15.96	5.715
25.00	1.398	30	16	53.333	53	5.08	5.119	5.065	19.02	5.108
12.50	1.097	30	10	33.333	33	4.56	4.506	4.544	17.43	4.501
6.25	0.796	30	4	13.333	13	3.87	3.893	3.873	11.10	3.894

Results:

Y = 2.287933 + 2.017423 X
Chi-squared is 7.806015e-02 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.344323
LD_{50} is 22.09646 µg/insect
95% confidence limits are 16.60753 to 29.39955 µg/insect

Appendix Table VI. Probit mortality of *T. castaneum* by $CHCl_3$ extract of root-bark of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.00	1.699	30	24	80.000	80	5.85	5.824	5.800	15.09	5.798
25.00	1.398	30	17	56.667	57	5.18	5.233	5.202	18.81	5.216
12.50	1.097	30	11	36.667	37	4.67	4.642	4.659	18.03	4.634
87.5	0.796	30	5	16.667	17	4.05	4.051	4.037	13.17	4.053

Results:

Y = 2.514534 + 1.93263 X
Chi-squared is 1.788712E-02 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.286054
LD_{50} is 19.32208 µg/insect
95% confidence limits are 14.45491 to 25.82807 µg/insect

Appendix Table VII. Probit mortality of *T. castaneum* by $CHCl_3$ extract of stem-bark of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.00	2.000	30	22	73.333	72	5.58	5.443	.5.564	18.03	5.444
50.00	1.699	30	11	36.667	34	4.59	4.771	4.584	18.48	4.752
25.00	1.398	30	6	20.000	17	4.05	4.099	4.037	13.17	4.060
12.50	1.097	30	3	10.000	7	3.52	3.427	5.540	7.14	3.367

Results:

Y = .8454337 + 2.299246 X
Chi-squared is 0.9991779 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.806926
LD_{50} is 64.10996 µg/insect
95% confidence limits are 47.81381 to 85.96024 µg/insect

Appendix Table VIII. Probit mortality of *T. castaneum* by $CHCl_3$ extract of stem-bark of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.00	2.000	30	24	80.000	79	5.81	5.772	5.798	15.69	5.768
50.00	1.699	30	16	53.333	52	5.05	5.069	5.050	19.11	5.063
25.00	1.398	30	8	26.667	24	5.29	4.366	4.298	15.96	4.357
12.50	1.097	30	4	13.333	10	3.72	3.663	3.730	9.06	3.652

Results:

Y = 1.080848 + 2.343688 X
Chi-squared is 0.1289635 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.672216
LD_{50} is 47.01277 µg/insect
95% confidence limits are 36.47686 to 60.59184 µg/insect

Appendix Table IX. Probit mortality of *T. castaneum* by MeOH extract of leaves of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.00	2.000	30	17	56.667	57	5.18	5.079	5.175	19.11	5.082
50.00	1.699	30	10	33.333	33	4.56	4.723	4.558	18.48	4.719
25.00	1.398	30	8	26.667	27	4.39	4.367	4394	15.96	4.356
12.50	1.097	30	5	16.667	17	4.05	4.011	4.037	13.17	3.993

Results:

Y = 2.669598 + 1.206187 X
Chi-squared is 0.6929245 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.932041
LD_{50} is 85.51466 µg/insect
95% confidence limits are 44.35533 to 164.8676 µg/insect

Appendix Table X. Probit mortality of *T. castaneum* by MeOH extract of leaves of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.00	2.000	30	22	73.333	73	5.61	5.424	5.591	18.03	5.411
50.00	1.699	30	11	36.667	37	4.47	4.928	4.665	19.02	4.911
25.00	1.398	30	8	26.667	27	4.39	4.432	4.390	16.74	4.412
12.50	1.097	30	5	16.667	17	4.05	3.936	4.062	12.15	3.913

Results:

Y = 2.093695 + 1.658553 X
Chi-squared is 2.019415 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.752314
LD_{50} is 56.53458 µg/insect
95% confidence limits are 38.91393 to 82.13403 µg/insect

Appendix Table XI. Probit mortality of *T. castaneum* by MeOH extract of nut shell of *A. occidentale* after 24 h of exposure

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.00	1.699	30	26	86.667	87	6.13	6.005	6.087	13.17	5.958
25.00	1.398	30	16	53.333	53	5.08	5.275	5.098	18.81	5.242
12.50	1.097	30	10	33.333	33	4.56	4.545	4.544	17.43	4.526
6.25	0.796	30	4	13.333	13	3.87	3.815	3.873	11.10	3.810

Results:

Y = 1.917841 + 2.377903 X
Chi-squared is 0.6589203 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.296167
LD_{50} is 19.77728 µg/insect
95% confidence limits are 15.47777 to 25.27114 µg/insect

Appendix Table XII. Probit mortality of *T. castaneum* by MeOH extract of nut shell of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.00	1.699	30	27	90.000	90	6.28	6.103	6.270	12.15	6.042
25.00	1.398	30	17	56.667	57	5.18	5.416	5.159	18.03	5.371
12.50	1.097	30	11	36.667	37	4.67	4.729	4.662	18.48	4.699
6.25	0.796	30	6	20.000	20	4.16	4.042	4.160	13.17	4.028

Results:

Y = 2.254093 + 2.229367 X
Chi-squared is 1.694714 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.231698
LD_{50} is 17.04898 µg/insect
95% confidence limits are 13.17832 to 22.0565 µg/insect

Appendix Table XIII. Probit mortality of *T. castaneum* by MeOH extract of root-bark of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.00	1.699	30	24	80.000	80	5.85	5.784	5.830	15.96	5.781
25.00	1.398	30	18	60.000	60	5.25	5.288	5.280	18.81	5.283
12.50	1.097	30	11	36.667	37	4.67	4.792	4.662	18.48	4.785
6.25	0.796	30	8	26.667	27	4.39	4.296	4.388	15.09	4.287

Results:

Y = 2.970249 + 1.654154 X
Chi-squared is 0.4717636 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.227063
LD_{50} is 16.86798 µg/insect
95% confidence limits are 12.1127 to 23.49013 µg/insect

Appendix Table XIV. Probit mortality of *T. castaneum* by MeOH extract of root-bark of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.00	1.699	30	25	83.333	83	5.95	5.844	5.902	15.09	5.794
25.00	1.398	30	18	60.000	60	5.25	5.353	5.240	18.48	5.324
12.50	1.097	30	12	40.000	40	4.75	4.862	4.760	18.81	4.854
6.25	0.796	30	9	30.000	30	4.48	4.371	4.490	15.96	4.384

Results:

Y = 3.140641 + 1.561937 X
Chi-squared is 0.6518001 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.190419
LD_{50} is 15.50311 µg/insect
95% confidence limits are 10.88405 to 22.08246 µg/insect

Appendix Table XV. Probit mortality of *T. castaneum* by MeOH extract of stem-bark of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.00	2.000	30	13	43.333	43	4.82	4.804	4.838	18.81	4.807
50.00	1.699	30	9	30.000	30	4.48	4.573	4.460	17.43	4.577
25.00	1.398	30	9	30.000	30	4.48	4.342	4.490	15.96	4.346
12.50	1.097	30	5	16.667	17	4.05	4.111	4.056	14.13	4.116

Results:
Y = 3.27767 + 0.7644957 X
Chi-squared is 0.6355081 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 2.252898
LD_{50} is 179.0186 µg/insect
95% confidence limits are 35.28441 to 908.266 µg/insect

Appendix Table XVI. Probit mortality of *T. castaneum* by MeOH extract of stem-bark of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.00	2.000	30	15	50.000	50	5.00	4.930	4.990	19.02	4.922
50.00	1.699	30	9	30.000	30	4.48	4.645	4.470	18.03	4.638
25.00	1.398	30	9	30.000	30	4.48	4.360	4.490	15.96	4.354
12.50	1.097	30	5	16.667	17	4.05	4.075	4.037	13.17	4.070

Results:

Y = 3.035686 + 0.9431001 X
Chi-squared is 0.9063111 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 2.082826
LD_{50} is 121.0115 µg/insect
95% confidence limits are 42.22026 to 346.8423 µg/insect

Appendix Table XVII. Probit mortality of *T. castaneum* by nut-shell compound NS-1 of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
25.000	1.398	30	25	83.333	83	5.95	5.876	5.902	15.09	5.873
12.500	1.097	30	18	60.000	60	5.25	5.292	5.280	18.81	5.286
6.250	0.796	30	12	40.000	40	4.75	4.709	4.740	18.48	4.699
3.120	0.494	30	4	13.333	13	3.87	4.124	3.904	14.13	4.110
1.560	0.193	30	3	10.000	10	3.72	3.540	3.750	8.07	3.523

Results:

Y = 3.146098 + 1.950926 X
Chi-squared is 1.061325 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is .9502675
LD_{50} is 8.918 µg/insect
95% confidence limits are 6.784049 to 11.7232 µg/insect

Appendix Table XVIII. Probit mortality of *T. castaneum* by nut-shell compound NS-1 of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
25.000	1.398	30	26	86.667	87	6.13	5.954	6.136	14.13	5.947
12.500	1.097	30	18	60.000	60	5.25	5.382	5.240	18.48	5.368
6.250	0.796	30	12	40.000	40	4.75	4.811	4.760	18.81	4.790
3.120	0.494	30	5	16.667	17	4.05	4.238	4.048	15.09	4.210
1.560	0.193	30	4	13.333	13	3.87	3.666	3.931	9.06	3.632

Results:
Y = 3.260723 + 1.921474 X
Chi-squared is 2.035477 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is .9051789
LD_{50} is 8.038571 µg/insect
95% conf limits are 6.118211 to 10.56169 µg/insect

Appendix Table XIX. Probit mortality of *T. castaneum* by nut-shell compound NS-2 of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
25.000	1.398	30	22	73.333	73	5.61	5.534	5.584	17.43	5.530
12.500	1.097	30	15	50.000	50	5.00	5.043	5.000	19.11	5.033
6.250	0.796	30	10	33.333	33	4.56	4.552	4.544	17.43	4.535
3.120	0.494	30	4	13.333	13	3.87	4.061	3.873	13.17	4.037
1.560	0.193	30	3	10.000	10	3.72	3.570	3.750	8.07	3.539

Results:

Y = 3.220169 + 1.652573 X
Chi-squared is 0.7833176 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.077006
LD_{50} is 11.94005 µg/insect
95% confidence limits are 8.433496 to 16.90459 µg/insect

Appendix Table XX. Probit mortality of *T. castaneum* by nut-shell compound NS-2 of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
25.000	1.398	30	23	76.667	77	5.74	5.634	5.730	16.74	5.623
12.500	1.097	30	15	50.000	50	5.00	5.135	4.990	19.02	5.123
6.250	0.796	30	11	36.667	37	4.67	4.636	4.659	18.03	4.622
3.120	0.494	30	5	16.667	17	4.05	4.137	4.056	14.13	4.121
1.560	0.193	30	3	10.000	10	3.72	3.638	3.730	9.06	3.621

Results:

Y = 3.299809 + 1.661833 X
Chi-squared is 0.7185936 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.023082
LD_{50} is 10.54586 µg/insect
95% confidence limits are 7.575519 to 14.68086 µg/insect

Appendix Table XXI. Probit mortality of *T. castaneum* by root-bark compound RB-1 of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.000	1.699	30	24	80.000	80	5.85	5.783	5.830	15.96	5.761
25.000	1.398	30	18	60.000	60	5.25	5.371	5.240	18.48	5.353
12.500	1.097	30	15	50.000	50	5.00	4.959	4.990	19.02	4.945
6.250	0.796	30	10	33.333	33	4.56	4.547	4.544	17.43	4.537

Results:

Y = 3.45836 + 1.355214 X
Chi-squared is 0.3514137 with 2 degrees of freedom
No significant heterogeneity
Log $LD5_0$ is 1.137562
LD_{50} is 13.72656 µg/insect
95% confidence limits are 9.080414 to 20.74999 µg/insect

Appendix Table XXII. Probit mortality of *T. castaneum* by root-bark compound RB-1 of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr %	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
50.000	1.699	30	25	83.333	83	5.95	5.849	5.902	15.09	5.802
25.000	1.398	30	18	60.000	60	5.25	5.458	5.240	18.03	5.429
12.500	1.097	30	17	56.667	57	5.18	5.067	5.175	19.11	5.055
6.250	0.796	30	11	36.667	37	4.67	4.676	4.659	18.03	4.681

Results:

Y = 3.692723 + 1.241786 X
Chi-squared is 1.076078 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.05274
LD_{50} is 11.29119 µg/insect
95% Confidence limits are 6.91413 to 18.43918 µg/insect

Appendix Table XXIII. Probit mortality of *T. castaneum* by stem-bark compound SB-2 of *A. occidentale* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr%	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
30.000	1.477	30	22	73.333	72	5.58	5.530	5.556	17.43	5.516
15.000	1.176	30	16	53.333	52	5.05	5.050	5.050	19.11	5.036
7.500	0.875	30	9	30.000	28	4.42	4.570	4.404	17.43	4.555
3.750	0.574	30	7	23.333	21	4.19	4.090	4.201	13.17	4.075

Results:

Y = 3.158351 + 1.596216 X
Chi-squared is 0.6403599 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.15376
LD_{50} is 14.2482 µg/insect
95% confidence limits are 9.941386 to 20.42081 µg/insect

Appendix Table XXIV. Probit mortality of *T. castaneum* by stem-bark compound SB-2 of *A. occidentale* after 48 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr%	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
30.000	1.477	30	23	76.667	76	5.71	5.583	5.668	17.43	5.550
15.000	1.176	30	16	53.333	52	5.05	5.141	5.040	19.02	5.115
5.700	0.756	30	10	33.333	31	4.50	4.699	4.497	18.03	4.680
3.750	0.574	30	9	30.000	28	4.42	4.257	4.422	15.09	4.245

Results:

Y = 3.414889 + 1.445666 X
Chi-squared is 1.426079 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.096458
LD_{50} is 12.48698 µg/insect
95% confidence limits are 8.545703 to 18.24598 µg/insect