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Bioactive Properties of The Toothbrush Plant, Glycosmis Pentaphylla (Retz.) with Special Reference to Stored Product Pests

Pramanik, Md. Shahidullah

University of Rajshahi

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Ph.D. THESIS

A thesis submitted to the University of Rajshahi in fulfillment of the requirements for the degree of Doctor of Philosophy

Submitted by

Md. Shahidullah Pramanik

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

Third Science Building
Motihaar Green
December 2007

Crop Protection and Toxicology Laboratory
Department of Zoology
University of Rajshahi
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DEDICATED TO

MY

BELOVED PARENTS

Declaration

I hereby declare that the entire work submitted as a thesis towards the fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology at the University of Rajshahi 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.

Md. Shahidullah Pramanik 13.12.07

(Md. Shahidullah Pramanik)

December 2007
Rasjshai University
Rajshahi, Bangladesh

UNIVERSITY OF RAJSHAHI
Department of Zoology
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CERTIFICATE

This is to certify that the thesis entitled "Bioactive properties of the toothbrush plant, *Glycosmis pentaphylla* (Retz.) with special reference to stored product pests" is a bonafide original research work of Md. Shahidullah Pramanik.

Supervisor

13.12.07

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Md. Shahidullah Pramanik

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LIST OF ABBREVIATIONS

#U	=	Number of insects used
% kill	=	Insects killed per cent
+ve	=	Positive
µg	=	microgram
µl	=	micro liter
$CHCl_3$	=	Chloroform
cm^2	=	centimeter square
Cr %	=	corrected mortality percent.
df	=	degree of freedom.
E. Pr	=	Empirical Probit.
et al.,	=	and others (author)
EtOAc	=	Ethyl Acetate
Ex Pr	=	Expected Probit
F Pro	=	Final Probit
Fig.	=	Figure
fr	=	factor (s)
h	=	hour (s)
HPLC	=	High Perform Liquid Chromatography
i.e.	=	that is
KI	=	Number of insects killed
LC_{50}	=	concentration required to kill 50% of test organisms
LD_{50}	=	dose required to kill 50% of test organisms
LDose	=	Log dose
MeOH	=	Methanol
mg	=	milligram (s)
ml	=	milliliter
mm	=	millimeter
mp	=	melting point
nm	=	nanometer
NMR	=	Nuclear Magnetic Resonance
PDA	=	Potato Dextrose Agar
Rf	=	Retention factor
TLC	=	Thin Layer Chromatography
-ve	=	Negative
Weight	=	Weighting coefficient
Wk Pro	=	Working probit
χ^2	=	Chi-squared

ABSTRACT

In the antimicrobial activity test both the antibacterial and antifungal tests offered promising outputs and the results were supported by the results achieved by the previous workers (Rahmani *et al.,* 2004; Greger, 1993) dealt with this plant. In the disc diffusion method the leaf extract gave the inhibition zones 17-, 16-, 16-, 14-, 16-, 20-, 12- and 7 mm for *B. megaterium*, *B. subtilis, S. lutea, S. - β-haemolyticus, S. typhi, S. sonnei, S. boydii* and *P. aeruginosa* respectively, while in comparison the inhibition zones for the standard were 28-, 33- 30-, 31-, 35-, 33-, 34- and 31 mm respectively. For the stem bark extract the inhibition zones were 14-, 15-, 15-, 11-, 15-, 10- and 10 mm for *S. aureus, B. megaterium, S. lutea, S. typhi, S. sonnei, S. boydii* and *P. aeruginosa* respectively, while the same for the standard were 31-, 28-, 30-, 35-, 33-, 34-, and 31 mm for the same test agents respectively. For the stem wood extract the inhibition zones were 7-, 14-, 12-, 10- and 12 mm respectively for *B. megaterium*, *B. subtilis, S.- β-haemolyticus, S. boydii* and *P. aeruginosa,* while the inhibition zones for the standard were 28-, 33-, 31-, 34- and 31 mm; and for the root extract the inhibition zones were 9-, 9-, 10-, 8- and 7 mm for *S. aureu, B. megaterium, S. sonnei, S. boydii* and *P. aeruginosa* respectively, while the inhibition zones for the standard were 31-, 28, 33-, 34- and 31 mm for the above mentioned test agents respectively. Many previous works support this output. Arborinine, and acridone alkaloid obtained from *G. pentaphylla*, exhibited significant inhibition of crown gall tumors produced by *Agrobacterium tumefaciens* in a potato disc bioassay (Quader, *et al.,* 1999).

In the antifungal activity tests the leaf extract (chloroform) of *G. pentaphylla* offered promising activity, while the inhibition zones were 20 mm for *A. fumigatus and Mucor* sp. after 24 h of exposure, however both of them remained responsive after 48 h with 12 mm of the inhibition zones; while the inhibition zones for the standard were 32- and 30 mm for the above mentioned test fungi respectively. For the stem bark extract the inhibition zones 19-, 7-, 17- and 12 mm for *A. fumigatus, A. flavus, Mucor* sp. and *C. albicans* after 24 h of exposure, however after 48 h only the *A. fumigatus, Mucor* sp. and *C. albicans* remained responsive to the same with 12-, 13- and 9 mm of inhibition zones; while the inhibition zones for the standard were 32, 28-, 30-and 31mm for the above mentioned test fungi. The stem wood extract offered inhibition

zones 7-, 12-, 10-, 17- and 10 mm after 24 h of exposure against *F. vasinfectum, A. fumigatus, A. flavus, Mucor* sp. and *C. albicans,* among them only the *Mucor* sp. remained responsive to the same with 13 mm of the inhibition zone after 48 h of exposure; while the inhibition zones for the standard were 29-, 32-, 28-, 30- and 31 mm for the above mentioned test fungi. For the root extract the inhibition zones were 7-, 15- and 12 mm for *F. vasinfectum, A. fumigatus* and *Mucor sp.* after 24 h of exposure, however after 48 h the *A. fumigatus and Mucor* sp. remained responsive to the same with 9 mm of the inhibition zones; while the inhibition zones for the standard were 29-, 32-, 31- and 30 mm for the above mentioned test fungi respectively.

The crude extracts showed cytotoxic activity while tested on the brine shrimp nauplii, *A. salina.* The LC_{50} values established were 28.579-, 28.659-, 57.213- and 84.111ppm for leaf, stem bark, stem wood and root (root bark and root wood were not separated) extracts respectively; while the efficacy could be arranged in a descending order Leaf>stem bark> stem wood> root. Findings of Muthukrishnan and his group (Muthukrishnan *et al.,* 1999) resembles with this result, while the addition of 10±4 to 10±5 M the quinazolone of arborine isolated from the ethyl acetate fraction of *G. pentaphylla* leaf extract to water resulted in 83 to 100% mortality of *C. quinquefasciatus* larvae.

Since only the root extract was found active in the *Ad hoc* experiment it was subjected to dose-mortality assay on *T. castaneum* larvae. All through the experiment almost weak mortality was traced, however, no useful data was able to read and subject to analysis. Where as, moulting was delayed and thus prolongation of the instars took place, however, during the 3rd instar a huge number of larvae and pupae died. Larvicidal assay on *M. domestica* larvae didn't offer any mortality, while prolongation of the developmental period was traced so far.

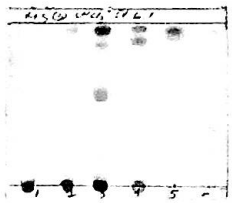
However, repellents, fumigants, feeding deterrents, growth inhibitors and insecticides of natural origin are rational alternatives to synthetic insecticides *G. pentaphylla* draws a special attention for its low lethal and other environment-friendly activities. Of course, this investigation reveals its special type of potentials that the chloroform extracts of the different parts of this plant shows repellent activity. The F values have been established through ANOVA with the arcsin transformed data were 60.983, 14.177, 19.437, 15.429 and 1.082 for the analysis between doses and 2.52, 1.806, 1.314, 3.468 and 1.272 for the analysis between time interval for leaf, stem bark, stem wood, root bark and root wood extracts respectively. Except the root extract

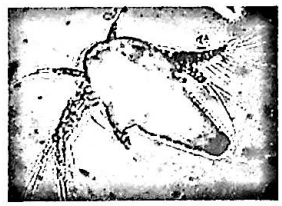
strong repellent activity was found, while repellency due to differences between doses were highly significant (P<0.001). The P values were established as 2.03E-11, 5.41E-6, 4.62E-7, 2.48E-6 and 0.4002 for the analysis between doses and 0.0733, 0.1673, 0.2989, 0.0262 and 0.3139 for the analysis between time interval for the leaf, stem bark, stem wood, root bark and root wood extracts respectively, and thus the intensity of repellent activity could be arranged in a descending order of leaf>stem wood>root bark>stem bark. This finding has been supported by Chopra *et al.* (1956) who mentioned that the leaves of this plant are used to keep insects away from sweets and other edible items are taken by natives in India and Australia.

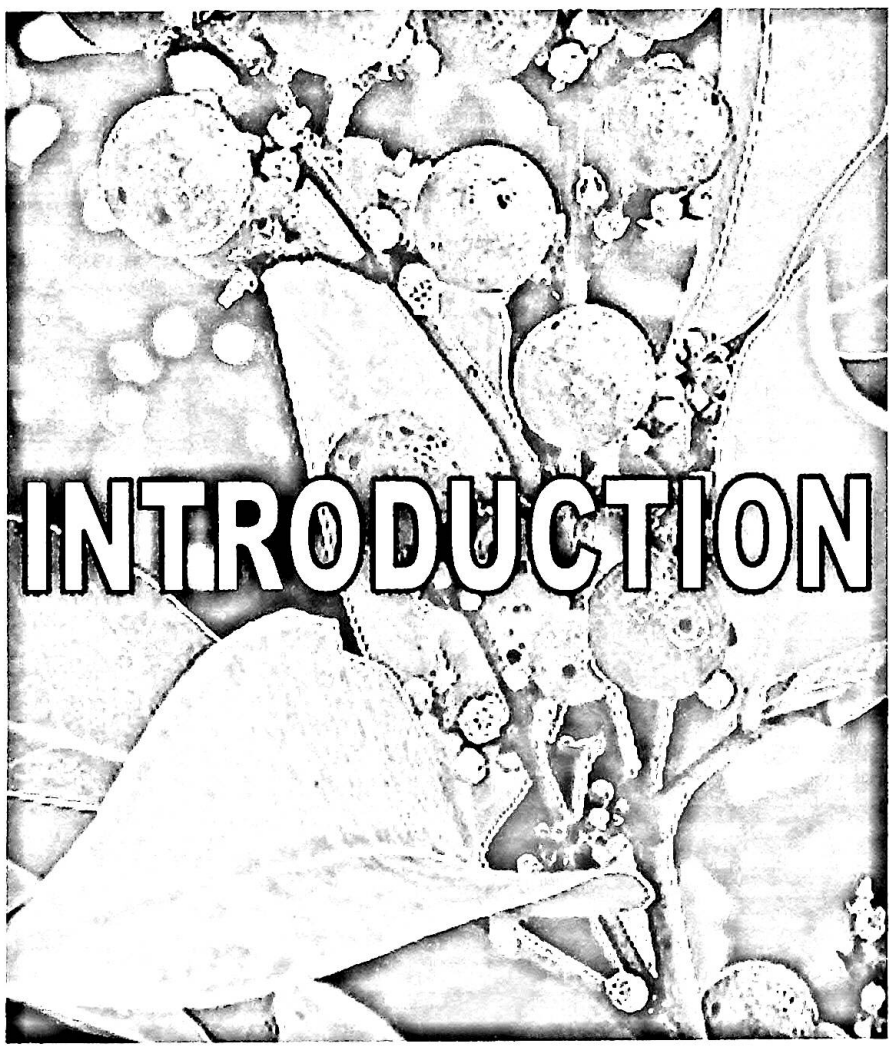
Anyway, the results indicate that the test plant possess a very special type of potentiality other than killing of organisms in the bioassays. The results revealed that this plant opens no mentionable insecticidal value since no mortality was recorded in case of the treatments carried out against the housefly, *M. domestica* larvae; the rust red flour beetle, *T. castaneum* adults and the lesser mealworm, *Alphitobius diaperinus* adults; while a very weak activity was traced against the *t. castaneum* larvae and against the cowpea weevil *C. maculatus* adults, while the LD_{50} values for root extract against *C. maculatus* were 1425.036-, 213.523- and 91.782 $\mu g/cm^2$ for 24, 48 and 78 hours of exposure respectively. However, the treated living *T. castaneum* larvae got abnormality in shape, size and colouration, as well as, vulnerable physical condition, so that the pupae that evolved after the 3rd week of treatment died shortly.

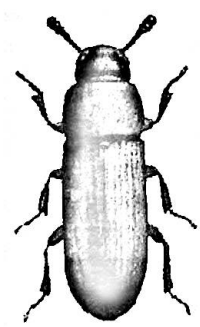
The main target of this research revolves no doubt in tracing out and isolate bioactive potentials of the test plant, and it offered two pure compounds GP1 (12 mg) and GP2 (14mg) which were glycozoline and glycoborinine respectively. Due to insufficiency in amount it was impossible to go through all the biological assays (used in this investigation) for these two pure compounds, while only antibacterial test was possible to carry out. These two compounds found active against the test bacteria used in this investigation.

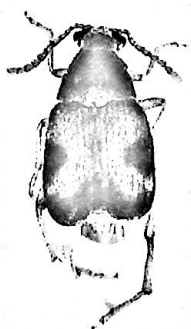
The findings of this work along with the findings of the previous researchers triggered a hope for further progress in research with this promising plant *G. pentaphylla* towards a molecular level investigation of its pesticidal potentials for an environment friendly protection of crops and stored products.

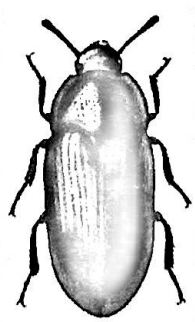





INTRODUCTION







1. Introduction

Plants are like natural laboratories where a great number of chemicals are biosynthesized and in fact they may be considered the most important source of chemical compounds. Primary plant metabolism synthesizes essential compounds, which are present in all plant species. 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, *et al.*, 2001).

Documentation of the ancient use of chemical controls for the insects appears 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. On the other hand, the end products of secondary metabolism are neither essential nor universally present in all plants. Common among these metabolites are compounds with protective action against insects, such as alkaloids, non-proteic amino acids, steroids, phenols, flavonoids, glycosids, glucosinolates, quinones, tannins and terpenoids. 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).

The first botanical insecticide used, as such, dates back to the Seventeenth Century when it was shown that nicotine, obtained from tobacco leaves, would kill plum beetles. Around 1850 a new plant insecticide known as rotenone was introduced. It was obtained from the roots of plants called Timbó (*Lonchocarpus nicou*) and Luba (*Derris* spp.). Up to that time this plant

was used for fishing purposes only as natives had known for a long time that throwing root pieces to the water caused fish to start floating a few minutes later, making them very easy to catch. Later on plants with irritating properties like incense (composed of aromatic organic materials that releases fragrant smoke when burned) and sabadilla (*Schoenocaulon officinale*) were used and extracts from the latter plant were also used as decongestants. These plants did not kill insects directly but it was said that they scared them off. More recently other plants used are Quasia (*Quaisa amara,* Simaroubaceae), Neem or Margosa (*Azadirachta indica*) mentioned above, which besides giving excellent results for insect control are also a source of compounds used against cancer. Use of plant extracts and powdered plant parts as insecticides goes back at least as far as the Roman Empire. For instance, there are reports that in 400 B.C. during Persian King Xerxes' reign, the delousing procedure for children was with a powder obtained from the dry flowers of a plant known as Pyrethrum (*Tanacetum cinerariaefolium,* Compositae).

However, the earth is inhibited by about one million insect species, roughly a half of them feed on plants (Schoonhoven, 1982). Only plant species, which during millions of years have developed strong chemical defense system that have survived the heavy selection pressure by greedy early created animals. Although, each of the defense systems having own intrinsic merit, some of them have featured which render them 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.

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. There are many species of plants of these families in Bangladesh that are used as traditional medicine by the native people from the remotest antiquity.

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; Waises *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 multiresistant 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). Furthermore, synthetic insecticides are not only a threat to the farmers' major resources but also used beyond the permissible limits (Lepigre and Pointel, 1971).

Natural products derived from plants, as an alternative to conventional insecticides for insect control is now a day very popular among the IPM practitioners. Plant-derived pesticides are more readily biodegradable. Therefore they are less likely to contaminate the environment and may be less toxic to mammals. 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' indifferent ways.

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 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 he pest's natural enemies to poisoned food or starvation.

Now a day, a number of traditionally used plant preparations have found in local commercial markets, for example *Ryania speciosa* (Ryania) (Flaccourtiaceae), which contain an insecticidal alkaloid. *Halophyton* 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. Also, 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 items of crops biological assays could be of different types. For field crops infesting agents might not be the same to that of stored product pests. However, there are some test organisms, use 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.

The use of plants as repellents is very old but has not received the necessary attention for proper development. Use has been achieved with compounds having bad odor or irritant effect, as in the case for garlic and hot peppers. A clear example can be seen in uses by Guatemalan and Costa Rican natives, who used to paint containers or sprinkle them with garlic powder before using them to store corn or beans to prevent the grain from being damaged by weevils (weevil punctures) and also to shoo away rodents. Lastly, it is not

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uncommon to hear of home recipes mentioning the use of fennel (*Foniculum vulgare*), rue (*Ruta graveolens*) and eucalyptus (*Eucaliptus globolus*) among other aromatic plants to repel clothes' moths.

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). Recently, 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 chemical 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; Sudhakar *et al.*, 1978; Carpentier *et al.,* 1979; Warthen, 1979; Jurd and Manners, 1980; Menn, 1980). Renewed interest in botanical pest control agents is motivated by three major objectives: i) to encourage traditional use of simple formulation of locally available plant materials by farmers who can not afford commercial insecticides; ii) to identify sources of new botanical pesticides for commercial extraction; and iii) to elucidate the chemical structure of active principles. Botanical pest control agents extracted on large scale may also be used to replace for supplement the activity of existing synthetic pesticides against refractory pests. Structural elucidation of the active constituents may provide further insight into structure-activity relationships. Novel metabolites identified may serve as models for chemical synthesis of new pesticides with more desirable properties. The general pathway of the whole work is given in the Fig. 1a and Fig. 1b; however, the present work could be ahead of purification and pure compound bioassay according to the facilities available here.

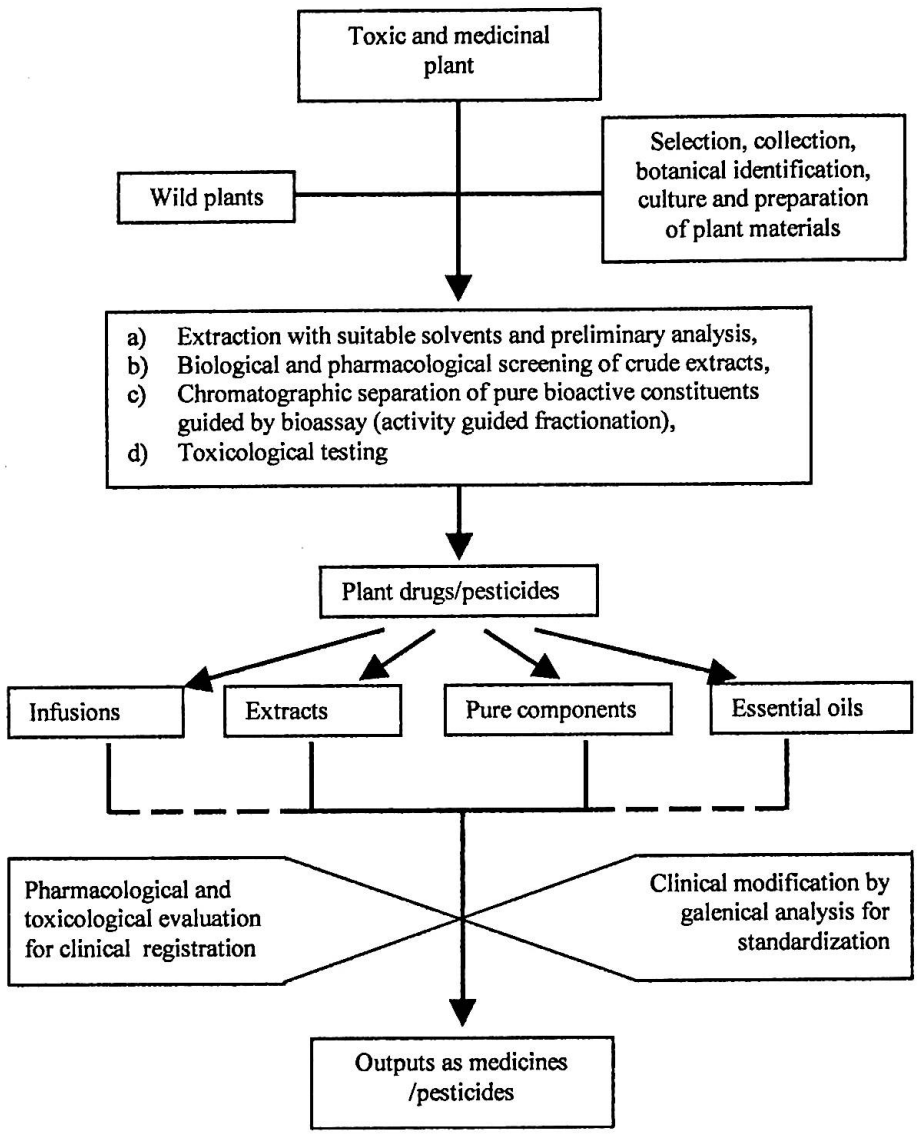


Fig. 1a: Phytochemical investigation towards the outputs.

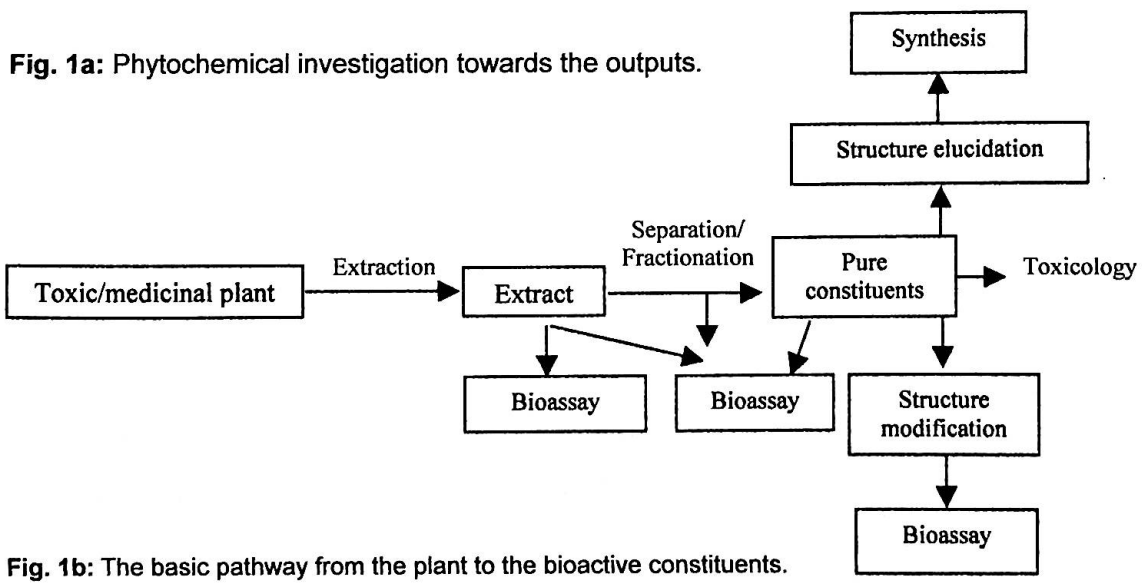


Fig. 1b: The basic pathway from the plant to the bioactive constituents.

In this proposition different parts, i.e. leaves, stem bark, stem wood, root bark and root wood of *Glycosmis pentaphylla* (Retz.) (Rutaceae) were subjected to biological screening and phytochemical investigation to trace presence of bioactive potentials and to isolate, purify and characterize them for their possible use in the pest control sector.

1.1. Background information on the title plant species

G. pentaphylla is a thornless shrub or small tree that is native to southeastern Asia and northeastern Australia. This plant grows from 1 to 5 meters in height. The leaves usually have from 3 to 5 pinnately arranged leaflets can be 1-, 2-, 3-, 4-, or 5-foliate, through these are sometimes reduced to one or two, and all forms being often found on the same plant. The leaflets are oblong-lanceolate to lanceolate, 5 to 18 centimeters long, and 2 to 7 centimeters wide. The flowers are small, white followed by translucent pink to red berries, about 6 millimeters in diameter, born in axillary, solitary or paired, interrupted, narrow, cymose panicles which are 5 centimeters or less in length. The fruit is fleshy, pink or reddish, rounded, 1 centimeter in diameter, and contains a single nearly spherical seed, which is 5 millimeter in diameter. The fleshy mesocarp is sweet (Plate 1).

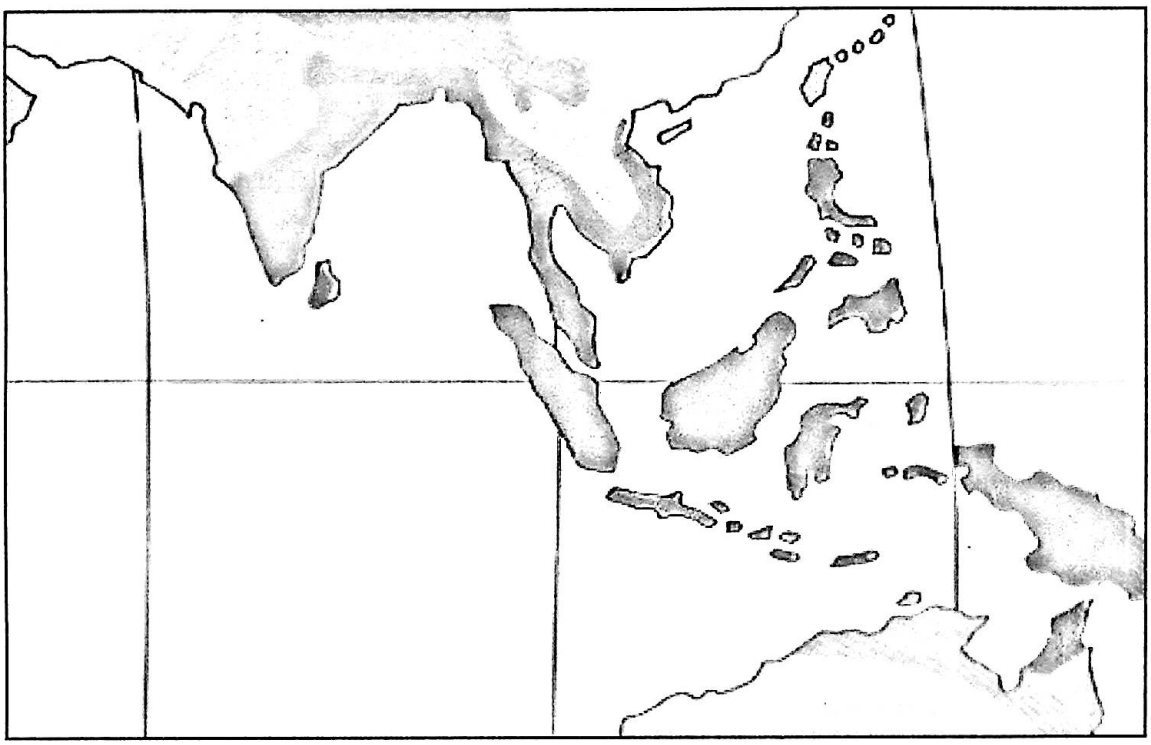
Since it is growing wild in India it has a popular position among the Indians. In Hindu medicine it has been used traditionally in bilious complaints, cough, worms, jaundice and fever. Its medicinal virtues came into light when it was found that people who were cleansing their teeth with little stick of Ash-sheora (*G. pentaphylla)* did not suffer from Kala azar fever.

The roots of Rutaceae in the subfamily Aurantioidea contain several classes of natural products that exhibit insecticidal or other biological activity. Among those coumarins, alkaloids, amides, flavonoids, limonoids, and terpenoids are well known (Shapiro, 1991). Growth-inhibiting activity against larvae of *Diaprepes abbreviatus* has been found in both live and milled roots from seedlings of two citrus relatives, *G. pentaphylla* or orangeberry (Shapiro *et al.*,

1997; Bowman *et al.*, 1999; Lapointe and Shapiro, 1999) and *Murraya koenigii* (Lapointe and Shapiro, 1999). Species in the genus *Glycosmis* (Stone, 1985) contain a wide variety of compounds with potential biological activity (Fig. 3). These include terpenoids (Chakravarty *et al.*, 1996), amides (Greger *et al.*, 1992, 1993a,b, 1994, 1996; Hofer *et al.*, 1995a, 1998), imides (Hofer *et al.*, 1995b), alkaloids (Wu *et al.*, 1983; Wurz *et al.*, 1993; Ono *et al.*, 1995), coumarins (Rahmani *et al.*, 1998), and flavonoids (Wu *et al.*, 1995). Compounds exhibiting antifungal and insecticidal activities (Greger *et al.*, 1996) have already been isolated from several *Glycosmis* species. A screening of foliage against the citrus leafminer, *Phyllocnistis citrella*, demonstrated activity in both *M. koenigii* and *G. pentaphylla* (Jacas *et al.*, 1997). In addition to inhibiting juvenile hormone III *in vitro* synthesis in the cricket *Gryllus bimaculatus*, the addition of 10±4 to 10±5 M the quinazolone of arborine isolated from the ethyl acetate fraction of *G. pentaphylla* leaf extract to water resulted in 83 to 100% mortality of *C. quinquefasciatus* larvae (Muthukrishnan *et al.*, 1999).

1.1.1. Distribution and ecology of *G. pentaphylla*

G. pentaphylla is a wild, oriental in origin and an unarmed shrub or small tree distributed throughout the roadsides, under large trees or on uncultivated lands in Bangladesh, Srilanka, Eastern part of India, Southern Indochina, Malaysia, Indonesia and in Eastern Australia. A single species *G. parviflora* is found in Miami in the USA. In Bangladesh and India it is popularly known as Ash-sheura, Bon-nimbu, Bon-nebu, Atishoti, Vat, Dantan-gaas, (Rastogi *et al.*, 1980).



Region where *G. pentaphylla* grows in the World.

Fig. 2: Geographical distribution of *G. pentaphylla* (Retz.)

1.1.2. Taxonomic position of the test plant *G. pentaphylla* (Retz.)

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Sapindales
Family	Rutaceae
Genus	*Glycosmis*
Species	*Glycosmis pentaphylla*

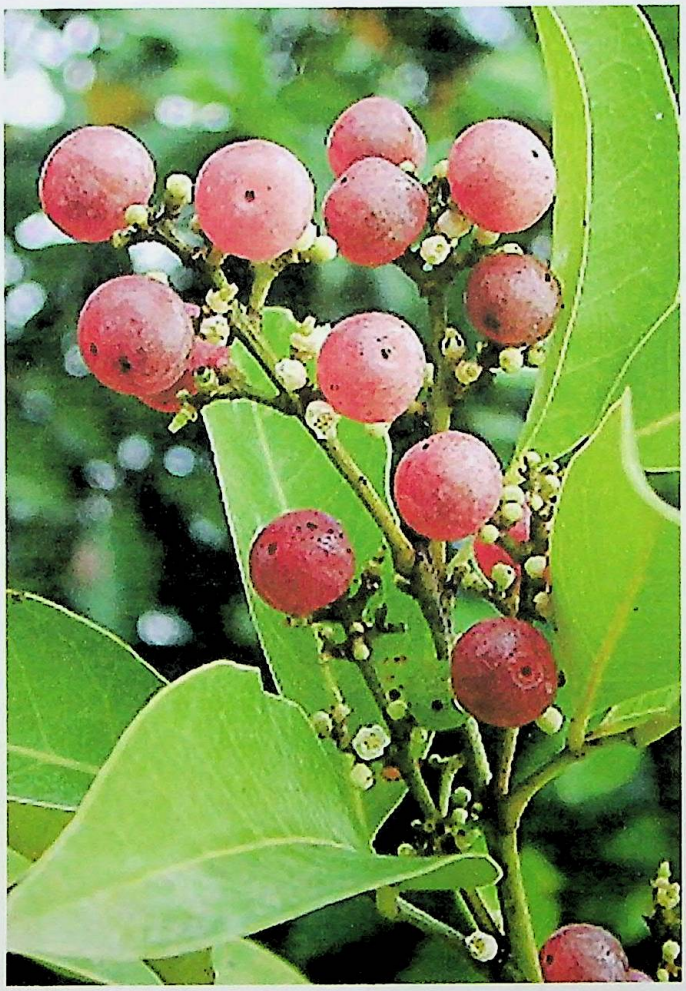


Plate 1: *Glycosmis pentaphylla* (Retz.)

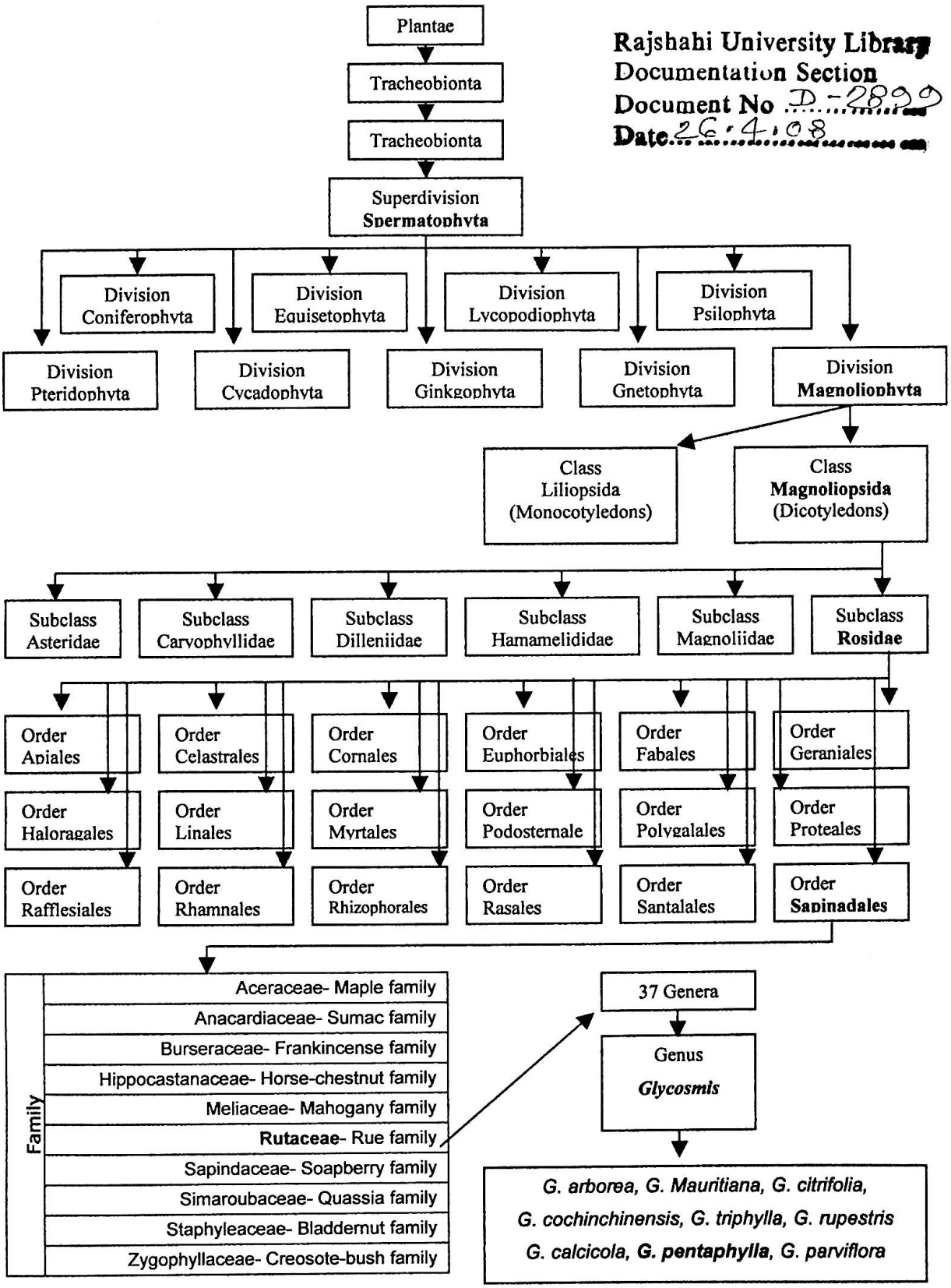


Fig. 3: Systematic position of *G. pentaphylla*

1.1.3. Previous findings on bioactive properties of the test plant

Investigation of bioactive properties for their possible use as ingredients of medicine and pesticide from plants has been a history for decades and a number of potential sources has also been traced so far. Medicinal plants are promising to contain such properties. Since antifungal and antibacterial or antimicrobial activity indicates efficacy against certain living beings, it also shows activity against other animals, such as, insects, nematodes, mollusks, etc. Chinese researchers reviewed that the plants used for pest insect control in China and found that there was a strong connection between medicinal and pesticidal plants. A large percentage of the drugs that exist today originally derived from natural sources, in which the active principles were found biologically active.

The genus has provided various alkaloids belonging to furoquinoline, acridone, quinazolone and carbazole groups, which are of taxonomic significance (Chakravarty *et al.*, 1953, Chakraborty, 1969; Chakraborty *et al.*, 1974). Being a medicinal plant and originally being confined to a certain area of Asia and Australia micropropagation of this plant is important. Furthermore, being *Citrus* related Genera, *Glycosmis* could have special attention, particularly when viewed as potentially valuable sources of novel genetic variation, such as, disease resistance for use in *Citrus* improvement programs (Akihama *et al.*, 1985).

The alkaloid quinazolone group isolated and identified from the EtOAc soluble fraction of a leaf extract of *G. pentaphylla* shown to inhibit juvenile hormone III-biosynthesis *in vitro* in the corpora allata from 3 day old virgin females of *Gryllus bimaculatus* in a dose-dependent manner (Muthukrishnan *et al.*, 1999).

Manipulation of endocrine control of development, growth, metamorphosis and reproduction is one of the mechanisms of action of these secondary plant substances (Richter and Koolman, 1991; Couillaud and Peypelut, 1995). A few plant species, such as, *Calophyllum inophyllum* Linn. (Clusiaceae), *G. pentaphylla* (Retz.) DC. (Rutaceae), *Rhinacanthus nasutus* Kurz. (Acanthaceae), *Solanum surratense* Burm. (Solanaceae) and *S. trilobatum* Linn. (Solanaceae) had been screened for their insecticidal activity in the laboratories (Pushpalatha, 1997; Muthukrishnan *et al.,* 1999). Exposure to partially purified leaf extract of *G. pentaphylla* in the medium extended the larval duration, inflicted very high larval mortality and induced developmental deformities in larvae of mosquitoes, *C. quinquefasciatus*, *Anopheles stephensi,* and *Aedes aegypti* (Pushpalatha and Muthukrishnan, 1995).

Oral administration of *G. pentaphylla* leaf extract to the penultimate and final instar larvae of the castor semilooper, *Achaea janata*, decreased the weight gain and inhibited larval±pupal and pupal±adult metamorphosis, respectively (Muthukrishnan and Ananthagowri, 1994). Besides, leaves of this plant are used to keep insects away from sweets and from other edible items that are taken as food by the natives in India, South Africa and Australia (Chopra *et al.,* 1956). Ethyl acetate extracts of *G. pentaphylla* leaves significantly reduced hatchability of *C. quinquefasciatus* eggs, prolonged developmental duration of mosquito larvae, reduced the number of adults emerged and induced malformation during development and metamorphosis at sublethal concentrations (2±50 ppm) (Naik *et al.*, 1979).

1.1.4. Phytochemical and biomedical properties from *G. pentaphylla* and some of its related species

A number of researchers investigated this plant phytochemically and found versatile openings mostly on biologically active components. An active principle, *glycosmin*, which is a crystalline glucoside, is present throughout the plant in traces only, its greatest concentration being found in the new leaves and buds, where it reaches the extent of 0.2 percent of sugars, while in the

mature leaves and soft stems, the percentage varies from 0.08 to 0.1 percent. Along with glycosmin, tannin, a phlobaphene, traces of salicin, and about 2.1 percent of sugars (both reducing and not reducing) have been found to be present in the leaves. Glycosmin, therefore is quite analogous to populin isolated from poplar buds, which is the benzoyl derivative of salicin. Unlike salicin (which is very bitter), glycosmin has only a slightly bitter taste that becomes apparent only after keeping the substance on the tongue for some time. However, the genus has provided various alkaloids belonging to furoquinoline, acridone, quinazolone and carbazole groups, which are of taxonomic significance (Chakravarty *et al.*, 1953, Chakraborty, 1969; Chakraborty *et al.*, 1974).

Four hydroquinone diglycoside acyl esters, glypentosides A-C and seguinoside F, were isolated from the stems of *G. pentaphylla*. Glypentosides A-B were identified as compounds are designated as methoxyquinol 4-O-[(5-O-trans-p-coumaroyl)-beta-d-apiofuranosyl-(1-->2)-beta-d-glucopyranoside] and 4-demethylantiarol 4-O-[(3-methoxy-4-hydroxy-benzoyl)-beta-d-apiofura nosyl-(1-->2)-beta-d-glucopyra noside]. Glypentoside C is a hydroquinone di glycoside acyl ester with a neolignan moiety in the acyl unit (Wang *et al.*, 2006).

Six apiosyl-(1→6)-glucosyl isoflavones and four known ones were isolated from the stems of *G. pentaphylla*. The structures of the new glycosides are 3¢,7-dihydroxy-4¢,5,6-trimethoxyisoflavone7-O-(5-O-trans-pcoumaroyl)-â-D-apiofuranosyl-(1→6) â-D-glucopyranoside, 2¢,7-dihydro xy-4¢,5¢,5,6-tetra methoxyisoflavone 7-O-(5-O-trans-p-coumaroyl)-â-D-apio furanosyl-(1f6)-â-D-glucopyranoside , 2¢,7-dihydroxy-4¢,5¢,5,6-tetrame thoxyisoflavone 7-O-â-D-apiofuranosyl-(1→6)-â-D-glucopyranoside, 7-hy droxy-4¢,8-dimethoxyisofla vone 7-O-â-D-apiofuranosyl- (1→ 6)-â-D-gluco pyranoside, 7-hydroxy-4¢,6-dimethoxyisoflavone 7-O-â-D-apiofuranosyl-(1→6)-â-D-glucopy rano side, and 4¢,5-dihydroxy-3¢,7-dimethoxyisoflavone 4¢-O-â-D-apiofuranosyl-(1→ 6)-

â-D-glucopyranoside. Their structures were established primarily by NMR experiments and chemical methods (Wang *et al.,* 2006).

Glycozolidol, $C_{14}H_{13}NO_2$ ($[M]^+$ 227), mp 240° has been isolated from the roots of *G. pentaphylla* (Bhattacharyya *et al.,* 1985). Chowdhury *et al.* (1987) in continuation of their search for carbazole alkaloides detected carbazole and 3-methylcarbazole from the root bark of *G. pentaphylla*. The compound 3-methylcarbazole was also furnished by Zinc dust distillation of glycozolicine, $C_{14}H_{13}NO$; ($[M]^+$ *m/z* 211) (mp 135°). (Jash *et al.*, 1992).

A new flavonoid, dihydroglychalcone-A, was isolated from the leaves extract of *G. chlorosperma* in addition to two known sulphur-containing amides, dambullin and gerambullin. The structure of the new compound was assigned as 2'-hydroxy-4, 6'-dimethoxy-3', 4'-(2", 2"-dimethylpyrano) dihydrochalcone. The extract of the leaves was also found to exhibit antimicrobial and cytotoxic activities.

Tetracyclic triterpenoids were isolated by Chakravarty *et al.* (1996) from the over ground (aerial) part of *G. arborea*. The overground part (10 kg) was dried, milled and extracted with petrol in a soxhlet apparatus for 24 h. The extract was concentrated and subjected to column chromatography (CC) in silica gel to obtain a triterpenoid fraction, which was acetylated with Ac_2O-pyridine at room temperature for 24 h. The acetate mixture on repeated CC over silica gel and neutral alumina yielded (24S)-24-methyl-5α-lanosta-9(11),25-dien-3α-ol, (24S)-24-methyl-5α-lanosta-9(11),25 -dien-3β-ol,24,24-dimethyl-5α -lanosta-9(11) 25-dien-3α-ol and 24,24-dimethyl-5α-lanosta-9(11), 25-dien-3β-ol, besides arborinyl acetate and isoarborinyl acetate, a sticky mass which on repeated preparative HPLC furnished (24S)-24-methyl-5α-lanosta-9(11),25-dien-3α-ylacetate,(24S)-24-methyl-5α-lanosta-9 (11),25-dien -3β- ylacetate, 24,24- dimethyl-5α- lanosta-9(11),25-dien-3α- ylacetate and 24,24-di methyl-5α-lanosta-9(11),25-dien-3β- ylacetate in pure form.

Chakravarty *et al.* (1999) added more alkaloids in the list of compounds derived from roots of *G. arborea*. Air-dried and milled roots of the plant were successively extracted with petrol (60-80°C) and MeOH at room temperature. The extracts were evaporated to dryness under reduced pressure before fractionation. The petrol extract yielded glycozoline (recrystallized from petrol-$CHCl_3$, mp 177-178°C, EI-MS *m/z* 211.0978 $[M]^+$), glycozolidine (recrystallized from benzene-MeOH, mp 166-167°C, EI-MS *m/z* 241.1108 $[M]^+$) and 3-(3',3'-Dimethylallyl)-4,8-dimethoxy -N-methyl-quinolin-2-one (EI-MS *m/z* 287.1513 $[M]^+$) by repeated chromatography over neutral alumina. The MeOH extract on chromatographic purification over the same absorbent furnished glycoborinine (recrystallized from benzene, mp 220-221°C, EI-MS *m/z* 279.1269 $[M]^+$), Skimmianine (recrystallized from benzene-MeOH, mp 177-178°C, EI-MS *m/z* 259.0834 $[M]^+$) and also glycozoline. Glycozolidine was also reported from root bark extract of *G. pentaphylla* by Chakraborty and Das (1966), where they have indicated mp 161-162°C and molecular formula as $C_{15}H_{15}NO_2$ and mass spectral measurements 241 $[M]^+$. In the same communication it was also reported that glycozoline and 3-methyl-6-methoxy carbazole were isolated from the same source by Chakraborty (1966). The structure of glycozolidine was further taken into discussion by Chakraborty *et al.* (1974), where the mp was indicated as 160-162°C.

Skimmianine has been isolated from the root bark of *G. pentaphylla* along with dictamnine and γ-fagarine. A crystalline alkaloid (mp 176°C) was isolated from the eluate, R_f 0.41 in 30% aqueous alcohol and the analytical data corresponded to the formula $C_{14}H_{13}NO_4$ which were identical with those of skimmianine. Another crystalline alkaloid dictamnine was isolated from the same source, mp 130-132, R_f 0.36 in 30% aqueous alcohol and the formula was established as $C_{12}H_9NO_2$ (Chakraborty and Barman, 1961).

Jash *et al.* (1992) reported glycozolicine, 3-formylcarbazole and glycosinine from *G. pentaphylla*. From the C_6H_6 eluate glycozolicine was obtained, which

was crystallized from C_6H_6-$CHCl_3$, 1:1; R_f 0.39. The formula of the compound was found $C_{14}H_{13}NO$. From the C_6H_6-$CHCl_3$ eluate, a colourless solid was obtained which showed the presence of two compounds by TLC on silica gel (Benzene-hexane-EtOAc, 8:9:3; R_f 0.26 and 0.22). These two compounds were separated by repeated preparative TLC. From R_f 0.26 a compound, mp 158°C, was obtained which was identified as 3-formylcarbazole, $C_{13}H_9NO$. From R_f 0.22 another compound, mp 185°C was obtained, which was identified as glycosmine, $C_{14}H_{11}NO_2$ (Fig. 4).

Sinhababu and Thakur, (1995) dealt with the flower of the test plant *G. pentaphylla* for isolation and characterization of some alkaloides and an amide. Elution of the chloroform extract on alumina with benzene-chloroform, 9:1 gave arborine (recrystallized from benzene-hexane), glycorine (recrystallized from benzene) and benzamide-2-methylamine, mp 160-161°C, EI-MS M^+ 150. Further elution of the same with EtOAc-benzene, 30:70 gave glycosmicine (recrystallized from MeOH). Wurz *et al.,* (1993), isolated schinifoline and phenaglydon from the leaves of G. *cyanocarpa*. It has also some sterols (β-sitosterol and stigmesterol), sugars 2.1% and aphlobephene and tannin.

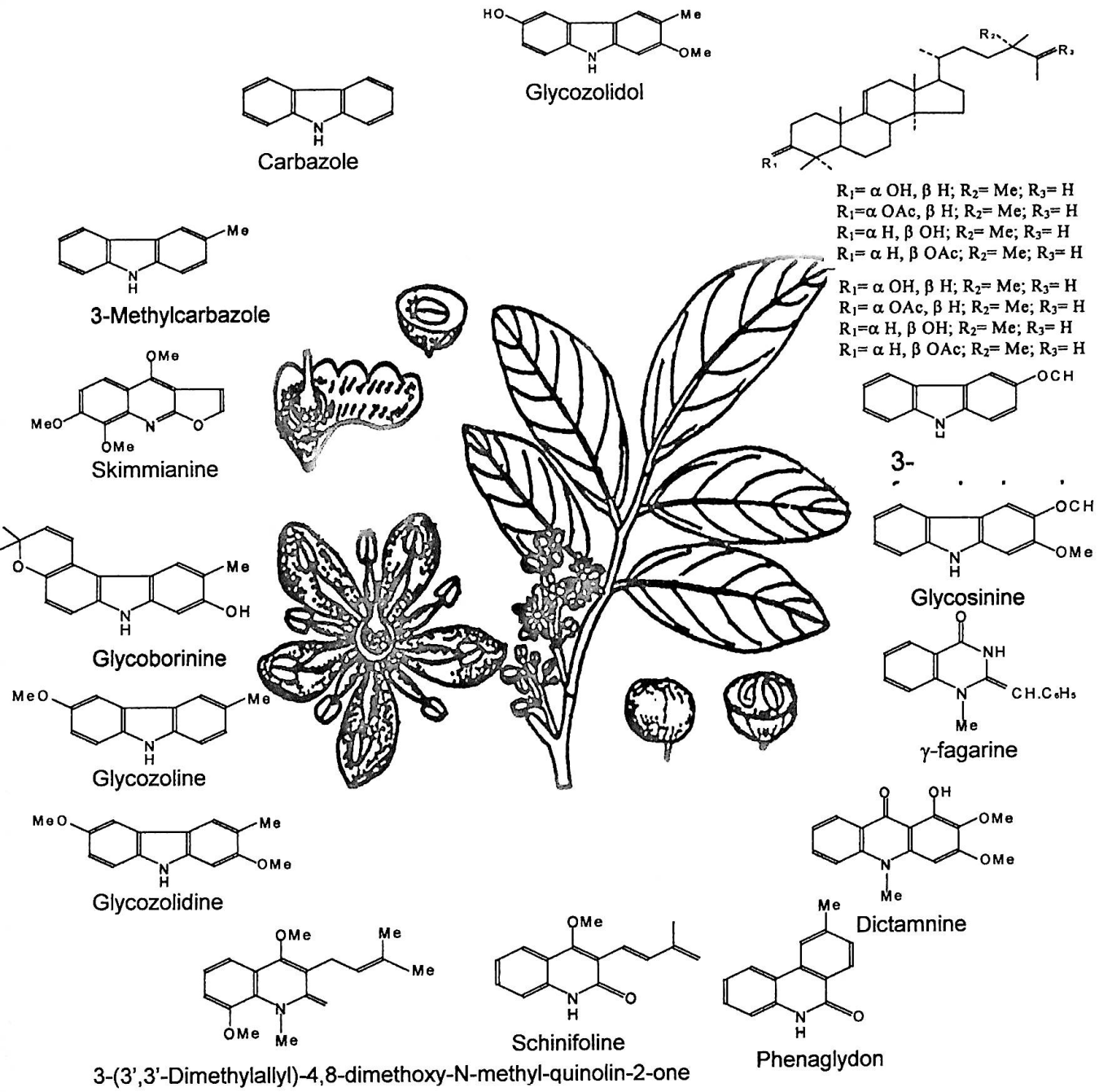


Fig. 4: Some of the compounds isolated from different parts of *G. pentaphylla*

Table 1: Previous findings of the *G. pentaphylla* components

Name of the compound	Authors	Year	Journal	Title
Glycozolidol	Bhattacharyya *et al.*	1985	Phytochemistry, **24** (4): 882-883	Glycozolidol, an antibacterial carbazole alkaloid from *Glycosmis pentaphylla*
Carbazole	Chowdhury *et al.*	1987	Phytochemistry, **26** (7): 2138-2139	Carbazole and 3-methylcarbazole carbazole from *Glycosmis pentaphylla*
3-methylcarbazole	Chowdhury *et al.*	1987	Phytochemistry, **26** (7): 2138-2139	Carbazole and 3-methylcarbazole carbazole from *Glycosmis pentaphylla*
3-methylcarbazole	Jash *et al.*	1992	Phytochemistry, **31**(7): 2503-2505	Carbazole alkaloides from *Glycosmis pentaphylla*
(24S)-24-methyl-5α-lanosta-9(11),25-dien-3α-ol	Chakravarty *et al.*	1996	Phytochemistry, **42** (4): 1109-1113	Tetracyclic triterpenoids from *Glycosmis arborea*
(24S)-24-methyl-5α-lanosta-9(11),25-dien-3β-ol	Chakravarty *et al.*	1996	Phytochemistry, **42** (4): 1109-1113	Tetracyclic triterpenoids from *Glycosmis arborea*
24,24-dimethyl-5α-lanosta-9(11),25-dien-3α-ol	Chakravarty *et al.*	1996	Phytochemistry, **42** (4): 1109-1113	Tetracyclic triterpenoids from *Glycosmis arborea*
24,24-dimethyl-5α-lanosta-9(11),25-dien-3β-ol	Chakravarty *et al.*	1996	Phytochemistry, **42** (4): 1109-1113	Tetracyclic triterpenoids from *Glycosmis arborea*
(24S)-24-methyl-5α-lanosta-9(11),25-dien-3α-ylacetate	Chakravarty *et al.*	1996	Phytochemistry, **42** (4): 1109-1113	Tetracyclic triterpenoids from *Glycosmis arborea*
(24S)-24-methyl-5α-lanosta-9(11),25-dien-3β- ylacetate	Chakravarty *et al.*	1996	Phytochemistry, **42** (4): 1109-1113	Tetracyclic triterpenoids from *Glycosmis arborea*

24,24-dimethyl-5α-lanosta-9(11),25-dien-3α- ylacetate	Chakravarty *et al.*	1996	Phytochemistry, **42** (4): 1109-1113	Tetracyclic triterpenoids from *Glycosmis arborea*
24,24-dimethyl-5α-lanosta-9(11),25-dien-3β- ylacetate	Chakravarty *et al.*	1996	Phytochemistry, **42** (4): 1109-1113	Tetracyclic triterpenoids from *Glycosmis arborea*
Glycoborinine	Chakravarty *et al.*	1999	Phytochemistry, **50**: 1263-1266	Carbazole alkaloids from roots of *Glycosmis arborea*
glycoquinone (**1**)	Chihiro ITO et al	November 1999	Chem. Pharm. Bull. **47**(11) 1579—1581	Chemical Constituents of *Glycosmis pentaphylla*. Isolation of A Novel Naphthoquinone and A New Acridone Alkaloid
glycocitrine-III (**2**).	Chihiro ITO et al	November 1999	Chem. Pharm. Bull. **47**(11) 1579—1581	Chemical Constituents of *Glycosmis pentaphylla*. Isolation of A Novel Naphthoquinone and A New Acridone Alkaloid
Glycozoline	Chakravarty *et al.*	1999	Phytochemistry, **50**: 1263-1266	Carbazole alkaloids from roots of *Glycosmis arborea*
Glycozolidine	Chakravarty *et al.*	1999	Phytochemistry, **50**: 1263-1266	Carbazole alkaloids from roots of *Glycosmis arborea*
Glycozolidine	Chakraborty & Das	1966	Sci. & Cult., **32**: 181-182	Glycozolidine, a new carbazole derivative from *Glycosmis pentaphylla* (Retz.) DC.
Glycozolidine	Chakraborty *et al.*	1974	The Plant Biochem. J. **1**(2):73-77	Structure of Glycozolidine
Skimmianine	Chakravarty *et al.*	1999	Phytochemistry, **50**: 1263-1266	Carbazole alkaloids from roots of *Glycosmis arborea*
Skimmianine	Chakraborty & Barman	1961	Trans. Bose Res. Inst., **24**(3): 121-123	Alkaloides of the root bark of *Glycosmis pentaphylla* Retz. (D.C.)
3-(3',3'-Dimethylallyl)-4,8-dimethoxy-N-methyl-quinolin-2-one	Chakravarty *et al.*	1999	Phytochemistry, **50**: 1263-1266	Carbazole alkaloids from roots of *Glycosmis arborea*
γ-fagarine	Chakraborty & Barman	1961	Trans. Bose Res. Inst., **24**(3): 121-123	Alkaloides of the root bark of *Glycosmis pentaphylla* Retz. (D.C.)
Dictamnine	Chakraborty & Barman	1961	Trans. Bose Res. Inst., **24**(3): 121-123	Alkaloides of the root bark of *Glycosmis pentaphylla* Retz. (D.C.)

Glycozolicine	Jash *et al.*	1992	Phytochemistry, **31**(7): 2503-2505	Carbazole alkaloides from *Glycosmis pentaphylla*
3-formylcarbazole	Jash *et al.*	1992	Phytochemistry, **31**(7): 2503-2505	Carbazole alkaloides from *Glycosmis pentaphylla*
Glycosinine	Jash *et al.*	1992	Phytochemistry, **31**(7): 2503-2505	Carbazole alkaloides from *Glycosmis pentaphylla*
Benzamide-2-methylamine	Sinhababu & Thakur	1995	Asian J. Chem. **7**(1): 221-222	Constituents of the flower of *Glycosmis pentaphylla* Retz. Correa
Glycorine	Sinhababu & Thakur	1995	Asian J. Chem. **7**(1): 221-222	Constituents of the flower of *Glycosmis pentaphylla* Retz. Correa
Glycoplymine	Sinhababu & Thakur	1995	Asian J. Chem. **7**(1): 221-222	Constituents of the flower of *Glycosmis pentaphylla* Retz. Correa
Glycosmicine	Sinhababu & Thakur	1995	Asian J. Chem. **7**(1): 221-222	Constituents of the flower of *Glycosmis pentaphylla* Retz. Correa
Schinifoline	Wurz *et al.*	1993	Nat. Prod. Let. **3**(3): 177-182	Structure and synthesis of phenaglydon, a new quinolone derived phenanthridine alkaloid from *Glycosmis cyanocarpa*
Phenaglydon	Wurz *et al.*	1993	Nat. Prod. Let. **3**(3): 177-182	Structure and synthesis of phenaglydon, a new quinolone derived phenanthridine alkaloid from *Glycosmis cyanocarpa*
methoxyquinol 4-O-[(5-O-trans-p-coumaroyl)-beta-d-apiofuranosyl-(1-->2)-beta-d-glucopyranoside	Wang et al	2006 Jan17	Phytochemistry.; : 16426648	Hydroquinone diglycoside acyl esters from the stems of *Glycosmis pentaphylla*
4-demethylantiarol 4-O-[(3-methoxy-4-hydroxy-benzoyl)-beta-d-apiofuranosyl-(1-->2)-beta-d-glucopyranoside	Wang et al	2006 Jan17	Phytochemistry.; : 16426648	Hydroquinone diglycoside acyl esters from the stems of *Glycosmis pentaphylla*
Glypentoside C (3) is a hydroquinone diglycoside acyl ester with a neolignan moiety in the acyl unit	Wang et al	2006 Jan17	Phytochemistry.; : 16426648	Hydroquinone diglycoside acyl esters from the stems of *Glycosmis pentaphylla*

seguinoside F (4),	Wang et al	2006 Jan17	Phytochemistry.; : 16426648	Hydroquinone diglycoside acyl esters from the stems of *Glycosmis pentaphylla*
3′,7-Dihydroxy-4¢,5,6-trimethoxyisoflavone 7-*O*-(5-*O*-*trans*-*p*-coumaroyl)-â-D-apiofuranosyl-(1f6)-â-D-glucopyranoside	Wang et al.	2006	J. Nat. Prod., 69, 778-782	Isoflavone Diglycosides from *Glycosmis pentaphylla*
2′,7-Dihydroxy-4¢,5¢,5,6-tetramethoxyisoflavone 7-*O*-(5-*O*-*transp*-coumaroyl)-â-D-apiofuranosyl-(1f6)-â-D-glucopyranoside	Wang et al.	2006	J. Nat. Prod., 69, 778-782	Isoflavone Diglycosides from *Glycosmis pentaphylla*
2′, 7-Dihydroxy-4′,5′ë,5,6-tetramethoxyisoflavone 7-*O*-â-D-apiofuranosyl-(1f6)-â-D-glucopyranoside	Wang et al.	2006	J. Nat. Prod., 69, 778-782	Isoflavone Diglycosides from *Glycosmis pentaphylla*
7-Hydroxy-4′,8-dimethoxyisoflavone 7-*O*-â-D-apiofuranosyl-(1f6)-â-D-glucopyranoside	Wang et al.	2006	J. Nat. Prod., 69, 778-782	Isoflavone Diglycosides from *Glycosmis pentaphylla*
7-Hydroxy-4′,6-dimethoxyisoflavone 7-*O*-â-D-apiofuranosyl-(1f6)-â-D-glucopyranoside	Wang et al.	2006	J. Nat. Prod., 69, 778-782	Isoflavone Diglycosides from *Glycosmis pentaphylla*
4′, 5 -Dihydroxy-3¢,7-dimethoxyisoflavone 4¢-*O*-â-D-apiofuranosyl-(1f6)-â-D-glucopyranoside	Wang et al.	2006	J. Nat. Prod., 69, 778-782	Isoflavone Diglycosides from *Glycosmis pentaphylla*

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Indian Herbal Research & Methods By Pankaj Oudhia (http://www.botanicalboards.org/poudhia)

1.1.5. Uses of the source plant in traditional medicine and in public

Different parts of *G. pentaphylla* are used as therapeutic agents in the indigenous system of Indian traditional medicine (Kirtikar and Basu, 1935). The roots pounded and mixed with sugar are given in cases of low-fever, for liver complaints, and certain other diseases. The wood ground with water and administered internally as an antidote for snake-bite. In some areas of China, an infusion of the dried leaves is given as a tonic and appetizer to women after delivery. Water extract of fresh stem-bark is used as an ingredient in a folk medicine against cough and problems in respiration. Folk use of its leaves, stem-bark or twigs to heal up wounds is still prevalent in Bangladesh.

The test plant *G. pentaphylla* has been reported to elaborate carbazole alkaloids (Chakraborty, 1969, 1977; Chakraborty *et al.,* 1974; Bhattacharyya *et al.,* 1985). According to the Indian Medicinal Plants having liver protection properties against chemical-induced liver damage in experimental animals (Mitra *et al., 1997.*) different parts of this plant are used as therapeutic agents in the indigenous systems of medicine (Kirtikar and Basu, 1935); the roots pounded and mixed with sugar are said to be effective in low fever (Chopra *et al.,* 1956). Very recently, paraffin-oil soluble part of water decoction of the roots of this plant has been found to be effective against haemorrhoids. It is an age-old practice in Bangladesh and West Bengal to use branches of this shrub as toothbrush, while the stems are largely used as toothbrushes in eastern Bengal on account of their fibrous nature and slightly astringent, bitter taste. Their constant use not only makes the teeth clean but also keeps them strong.

The bitter juice of the leaves of this plant is very widely used for fevers, liver complaints and intestinal worms, particularly in the case of children's diseases. Occasionally the stem and the root of this plant are used on ulcers with good results. The leaves of this plant are good antidotes for eczema and other skin diseases. Its leaves, made into paste with a bit of ginger, are

applied over the affected part of the skin, and sometimes the paste of the leaves alone is applied over the navel for worms and for disorders of the bowels.

Arborinine, an acridone alkaloid obtained from *G. pentaphylla*, exhibited significant inhibition of crown gall tumors produced by *Agrobacterium tumefaciens* in a potato disc bioassay. Chemical constituents of MeOH-CH_2Cl_2 extract of the stem of *G. pentaphylla* collected in Papua New Guinea furnished a novel naphthoquinone and a new acridone alkaloid called glycoquinone and glycocitrine-III respectively, and they were isolated along with twelve known compounds. This is the first isolation of a naphthoquinone derivative from the genus *Glycosmis*. (Ito *et al.*, 2000).

The traditional healers of India consider the insect powder very useful in treatment of leucoderma [safed dag]. In combination with cow urine and bemchi (*Psordea coryliflia*) seeds, it is applied externally in the form of an aqueous paste and its application is very popular among the healers. Most of the healers consider it as a promising blood purifier. In normal cases of skin troubles it is given in combination with other medicinal herbs. The species *G. pentaphylla* is also sexually incompatible with citrus, but is partially graft-compatible with some citrus species (Bowman *et al.*, 2001).

1.1.6. Uses of the test plant in the homoeopathic system of medicine

Homoeopathic name: *Atista indica*; other names used in the homeopathic texts: common name- Ash sheora, sanskrit name- Venamenibuka or Ashvashakota, hindi name- Bannimbu, Bangla- Atishora, English name- Toothbrush plant or Orange berry.

A medicine produced with the *G. pentaphylla* ingredients has been found clinically useful against amoebiasis, dyspepsia, migraine and irritable bowel syndrome. This drug is proved to be effective in bilious complaints like nausea, vomiting, bitter taste in the mouth, heart-burn with desire for lime

juice which ameliorates and gastric symptoms are aggravated by eating. In gastric affections, colicky pain around navel is the keynote, and on this symptom it has helped in expulsion of worms. It is useful against *Leucorrhoea*- a white discharge with burning which runs down to heels and bring the patient towards lying down and also against *Migraine- a certain* pain in temples, throbbing type with bilious vomiting followed by weakness. This drug has been proved by the CCRH (Central Council for Research in Homeopathy) and a monograph has also been published in this regard.

1.1.7. Information on antimicrobial and pesticidal activities of the test plant

Leaf extracts of *G. mauritiana* and *G. pentaphylla* collected from different Sri Lankan provinces have been compared by HPLC analyses and tested for antifungal activity. All collections of *G. pentaphylla* are uniformly characterized by the known alkaloids arborine, skimmianine and arborinine, whereas *G. mauritiana* differ by displaying chemical polymorphism of different sulphur-containing amides. Three new amides, named illukumbin B, methylillukumbin B and methylillukumbin A, have been isolated and their structures are described on the basis of spectroscopic evidences. They show different antifungal activities in bioautographic tests and appear to be accumulated mainly in individuals collected from more humid and elevated habitats (Greger, 1993a).

The $CHCl_3$ extract of *G. calcicola* has been shown to be most effective in inhibiting mycelial growth, sporulation, and spore germination on three fungal pathogens of chili. The antifungal compounds were identified as flindersine and desmethoxyanthophylline and their structures were determined by spectroscopic methods and comparison with reported data. The Antifungal activity of *G. calcicola* and *G. rupestris* extracts have been traced successfully.

Species in the Genus *Glycosmis* (Stone, 1985) contain a wide variety of compounds with potential biological activity and these include terpenoids (Chakravarty *et al.*, 1996); amides (Greger *et al.*, 1992, 1993a,b, 1994, 1996; Hofer *et al.*, 1995a, 1998); imides (Hofer *et al.*, 1995b); alkaloids (Wu *et al.*, 1983; Wurz *et al.*, 1993; Ono *et al.*, 1995); coumarins (Rahmani *et al.*, 1998) and flavonoids (Wu *et al.*, 1995). Compounds exhibiting antifungal and insecticidal activities (Greger *et al.*, 1996) have already been isolated from several *Glycosmis* species. Recently, it is found in a screening of foliage against the citrus leafminer, *Phyllocnistis citrella*, demonstrated activity in both *Murraya koenigii* and *G. pentaphylla* (Jacas *et al.*, 1997). *G. pentaphylla* also exhibited antijuvenile hormone activity against the field cricket *Gryllus bimaculatus*, and activity was traced to the quinazolone alkaloid arborine (Muthukrishnan *et al.*, 1999).

Insecticidal and antifungal activities have been attributed to various bioactive amides from *Glycosmis* species and, specifically, to dehydrothalebanin B in preliminary tests (Greger *et al.*, 1996). Commercially used citrus rootstocks can all be seriously damaged by larvae of the sugarcane root weevil, *D. abbreviatus* (L.). The species *G. pentaphylla* (Retz.) Correa suffered as much damage from the weevil as common rootstock cultivars but significantly depressed growth of larvae feeding on them. One new hybrid rootstock, HRS-801, also significantly depressed *D. abbreviatus* larval growth.

In three of the six experiments, no significant differences in percentage root loss were observed between the cultivars/species tested. Plants of *Murraya paniculata*, *Citropsis gilletiana*, Smooth Flat Seville (*Citrus aurantium*), Sun Chu Sha (*Citrus reticulata*), *G. pentaphylla*, and the hybrid HRS-802 (*Citrus grandis*) did not suffer significant root loss from feeding of *D. abbreviatus* in at least one experiment (Shapiro *et al.*, 1997). In the diet-incorporation test against *Diaprepes*, with finely milled roots of *G. pentaphylla* at only 5% concentration in diet produced the same relative growth inhibition seen in whole live roots (Shapiro, 1992).

G. pentaphylla was resistant to *Diaprepes* weevil in greenhouse testing and diet feeding assays. Experiments are underway to identify suitable interstocks to allow use of *G. pentaphylla* rootstocks with commercial scions in areas infested by *D. abbreviatus*. The biochemical basis of resistance in *G. pentaphylla* has been investigated (Shapiro *et al.* 1997), and one component of this resistance has been identified as dehydrothalebanin (Shapiro *et al.*, 2000).

1.2. Background information on the test organisms

The whole project has been designed to carry on screening of the crude extracts of the test plant species on several test organisms for the detection of biological activity and isolation, purification and characterization of the bioactive compounds through chromatographic techniques, keeping an option to show extent of activity by analyzing the data statistically that read on various parameters during the course of the work. The following test steps have been taken into consideration:

1. Antimicrobial activity	Antibacterial	Gram positive bacteria 5
		Gram negative bacteria 6
	Antifungal	Plant and human pathogenic fungi 5
2. Cytotoxicity		*Artemia salina*
3. Larvicidal activity		*Musca domestica*
4. Repellent activity		*Tribolium castaneum*
5. Insceticidal activity		1. *Tribolium castaneum*
		2. *Callosobruchus maculatus*
		3. *Alphitobius diaperinus*

1.2.1. Selected bacteria and fungi for antimicrobial activity tests

It is very important to determine whether the crude chloroform extracts are active against various types of test organisms or not. And to do this a preliminary antibacterial and antifungal screening of the crude extract is very much necessary.

Tests	Target organisms
Antibacterial activity	Gram positive
	1. *Staphylococcus aureus*
	2. *Bacillus megaterium*
	3. *Bacillus subtilis*
	4. *Sarcina lutea*
	5. *Streptococcus-b -haemolyticus*
	Gram negative
	6. *Salmonella typhi*
	7. *Shigella dysenteriae*
	8. *Shigella sonnei*
	9. *Shigella boydii*
	10. *Escherichia coli*
	11. *Pseudomonas aeruginosa*
Antifungal activity	1. *Fusarium vasinfectum*
	2. *Aspergillus fumigatus*
	3. *Aspergillus flavus*
	4. *Mucor* sp.
	5. *Candida albicans*

Therefore, screening was done against various test pathogenic bacteria and fungi by disc diffusion assay (Bauer *et al.* 1966; Barry *et al.,* 1976) method. The fungal strains and bacterial isolates were used in the sensitivity test are mentioned above and pure cultures of the strains were collected from the Molecular Biology Laboratory, Institute of Biological Sciences, University of Rajshahi, Rajshahi, Bangladesh. The selected bacteria and fungi used in the investigation are well known and all of them are plant or human pathogenic in nature.

1.2.2. *A. salina* (brine shrimp) nauplii for cytotoxicity test

Brine shrimp lethality bioassay is a recent development in the bioassay for the bioactive compounds, which indicates cytotoxicity, as well as, a wide range of pharmacological activities e.g. anticancer, antiviral, pesticidal, AIDS, etc. of the compounds. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, anti-microbial and pharmacological activities of natural products. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. Here *in vivo* lethality of a simple zoological organism (brine shrimp) is used as a convenient monitor for screening in the discovery of new bioactive natural products. Generally, the median effective dose (ED_{50}) values for cytotoxicity are one tenth (1/10) of median lethal dose (LC_{50}) values in the brine shrimp test.

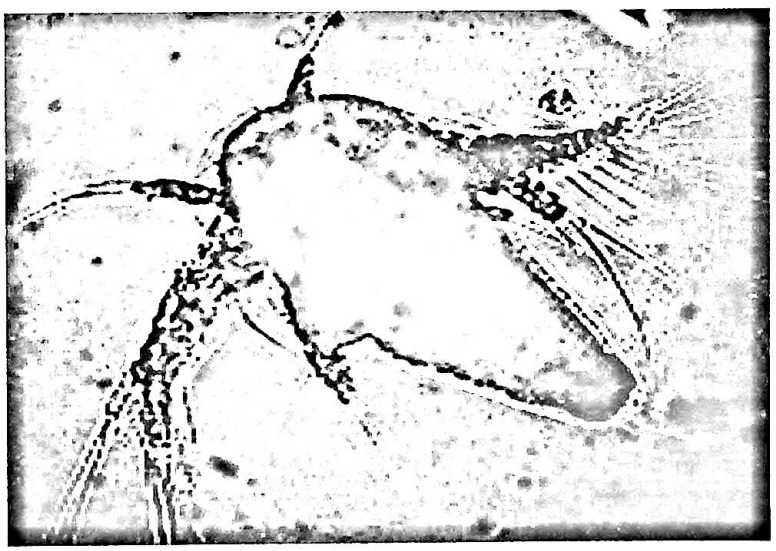


Plate 2: *A. salina* (brine shrimp) nauplius

The *A. salina* belongs to a genus of very primordial crustacean (crawfish - crayfish) - the *Anostraca* (Fairy Shrimps). Crawfish of this genus just have a divided exoskeleton made of chitin-enhanced protein, no usual crust of chitin (escutcheon) as other crawfish have. There are many species within the genus of *Anostraca*, but the *A. salina* are very nice to grow, since the rate of successful hatches is very high. It is very easy to collect from the market throughout the world since the *A. salina* nauplii are being used as aquarium fish feed, and it is easy to get the nauplii hatched out in the laboratory. Furthermore, it is easy to keep them in normal room temperature for a couple of days without providing any food so that in the mean time an experiment could be carried out.

1.2.3. Test agents for larvicidal activity tests

1.2.3.1. *M. domestica* L. larvae for larvicidal activity test

The housefly *M. domestica* was chosen for larvicidal activity tests because it is easy to rear, and it has a short developmental period (about 12 days at 26.5°C) and it has economic and medical significance. According to Crosskey and Lane (1993) the classification of *M. domestica* L. is as follows:

Phylum	Arthropoda
Sub-Phylum	Mandibulata
Class	Insecta
Sub-Class	Pterygota
Division	Endopterygota
Order	Diptera
Family	Muscidae
Genus	*Musca*
Species	*M. domestica* L.

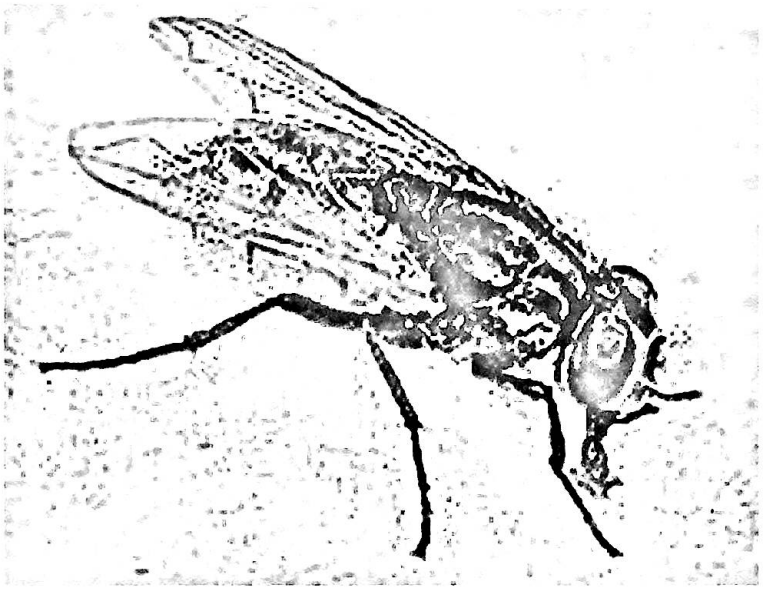


Plate 3: *M. domestica*

The fly that is typical of the Muscoidea is the best known and most notorious of all flies, *M. domestica* L., the housefly, or just the fly to millions of people (Oldroyd, 1964). Flies are abundant species that can be found almost everywhere. They usually occur in such large numbers as to constitute a nuisance. Muscidae also include the little housefly, face fly, stable fly and horn fly, all of which are pests of livestock. Houseflies are important pest from the family muscidae (Diptera). They are considered a nuisance and vectors of human and animal diseases. Houseflies transmit several diseases and pathogens that are harmful to livestock (Cumming and Cooper, 2000). These are commonly found where people work or live because of the warmer environment and ready supply of food. Eggs of houseflies are laid in moist or rotting matter, household rubbish compost of manure and once hatched the flies reach maturity in anything from two weeks in warmer weather. Flies are difficult to control because of their rapid rate of reproduction. One female fly can produce thousands offspring in a single breeding season. Flies also have the ability to develop in small quantities of food. Flies also have the typical insect ability to enter through and hide in openings the size of a pinhead (Snyder, 1991).

M. domestica is a well-known cosmopolitan pest both farm and home. This species is always found in association with humans or activities of humans. It is a highly versatile insect and suits its behavior of the local conditions as it finds them (Oldroyd, 1964). It is reported to transmit more than 20 human and animal diseases (Hicking 1974). Mastitis, Pinkeye, Anthrax, Typhoid fever, Amoebic dysentery, Tuberculosis, Cholera, Newcastle disease and salmonella are some of the diseases affecting man and animals that can be transmitted by these flies. Pathogenic organisms are picked up by flies from garbage, sewage and other sources of filth and then transferred on their mouthparts and other body parts through their vomits, feces and contaminated external body parts to human and animal food (Sanchez-Arryo, 1998).

1.2.3.2. *T. castaneum* (Hbst.) larvae for larvicidal activity test

This noble laboratory insect is good to carry on larvicidal assay, and information about it is given in the following step while an introduction has been made in connection with repellent activity test (at 1.2.4.).

1.2.4. *T. castaneum* (Hbst.) adults for repellent activity test

The rust-red flour beetle, *T. castaneum* (Herbst) is one of the most serious pests of stored products. It is commonly known as red flour beetle (Coleoptera: Tenebrionidae). Mouthparts of this pest insect are not adapted to feed on hard whole grains and they are thus found in almost any kind of flour, cracked grains etc. listed the specific food of *T. castaneum,* which includes whole-wheat flour, bran, rice flour, cornmeal, barley flour and oatmeal. It also feeds upon dried fruits, dried plant roots, nuts, chocolates, drugs, snuff, cayenne pepper, pulses and prepared cereal foods such as corn flakes (Metcalf and Flint, 1962). Not only pulses and millets, but also cereals are also been attacked by this beetle (Cotton, 1947; Purthi and Singh, 1950).

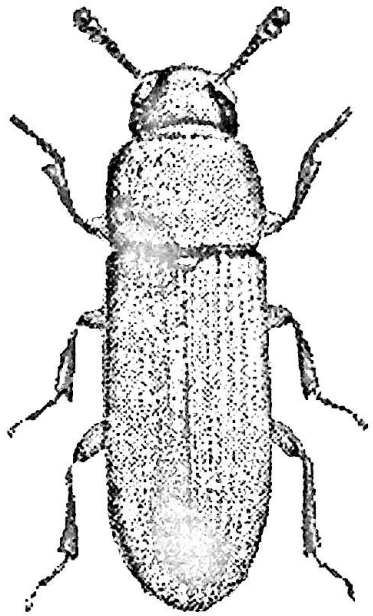


Plate 4: *T. castaneum* (Hbst.) adult

They are found in great numbers on infested materials and caused serious losses and considerable damage to flour and grains that have previously been attacked by other pests. Much of the damage done by *T. castaneum* is directly to kernels (germ and endoplasm). In case of severe infestation flour or other materials invaded may have a characteristics pungent odor as a result of the gaseous secretion exuded by the beetle. Such flour has an exceedingly low viscosity and its elasticity is markedly affected, which may cause gastric disturbance if used as food (Payne, 1925). In severe infestation, the flour turn grayish and moldy and has a pungent, disagreeable odor making it unfit for human consumption (Good, 1936). Infested material will show many elongate reddish brown beetles, about 1/7 inch long crawling over the material when it is disturbed and brownish white (somewhat flattened) six-legged larval bedding on the inside of the grain kernels and crawling over the infested seeds. They are generally known among millers as bran bugs. *T. castaneum* contaminates more than they consume. According to Khan and Mannan (1991) this contamination results from:

- The presence of living or dead insects or insect parts;
- Cast exuviae, egg shell and pupal cases;
- Fecal and persistent odour; and
- Webbing of food.

Both the larva and adults cause damage. The young larva is yellowish white and measures 1 mm in length. As it matures, it turns reddish yellow, becomes hairy and measures over 6 mm in length. Its head, appendages and the last abdominal segment are darker. The adult is a small reddish-brown beetle, measuring about 3.5 mm in length and 1.2 mm in width. Its antennae are bent and bear a distinct club formed by the three enlarged terminal joints. The last antennal segment is transversely rounded. The exact origin of *T. castaneum* is not known but according to Blair (1930) it was commonly found in the wild state in rotting wood and in loose bark of trees in India. This insect is now widely distributed all over the world mainly through commerce.

Tribolium species are major pests of stored grains and grain products in the tropics (Howe, 1965). Control of these insects relies heavily on the use of synthetic insecticides and fumigants, which has led to problems such as disturbances of the environment, increasing costs of application, pest resurgence, pest resistance to pesticides and lethal effects on non-target organisms in addition to direct toxicity to users (Jembere *et al.*, 1995). Thus, repellents, fumigants, feeding deterrents and insecticides of natural origin are rational alternatives to synthetic insecticides.

1.2.5. Isecticidal activity tests

1.2.5.1. *T. castaneum* (Hbst.) adults for insecticidal activity test

Information about this test organism is given in the preceding step while an introduction has been made in connection with repellent activity test (at 1.2.4.).

1.2.5.2. *C. maculatus* for insecticidal activity test

One of the target insects used in this investigation is the cowpea weevil, *C. maculatus* (F.). The cowpea weevil is an economically important storage pest (Singh and Jackai, 1985). Among the insects attacking stored products, Bruchidae and especially *C. maculatus* has involved the attention of many scientists not only because it can easily be manipulated but also because of its economic importance. Its common English name is cowpea weevil, spotted cowpea bruchid, and cowpea seed beetle and the scientific name is *C. maculatus* (F.), synonymous for *C. maculatus* are *Bruchus quadrimaculatus* F. and *Bruchus maculatus* F.

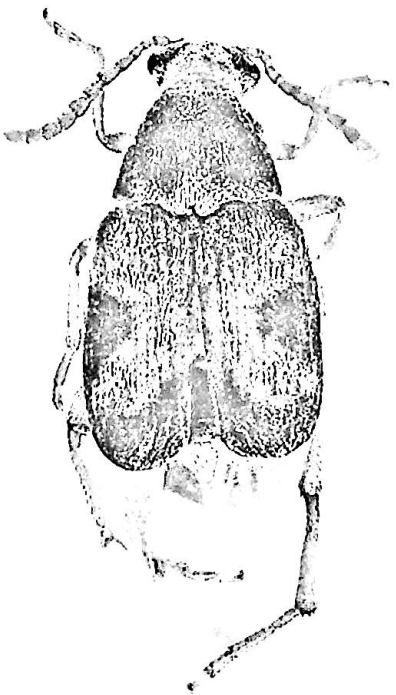


Plate 5: *C. maculatus* adult

1.2.5.3. *A. diaperinus* for insecticidal activity test

The darkling beetle or black bug or lesser mealworm, *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae) is a notorious and serious pest of a great variety of stored products. It is cosmopolitan in distribution and is associated with wheat, barley, rice, oatmeal, soybeans, cowpeas, peanuts, etc. and it has also been reported from linseed, cotton seed, oilseed products, tobacco,

skins, drugs, poultry litter, etc. (Lepesme, 1944; Lancaster and Simco, 1967; Spilman, 1987). It is also noted that this is a serious pest of poultry farms throughout the world (Ichinose, 1980; Nemeseri and Gesztessy, 1973; Vaughan and Turner, 1972; Wildey, 1983).

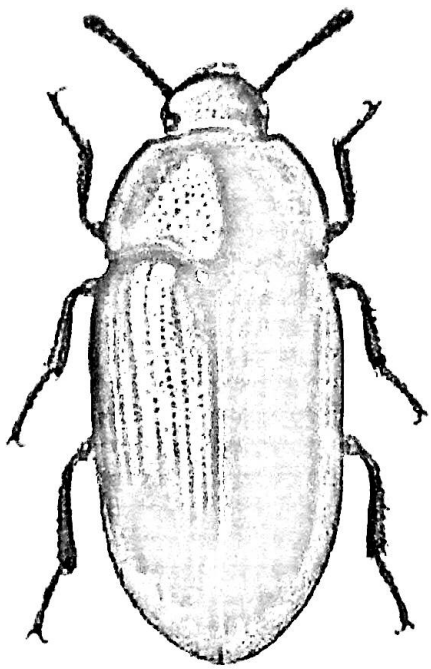


Plate 6: *A. diaperinus* adult

A. diaperinus is worldwide pest, and reaches immense populations in litter in broiler breeder and growout houses and, as well as, in the accumulated manure under caged layers and under the slates in breeder houses (Pfeiffer and Axttel, 1980; Rueda and Axtell, 1997). The beetle life cycle includes eggs, larvae (6-9 stages), pupae and adults, all of which are found in the litter or manure. A complete life cycle from egg to adult requires about 5 weeks, depending on the temperature (Rueda and Axtell, 1996). The adults are extremely long-lived (at least a year) and able to survive adverse conditions. Some of the beetle larvae in the presence of dense populations move into the building insulations to pupate and in the process destroy the insulating value (Geden and Axtell, 1987). Repair of damage to house is costly and the reduced insulation interferes with bird production and by making temperature

more difficult. In addition to this costly structural damage, the beetles are excellent reservoirs of disease organisms affecting both humans and birds and are a significant hazard to bird production. McAllister *et al.*, 1994,1995 and 1996). Young birds can eat large numbers of larvae, which interferes with normal feed consumption and growth, and provides an avenue for disease transmission (Despins and Axtell, 1994,1995; Despins *et al.* 1994). Adult beetles are capable of flying and flight takes place mostly at night. When beetle infested litter is removed from poultry houses and spread on fields, the adults quickly leave the unsuitable habitat and fly to nearby human dwelling causing great annoyance.

Current control measures against infections of *A. diaperinus* in animal houses frequently involve residual spray treatments of the building structure with organophosphorous insecticides. Spraying of pyrethrum or piperonyl butoxide with or without malathion in hot air in broiler houses was once found to control *A. diaperinus* effectively. Experiments on different chemical control measures against the beetle are going as reported. Cogan *et al.* (1996). However, such treatments rarely lead to eradication of the infestation, and the beetle also developed resistance to a number of commercial insecticides. For these reasons, there is a need to develop alternative control measures against *A. diaperinus*. In this regard, botanical like red mangrove was used. Recently, physical barriers like Styrofoam and Ethafoam, which are resistant to beetle infestation, are being used in the walls of the poultry houses together with paints impregnated with chlorpyrifos or other insecticides (Despins *et al.*, 1994).

The control of darkling beetle in the litter of growout houses is usually attempted by spraying the litter between flocks (Weaver, 1996). Although the beetles tend to churn the manure and assist in drying and may even prey to a minor extent on fly larvae, the characteristics of the beetles outweigh any benefits.

This beetle has been incriminated in the transmission of several diseases and disease agents. These include Newcastle disease, avian influenza, infectious bursal disease, Mark's disease, fowl pox, Salmonellasis, *Aspergillus* spp., Reovirus, Rotavirus, *Eimeria* (coccidiosis), tapeworms and caecal worms (De las Casas *et al.*, 1973, 1976; Despins *et al.*, 1994; McAllister *et al.*, 1994,1995,1996). *A. diaperinus* was also used to control other stored product insects, e.g., *Sitotroga cerealella, T. castaneum, C. chinensis, C. maculates, C. analis* and *Lasioderma serricorne* (Gautom, 1998). The adults preyed on larvae, and both the larvae and adults fed voraciously on the eggs of the mentioned insects. *A. diaperinus* larvae provided 68% crude protein and 21% fat of the young chicks and proved to be a good supplementary feed along with normal feed to the chickens (Despins and Axtell, 1994, 1995). The mouthparts of the lesser mealworm reveal that the adults are general feeders, while the larvae are adapted for feeding on cemented food substances.

1.3. Techniques for the isolation and purification of bioactive compounds

The key to any successful program involving the investigation of biologically active plant constituents is the availability and choice of chromatographic techniques for the separation of pure substances. 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 past decade or so, several new techniques have been introduced, leading to the 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.

The most important preparative separation techniques employed in the isolation and purification of plant constituents are as follows:

Preparative separation methods for plant constituents

Solid phase chromatography

Paper chromatography

Preparative TLC, Centrifugal TLC

Open-column chromatography

Vacuum liquid chromatography

Pressure column chromatography, flash chromatography

Low-pressure liquid chromatography (LPLC)

Medium-pressure liquid chromatography (MPLC),

High-pressure/High-performance liquid chromatography (HPLC)

Liquid liquid chromatography

Craig distribution

Droplet countercurrent chromatography (DCC)

Rotation locular countercurrent chromatography (RLCC)

Centrifugal partition chromatography (CPC)

Of the methods 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.

According to the laboratory set up and the availability of essentials in the Department of Zoology, Rajshahi University and certain other neighboring laboratories there were limitations in choosing out the preparative separation methods, while the thin layer chromatography and the open column

chromatography were used simultaneously in this investigation for the isolation and purification of the bioactive *G. pentaphylla* compounds.

1.4. Bioassay with the purified compound(s)

Bioassay with the purified compounds has been a major target in this investigation, however, the test organisms used in this activity guided investigation for isolation and purification were also considered for this bioassay.

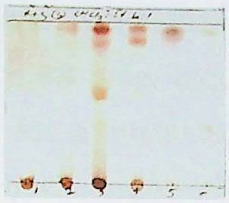
1.5. Aim of the work

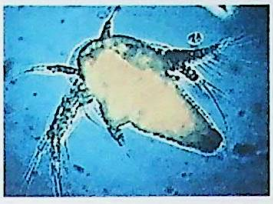
1. To trace presence of bioactive potentials in the toothbrush plant, *G. pentaphylla* through primary screening:

    - using chloroform extracts of its aerial part and roots against plant and human pathogenic bacteria and fungi;
    - using *A. salina*, the recognized test agent for cytotoxic effect to evaluate cytotoxicity of the extracts by establishing LC_{50} values;
    - using *M. domestica* larvae to evaluate larvicidal activity of the extracts;
    - using *T. castaneum* adults to evaluate efficacy of the extracts through repellent activity test;
    - using the stored product pests, *A. diaperinus, C. maculatus, T. castaneum* to evaluate efficacy of the extracts through dose-mortality tests by establishing LD_{50} values and to evaluate efficacy of the extracts on their biology.

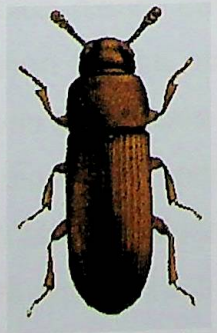
2. To isolate, purify and characterize the active compounds from the promising extract(s) and to evaluate efficacy of the purified compounds against selected test agents using any suitable assay.

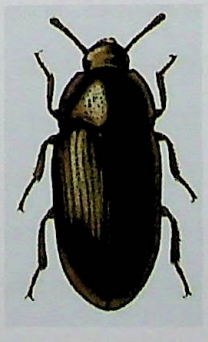
3. To comment on the future perspectives of the test plant depending on the achieved results.





MATERIALS AND METHODS





2. Materials and Methods

After development of multimedia techniques natural resources have been used to be the potential source for safe, biodegradable and more beneficial drugs, remedies or pesticides for a sustainable environment on the planet. Insects, mites, algae or even micro-organisms have also been subjected to yield active compounds in this regard. But plants are the most suitable source for such an interesting propagation in the field of pesticide technology while some plants in different parts of the world are considered toxic and some are used in the traditional medicine. A literature search on the title plant offered some essential openings that this species bears repellent, and toxicological properties which is subjected to go through screening and then isolation, purification and characterization of bioactive constituents to develop natural non-hazardous biodegradable pesticides.

The approach adopted to obtain an exploitable pure plant constituent involves interdisciplinary work in botany, zoology, pharmacognosy, pharmacology, chemistry and toxicology as described by Hostettman *et al.* (1995).

2.1. Selection of test materials

In order to arrive at useful compounds in the shortest possible time, careful selection of plant material is obviously very important. Random collection is one method but it is more judicious to base the selection on certain criteria. By way of illustration, plants used in traditional medicine are more likely to provide pharmacologically active compounds (Huxtable, 1992). Similarly, folk use of toxic plants could be taken with desirable output. However, in this investigation leaves, stem-bark, stem-wood, root-bark and root-wood of *G. pentaphylla* have been collected for the screening of 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 biologically active constituents. Incase of very small plants, such as herbs, shrubs, grass, etc. normally the whole plant is subjected for extraction, because the distribution of constituents generally

not vary too much. Being a relatively bigger bush type plant, the distribution of compounds in different parts of this plant is obviously different. The presence of constituents in the heart-wood may disappear in the leaves; similarly constituents in the roots may not be the same that present there in the fruits, and thus different parts of *G. pentaphylla* have been extracted separately in this investigation.

2.1.1. Preparation of test materials

The fresh leaves, stem-bark, stem-wood, root-bark and root-wood of *G. pentaphylla* were collected from the campus of the University of Rajshahi. After collection the materials were spread out to dry without heaping the materials together. It was done in the shade of the sun. After drying well the plant materials were powdered separately in a hand grinder machine.

2.1.2. Chemical extraction of the collected 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 vacuoles, 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 five different parts of *G. pentaphylla* separately.

For extraction, powdered materials i.e., leaves, stem-bark, stem-wood, root-bark and root-wood 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, stem-bark, stem-wood, root-bark and root-wood] separately. After filtration extraction by $CHCl_3$ (Merck, Germany) to collect 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 the components mostly from the cell vacuoles to yield the other ones [leaves, stem-bark, stem-wood, root-bark and root-wood] and thus extraction of all possible components has done. The extracts were then filtered (Plate-7) 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 bioassay tests. Process of extraction and collection of extracts from the plant materials have been shown in Fig. 5.

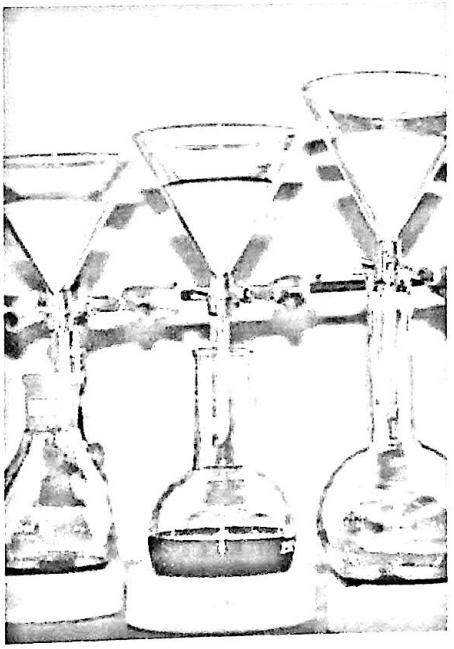


Plate 7: Extraction of *G. pentaphylla* materials

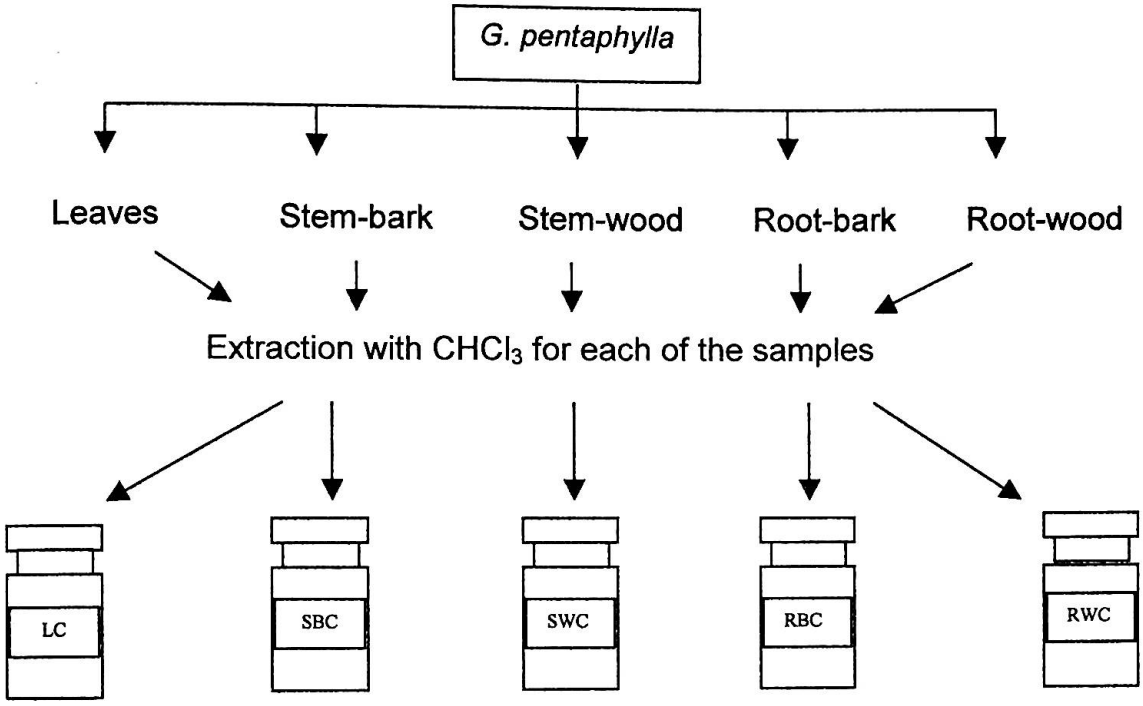


Fig. 5: Collection of extracts in chloroform from the plant materials.

2.2. Selection of test organisms for screening of bioactive principles in the test plant

Crucial to any investigation of plants with biological activities is the availability of suitable bioassays for monitoring the required effects. In order to cope with the number of extracts high sample throughput is necessary. The test systems should ideally be simple, rapid, reproducible, and inexpensive. If active principles are only present at low concentration in the crude extract then bioassay is to be high enough sensitive for their detection. Another factor of special relevance to plant extracts is the solubility of the sample, and finding a suitable system can pose problems.

For the selection of bioassays to employ in research on plant constituents, the first step is to choose suitable target organisms. The complexity of the bioassay has to be designed as a function of the facilities and resources available. A selection of these bioassays is shown in Table 2.

Table 2: List of test agents used in this investigation.

Test types	Methods
Antimicrobial activity test	Antibacterial activity test through disc diffusion method
	Antifungal activity test through disc diffusion method
Cytotoxicity test	Brine shrimp lethality test through WHO approved test-tube dose-mortality assay
Larvicidal test	Dose-mortality assay by V/V food and extract application on the 3rd instar larvae of *M. domestica*
Repellent activity test	Dose treated filter paper assay on *T. castaneum* based on the Method (No.3) of McDonald *et al.*, (1970)
Insecticidal activity test	On *T. castaneum* adults through residual film or surface film on petri dish; On *C. maculatus* through residual film on treated grains; On *A. diaperinus* through residual film on treated grains.

2.2.1. Screening through antibacterial activity test

The antimicrobial screening of an agent is essential to ascertain its spectrum against various types of pathogenic organisms. Antimicrobial activity of any plant or parts of a plant can be detected by observing the growth response of various microorganisms to the plant or parts of a plant extracts, which is placed in contact with them.

Eleven pathogenic bacterial isolates were selected for the test, five of which were Gram positive and the remaining six were Gram negative. These organisms as pure culture were collected from the Plant Pathology Laboratory of the Department of Botany, and from the Institute of Biological Sciences (I.B.Sc.), University of Rajshahi. The bacterial strains used in this investigation are shown in the Table 3.

Table 3: List of the pathogenic bacteria used as test agents

Serial No.	Name of test organism	Strain Number
Gram Positive		
1.	*Staphylococcus aureus*	ATCC-259233
2.	*Bacillus megaterium*	QL-38
3.	*Bacillus subtilis*	QL-40
4.	*Sarcina lutea*	-
5.	*Staphylococcus β-haemolyticus*	CRL
Gram Negative		
6.	*Salmonella typhi*	-
7.	*Shigella dysenteriae*	AL-35587
8.	*Shigella sonnei*	AJ-8992
9.	*Shiggella boydii*	AL-17313
10.	*Escherichia coli*	FPFC-1407
11.	*Pseudomonas aeruginosa*	-

2.2.1.1. *In vitro* antibacterial activity test

It is essentially a qualitative or semiqualitative test that indicates the sensitivity or resistance of microorganisms to the compound. However, this technique cannot be used to distinguish between bacteriostatic and bactericidal agents.

The primary assay can be done in three ways, such as-

A. Diffusion method
B. Dilution method and
C. Bioautographic method.

However, the diffusion method was used in this investigation.

2.2.1.2. Principle of diffusion method

Diffusion assay (Barry, 1976) is based on the ability of antibiotics to diffuse from a confined source through the nutrient agar gel and create a concentration gradient. If the agar is seeded or streaked with a sensitive

organism, a zone of inhibition will result where the concentration exceeds the minimum inhibitory concentration (MIC) for the particular organism.

In this method, measured amount of the test samples are dissolved in definite volumes of solvent to give solutions of known concentrations (μg/ml). The sterile (BBL, Cocksville, USA) filter paper (diameter 5 mm) disc are impregnated with known amounts of the test substances and dried. These test material discs are placed on plates containing a suitable medium (nutrient agar) seeded with the test organisms. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion. A number of events take place simultaneously which includes-

i) The dried discs absorb water from the agar medium and the material under test is dissolved.

ii) The test material diffuses from the discs to the surrounding medium according to the physical law that controls the diffusion of molecules through agar gel.

iii) There is a gradual change of test material concentration in the agar surrounding each disc.

To determine the most optimal concentration of extracts to be used in this study, sterile 7.5 mm filter paper disks were treated with 200 microliters of chloroform only (used as a control), and 200 microliters of each extract. The bacteria were inoculated on full-strength Nutrient Agar (Qualigens Fine Chemicals Prod # 58673) by suspending loops of bacteria in sterile de-ionized water. The bacterial suspension was then smeared on agar plates with a sterile glass-rod to ensure the entire surface of the agar had an even coating of the bacterial suspension. Plates were divided into several areas and one filter paper disk was placed in each area so that each plate had one disk of each treatment (control, 200 microliters of extract). Effects of the *G. pentaphylla* compounds on bacterial growth were quantified by measuring the diameter of the zones of inhibition less the size of the treated filter paper disks.

The plates are then kept in an incubator (37°C) for 12-18 h to allow the growth of the organisms. If the test material has antimicrobial activity, it will inhibit the growth of microorganisms, giving a clear, distinct zone called 'Zone of Inhibition'. Effects of the *G. pentaphylla* extracts on bacterial growth were quantified by measuring the diameter of the zones of inhibition in term of mm.

The size of the inhibitory zones depends principally on the following factors-

i) Intrinsic antimicrobial sensitivity of the test sample.
ii) Growth rate of the test microorganisms.
iii) Diffusion rate of the freshly seeded test organisms.
iv) Concentration of the freshly seeded test organisms.
v) Amount of test sample on disc.
vi) Thickness of the test medium in the Petri dishes.
vii) Composition of the culture medium.
viii) Inoculum size
ix) Incubation time
x) Temperature of incubation

2.2.1.3. Test materials used for the study

i) Chloroform extracts.
ii) Ciprofloxacin, (30 μg/disc) as standard disc.

2.2.1.4. Apparatus and reagents

i) Blank sterilized filter paper discs (diameter 5 mm)
ii) Petri dishes (diameter 120 mm)
iii) Test tubes
iv) Inoculating loop
v) Bunsen Burner
vi) Sterile forceps
vii) Sterile cotton
viii) Laminar air flow unit (Biocraft & Scientific Industries, India)

Table 4: Composition of nutrient agar medium.

Ingredient	Amount
Bactopeptone	0.5 gm
Sodium chloride	0.5 gm
Bactoyeast extract	1.0 gm
Bactoagar	2.0 gm
Distilled water q.s to	100 ml
pH	7.2±0.1 at 25°C

For demonstrating the antibacterial activity and subculture of the test organisms the nutrient agar media (DIFCO) was used.

2.2.1.7. Preparation of the nutrient agar (DIFCO) medium

The instant nutrient agar (DIFCO) medium was weighed and then reconstituted with distilled water in a conical flask according to specification (2.3% w/v). It was then heated in a water bath to dissolve the agar until a transparent solution was obtained.

2.2.1.8. Preparation of fresh culture of the pathogenic organisms

The nutrient agar medium was prepared and dispersed in a number of clean test tubes to prepare slants (5 ml in each test tube). The test tubes were plugged with cotton and sterilized in an autoclave at 121°C and 15 lbs./sq. inch pressure for 15 minutes. After sterilization, the test tubes were kept in an inclined position for solidification. These were then incubated at 37.5°C to ensure sterilization. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. Burning the loop after each transfer of microorganism was done to avoid contamination very carefully. The inoculated slants were then incubated at 37.5°C for 24 hours to assure the growth of test organisms. These fresh cultures were used for the sensitivity test.

ix) Micropipette (10 µl-100 µl)
x) Autoclave (ALP Co. Ltd. KT- 30L, Japan)
xi) Incubator (OSK- 9639A, Japan)
xii) Refrigerator (Ariston, Italy)
xiii) Punch machine
xiv) Beaker
xv) Nutrient agar media (DIFCO)
xvi) Solvent (methanol and chloroform) and
xvii) Vial.

2.2.1.5. Sterilization procedure

The antibacterial screening was carried out in a laminar air flow unit and all types of precautions were highly maintained to avoid any type of contamination during the test. UV light was switched on for half an hour before working in the laminar hood to avoid any accidental contamination. Petri dishes and other glass wares were sterilized in the autoclave at 121°C temperature and a pressure of 15 lbs./sq. inch for 15 minutes. Micropipette tips, culture media, cotton, forceps, blank discs etc. were also sterilized.

2.2.1.6. Culture media

A number of culture media are available to demonstrate the antibacterial activity. These are-

i) Nutrient agar medium
ii) Nutrient broth medium
iii) Mueller-Hinton medium
iv) Tryptic Soy broth (TSB) medium
v) Trypticase Soy agar medium
vi) Staphylococcus defined medium
vii) Adams and Roe medium
viii) NTH agar or broth medium

While, the nutrient agar medium was adopted to conduct this experiment.

Table 4: Composition of nutrient agar medium.

Ingredient	Amount
Bactopeptone	0.5 gm
Sodium chloride	0.5 gm
Bactoyeast extract	1.0 gm
Bactoagar	2.0 gm
Distilled water q.s to	100 ml
pH	7.2±0.1 at 25°C

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2.2.1.9. Preparation of the test plates

The test plates were prepared according to the following procedure:

(i) The nutrient agar medium prepared in the previous section was poured in 15 ml quantity in each in the clean test tubes and plugged with cotton.

(ii) The test tubes and a number of petri dishes were sterilized in an autoclave at 121°C and 15 lbs/sq. inch pressure for 15 minutes and were transferred into laminar air-flow unit and then allowed to cool to about 45°C to 50°C.

(iii) The test organism was transferred from the fresh subculture to the test tube containing 15 ml autoclaved medium with the help of an inoculating loop in an aseptic condition. Then the test tube was shaken by rotation to get a uniform suspension of the organism.

(iv) The bacterial suspensions were immediately transferred to the sterile petri dishes in an aseptic area. The petri dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous distribution of the test organisms. The media were poured into petri dishes in such a way as to give a uniform depth of approximately 4 mm.

(v) Finally, after medium was cooled to room temperature in laminar airflow unit, it was stored in a refrigerator (4°C).

2.2.1.10. Preparation of the sample containing discs

For the preparation of discs containing samples, following procedure was utilized:

(a) Sample discs

Sterilized filter paper discs (5 mm in diameter) were taken by the forceps in the plates. Sample solutions of desired concentrations were applied on the discs with the help of a micropipette in an aseptic condition. These discs were left for a few minutes in aseptic condition for complete removal of the solvent.

(b) Standard discs

These were used to compare the antibacterial activity of the test material. In the present study, Ciprofloxacin discs containing 30 μg/disc of antibiotic Ciprofloxacin were used as standard discs for comparison purpose.

2.2.1.11. Placement of the discs and incubation

For the placement of the discs, the following procedure was utilized:

(i) By means of a pair of sterile forceps, the sample impregnated discs were placed gently on the solidified agar plates seeded with the test organisms to ensure contact with the medium.

(ii) The plates were then kept in a refrigerator at 4°C for 24 hours in order to provide sufficient time to diffuse the antibiotics into the medium.

(iii) Finally, the plates were incubated at 37.5°C for 24 hours in an incubator.

Precaution: The discs were placed in such a way that they were not closer than 15 mm to the edge of the plate and for enough apart to prevent overlapping the zones of inhibition.

2.2.1.12. Measurement of the zones of inhibition

After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in term of mm with a transparent scale.

2.2.2. Screening through *In vitro* antifungal activity test

The increasing incidence of opportunistic systemic mycoses in man and the associated therapeutic difficulties require the search for new antifungal drugs. Plants derived compounds may offer potential leads for novel agents against systemic fungal diseases (Hufford and Clark, 1988).

Chloroform extracts of *G. pentaphylla* samples (leaves, stem-bark, stem-wood, root-bark and root-wood) were used for the investigation of antifungal activity. The extracts were dissolved in their solvent of extraction and used at a concentration of 500 µg/disc. For a better correlation of the antifungal activities Nystatin 50 µg/disc was used as a standard. The fungal strains used in the sensitivity test are given below and pure cultures of the strains were collected from the Institute of Biological Science (I.B.Sc.), University of Rajshahi, Rajshahi.

Table 5: List of the pathogenic fungi used as test agents

Serial No.	Name of test organism
1.	*Fusarium vasinfactum*
2.	*Aspergillus fumigatus*
3.	*Aspergillus flavus*
4.	*Mucor* sp.
5.	*Candida albicans*

2.2.2.1. Culture media

Potato dextrose agar (PDA) medium was used to perform the antifungal activity test and for subculture of the test organisms. The composition of the medium is as follows-

Table 6: Composition of PDA medium.

Ingredient	Amount
Potato	20.0 gm
Dextrose	2.0 gm
Agar	1.5 gm
Distilled water q.s. to	100.0 ml

2.2.2.2. Preparation of the medium

The constituents of the medium was accurately weighed and dispersed in a conical flask with distilled water. It was heated in water bath to dissolve the ingredients until a transparent solution was obtained. The pH of the medium was adjusted to 5.6. The volume was adjusted by adding distilled water and sterilized in an autoclave at 121°C and 15 lbs/sq-inch pressure for 15 minutes.

2.2.2.3. Preparation of the test plates

The test plates were prepared according to the following procedure:

(i) About 10 ml in quantity of distilled water was poured in several clean test tubes and plugged with cotton.

(ii) The test tubes, a number of petri dishes, glass rods, a piece of cotton and the medium were sterilized by autoclave and then transferred to the laminar air flow cabinet.

(iii) About 6 ml of the medium was poured carefully in the medium sized petri dishes in each. The petri dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous thickness of the medium and allowed to cool and solidify at about 30°C.

(iv) The test tubes containing distilled water were inoculated with fresh culture of the test fungi and were shaken gently to form a uniform suspension of the organism because of their high prevalence sporulation process.

(v) A piece of cotton was immerged in the test tubes with the help of individual glass rod and then gentle rubbed the medium and the cotton was discarded.

(vi) Finally, the plates were stored in a refrigerator (4°C) for overnight.

Note: Preparation of the discs, test samples, placement of the discs, diffusion incubation and measurement of zone of inhibition processes were almost same of the antibacterial screening. Here, only the incubation period was replaced by 48-72 hours at room temperature.

2.2.3. Screening through brine shrimp lethality bioassay

To conduct cytotoxicity test the brine shrimp nauplii were used because of its easy hatching and use in the experiment. The eggs (cysts) were collected from aquarium shops. For their easy hatching and use the requirements were as follows:

- Salt water: 1.5 - 3 tablespoons of marine salt every liter of water;
- Temperature: 26-28 degrees C (80-82F); 25-30C (77-86F);
- Light: The beaker was placed near a window with sunlight before hatching;
- Aeration: Picking up some water carefully with a spoon and let it drop back a few times once a day was done [but a small aquarium pump with a little air-stone is better];
- Helpful Hint: Brine shrimp egg is sometimes very bouyant. Swirl the water to knock down eggs;

The cysts absorb water and if the sun is shining (a signal for growing algae and other plankton) they hatch after 24-48 hours, depending on their environment. Fresh hatched *A. salina* are called nauplii and have a size of just 0.25mm (0.01inch). They molt like any other crawfish when they grow - to adult they molt about 17 times. If the breeding temperature is about 26-28°C (80-82°F), a nauplius hatches within 24-48 h, gets pubescent in 8-14 days and lives - depending on the concentration of salt - up to 4-5 weeks. The more salt, the less the life expectancy. Freshly hatched nauplii were used in this experiment.

2.2.3.1. Experimental design for the cytotoxicity test

Brine shrimp eggs are hatched in simulated sea-water to get nauplii. Test samples are prepared by the addition of calculated amount of DMSO (dimethyl sulfoxide) for obtaining desired concentration of test sample. The nauplii are counted by visual inspection and are taken in vials containing 5 ml

of sea-water. Then samples of different concentrations are added to the premarked vials through micropipette. The vials are left for 24 hours and then the nauplii are counted again to find out the cytotoxicity of the test agents and compared to the results with positive control.

Test materials:

(i) *A. salina* Leach (brine shrimp eggs)
(ii) Sea salt (non ionized NaCl)
(iii) Small tank with perforated dividing dam to hatch the shrimp
(iv) Lamp to attract the nauplii
(v) Pipette (1 ml and 5 ml)
(vi) Micropipette (10-200 µl adjustable)
(vii) Test tubes (5 ml)
(viii) Magnifying glass

2.2.3.2. Preparation of simulated sea-water (brine water)

Since the lethality test involves the culture of brine shrimp nauplii that is, the nauplii should be grown in the sea water. Sea water contains 3.8% of sodium chloride. Accordingly 3.8% sodium chloride solution was made by dissolving sodium chloride (38 gm) in distilled water (1000 ml) and was filtered off. The P^H of the brine water thus prepared was maintained between 8 and 9 using $NaHCO_3$.

2.2.3.3. Hatching of brine shrimp

Brine water was taken in a small tank and shrimp eggs (1.5 gm/l) were added to one side of the perforated divided tank with constant oxygen supply. Constant temperature (37°C) and sufficient light were maintained to give the sufficient aeration. After 48 hours, matured shrimp as nauplii (larvae) was collected and used for the experiment.

2.2.3.4. Preparation of sample solution

Chloroform extracts of the *G. pentaphylla* samples were applied against brine shrimp nauplii. For the leaf and stem bark samples 2 mg were initially dissolved in 100 µl of pure dimethyl sulfoxide (DMSO) to make hydrophilic before adding 19.98 ml of water to get a concentration of for each of 100 ppm which was used as stock solution-A. From the stock solution A, 10ml was taken (that was with a concentration of 100 ppm) and diluted up to 20 ml with brine water to obtain a concentration of 50 ppm and this is indicated a stock solution-B from this different other doses were made by serial dilution method. Then a series of following concentrations was made from the stock solution-A were 100-, 50-, 25- and 12.5 ppm. For the stem wood and root samples 500 µg were initially dissolved in 200 µl of pure dimethyl sulfoxide (DMSO) to make hydrophilic before adding 19.98 ml of water to get a concentration of for each of 250 ppm which was used as stock solution-A. Then a series of following concentrations was made from the stock solution-A of each of the samples were with 250-, 125-, 62.5-, 31.25- and 15.625 ppm.

During experiments according to the dose-mortality effects dose ranges were made different for some conveniences in achieving a good result since the extracts were active against the brine shrimp nauplii.

2.2.3.5. Preparation of the control group

For each concentration, one test tube containing the same volume of DMSO diluted up to 10 ml with sea-water and 30 shrimp nauplii was used as negative control group. It was used to verify the validity of the test. When the nauplii in the control showed a rapid mortality, then the test is considered to be invalid as the nauplii might die due to reasons other than the cytotoxicity of the compounds.

2.2.3.6. Application of test sample and brine shrimp nauplii to the test tubes

In each of the five test tubes, 10 ml brine water solution (3.8%) was taken, containing 30 brine shrimp nauplii with the help of a micropipette, specific volumes of each sample were transferred from the stock solution-B to the respective vials to get final concentrations of 100-, 50-, 25- and 12.5 ppm for leaf and stem bark extract and 250-,125-, 62.5-, 31.25- and 15.625 ppm for stem wood and root extracts.

The volumes of DMSO in these test tubes should not exceed 10 µl/ml of the brine solution, because above this concentration toxicity due to DMSO may arise.

2.2.3.7. Observation of data

After 24 hours, the test tubes were observed. The number of survived nauplii in each test tube was counted and the results were noted. From this, percentage of mortality of brine shrimp nauplii was calculated at each concentration for each sample and the results are given in Table 6 in the Results.

2.2.3.8. Analysis of the data

The dose mortality data were analyzed statistically by probit analysis. The effectiveness or the dose mortality relationship (concentration-mortality relationship) of plant product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

2.2.4. Screening through larvicidal assay

2.2.4.1. Screening through larvicidal assay by *T. castameum* larvae

Collection of beetles and culture of them for continuous supply of larvae for the experiment have been done simultaneously while the adults of this animal have been produced and used for the residual film assay (description is given at 2.2.5.).

2.2.4.2. Screening through larvicidal assay by *M. domestica* larvae

To carry on insecticidal test normally easy cultivable familiar insects are used. like mosquitoes, flies, ants, flour beetles, etc. The common housefly, *M. domestica,* L. is also used to screen candidate insecticides, chemosterilants by scientists, in public or private research institutions both here and abroad. In addition, all stadia have been utilized as a source of food for parasites and/or predators. Since, the immature stadia can survive in various substrates, entomologists have normally used materials that were available locally (Spiller, 1946, 1966). This has caused variation in the quality of flies that were produced, making it difficult to replicate test results. Here at the "Insect Genetics laboratory" Department of Genetics & Breeding, Rajshahi University, we have been able to produce a consistent quality insect at an economical cost by rearing the housefly according to the methods described.

The housefly *M. domestica* was selected for larvicidal assay. The house fly (local stain) adults were collected from a 'Butcher's shop' of Binodpur Bazar, Rajshahi, to make stock cultures, maintained in the 'Insect genetics laboratory' Genetics & Breeding department, Rajshahi University, Rajshahi, for the continuous supply of test insects to carry on the scheduled experiments in this investigation.

2.2.4.2.1. Culture of *M. domestica*

The house fly (local stain) adults were collected from a 'Butcher's shop' of Binodpur Bazar, Rajshahi, to make stock cultures, maintained in the 'Insect genetics laboratory' Genetics & Breeding department, Rajshahi University, Rajshahi, for the continuous supply of test insects to carry on the scheduled experiments in this investigation. Collected adults were released in 10 mesh cages measuring approximately 20 x 12 x 16 inch^3, made up of wood and fine ware nets with a door provided with muslin cloth for the easy collection of files during experiments.

2.2.4.2.2. Food for larvae and adults

The feeding for larvae was provided in plastic pots (10 cm deep) containing 9 gm of milk powder with 5 gm fresh yeast (dissolved in 100 ml of water) and 100 gm of wheat bran; then thoroughly stirred. The mixture put into the pots leaving 3 cm from the top. The pots were placed in inside the cages when the adults were engaged in mating and about to go laying eggs and the pots were kept for 24 hours.

2.2.4.2.3. Collection of larvae

The female files (after mating) laid eggs in batches, each of which contained 100-150 eggs, which were separated out and transferred to similar pots contained food mixture (also called rearing medium) covered with cloth-net, wrapped with paper and shielded with plastic lids. These pots are then placed in a box covering with net, within 6-8 days after eggs have been hatched out to produce larvae. After a certain period of time the larvae have been transformed into pupae and ultimately emerged as adults. The third instar larvae were used as test organisms in this investigation.

2.2.4.2.4. Preparation of doses for the larvicidal assay

To test the chloroform extracts of the selected plant *G. pentaphylla* against cultured larvae of *M. domestica* of same age were used provided with its food as a unit of volume by volume measurement. The food was prepared with 6.25 g of wheat bran and 0.5 g of milk powder (Red Cow) and 12 ml of water as a total of 19.5 g. For *G. pentaphylla* leaf extract 500 mg was dissolved in 1 ml of solvent (Chloroform) and mixed with the prepared food, however, being volatile the solvent was evaporated out shortly. To have a dose-effect to calculate toxicity by probit analysis 4 other successive doses were prepared and applied with ½ serial dilution and the doses were 25.641-, 12.821-, 6.410-, 3.205 and 1.603 mg/g in V/V.

2.2.4.2.5. Application of doses and observation of mortality

After application of doses changes of behavior of the treated organism along with effect on its growth and development, deformity in physical stature and certain other parameters have also been observed.

2.2.4.2.6. Application of doses and observation of mortality

Different doses (A, B, C, D and E with a control F) were applied to the same preparation of food with three replications and mortality was observed after 24- and 48 h of exposure.

2.2.4.2.7. Statistical analysis

The recorded mortality of the adults were corrected by the 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 = Control mortality (%), sometimes called natural mortality (%).

Then this percentage 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}).

2.2.5. Screening through repellent activity test

Extracts of plants have been used by humans for control of insects since before the time of the ancient Romans. In many areas of Africa and Asia, locally available plants and minerals are being widely used to protect stored products against damage by insect infestation, as an alternative to synthetic pesticides (Golob and Webley, 1980; Su *et al.*, 1972; Ahmed and Koppel, 1985; Khalique *et al.*, 1988.).

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 selected doses of all the $CHCl_3$ extract 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 petri dish (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 7). 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 $CHCl_3$. 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.

2.2.5.1. Collection of *T. castaneum*

To carry on tests for insect repellent activity and insecticidal properties of the extractives *T. castaneum* was selected a test organism, 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 their maintenance. Source of test insects- the *Tribolium* species, *T. castaneum* used in the present experiment were reared in the Crop Protection and Toxicology Laboratory, Department of Zoology, University of Rajshahi, Bangladesh.

2.2.5.2. Culture of the test insect *T. castaneum*

Mass cultures were maintained in plastic containers (1200ml) and sub-cultures in beakers (1000 ml) with the food medium. The beakers were kept in an incubator at $30^0C \pm 0.5^0C$ without light and humidity control. 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 increase 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.

2.2.5.3. Preparation of food medium for maintenance of the insect culture

The whole-wheat flour was used as the food medium for the insect species. The flour was sterilized at 60^0C 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 was sterilized at 60^0C 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 (Khan and Selman, 1981).

2.2.5.4. Collection of eggs of the test insect

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 $30^0C \pm 5^0C$. In regular interval the eggs were collected by sieving the food medium by two sieves of 500 and 250 mesh separating the adults and eggs respectively following the methods of Khan and Selman (1981). Eggs were then transferred to petri dish (90mm in diameter) and incubated at the same temperature.

2.2.5.5. Collection of newly hatched larvae of the test insect

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

2.2.5.6. Collection of mature larvae of the test insect

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 the day of hatching respectively. Depending on these days according to larval instar sixteen days old larvae have been considered as mature larvae. Larval cultures were maintained in an incubator in the same procedure at $30^0C \pm 5^0C$ without light and humidity control. The food medium was replaced by three days interval to a fresh one to avoid conditioning by the larvae (Park, 1934).

2.2.5.7. Collection of adults for the repellency test

A huge number of 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 from the food medium. For this purpose some pieces of filter paper were kept inside the beaker on the food. Adults crawled upon the paper and then the paper was taken out with a forceps. Beetles were then collected in a small beaker (100 ml) with the help of a fine camel-hair brush.

2.2.5.8. Preparation of doses for the repellency test

A general concentration for each of the extracts was selected as stock dose for surface film application to make other successive doses by serial dilution to give 78.634-, 39.317-, 19.659-, 9.829-, 4.915- and 2.457 $\mu g/cm^2$ concentrations for all the *G. pentaphylla* extracts.

2.2.5.9. Application of doses in the repellency test

To conduct surface film activity test 90 mm petri dishes were taken for all doses and their replications. One ml of each of the doses were poured into the lower part of the petri dish and allowed them to dry out. Being volatile the solvent was evaporated out within a few minutes. Ten insects were released in each of the treated petri dish. A control experiment by applying the only solvent into the petri dish was also set at the same time under the same condition.

2.2.5.10. Observation and preparation of repellency data

Repellency was observed for one-hour interval and up to five successive hours of exposure, just by counting the number if insects in the treated and non-treated part of the filter paper spread on the floor of the 90 mm petri dish. The values in the recorded data were then calculated for percent repellency, which was again developed by arcsin transformation for the calculation of ANOVA.

2.2.6. Screening through surface film method

2.2.6.1. Culture of the test insect *T. castaneum*

Collection of beetles and culture of them for continuous supply of larvae for the experiment have been done simultaneously while the adults of this animal have been produced and used for the residual film assay (description is given at 2.2.5.).

2.2.6.2. Culture of the test insect *C. maculatus*

The beetles used for this experiment were primarily collected from infested cowpea (*Vigna unguiculata* (L.) Walpers) found in a grocers' shop at Shaheb Bazar, Rajshahi City and were reared in the Crop Protection Laboratory, Rajshahi University, Rajshahi-6205, Bangladesh. For one generation prior to the start of the experimental beetles were reared at low density (1 egg/seed). These seeds were isolated to ensure that emerging beetles were virgin. All seeds were checked every 24 h for emerging adults. Thus, all females were 24 h old at the initiation of each experiment. Copulation occurs almost immediately and males were removed immediately after mating. Females were allowed to lay eggs. All beetles were maintained prior to and during the experiments in a laboratory growth chamber without humidity and temperature control. Subcultures were also maintained in separate beakers covered with muslin clothes marked with species name and date of culture. Normally three days old adults were used in this investigation.

2.2.6.3 Culture of the test insect *A. diaperinus*

The lesser mealworm, *A. diaperinus* is one of the insects used in the present study. This is the common pest of stored grains all over the tropical and sub-tropical countries of the world including Bangladesh. It is easily obtainable. About 1500 beetles were collected for culture. The beetles were sieved through U.S. standard No. 16 and 20 sieves. The healthy and active beetles

were taken in the plastic jars and covered with a cloth, secured with a rubber band at the top. Slices of potato were kept with in jars for humidity control, which were replaced when necessary. Several sub-cultures were maintained, each containing 100 beetles in 250g of food in a jar. For maintaining the culture of beetles, the following laboratory materials were used:

I. Plastic jars,	II. Beakers,	III. Petri dishes,
IV. Camel hair brush,	V. Pieces of cloth,	VI. Spoons,
VII. Sieves,	VIII. Rubber bands,	IX. Potato slices, etc.

All the materials mentioned above were sterilized by keeping in an oven for about six hours at 120°C (Khan, 1981).

2.2.6.3.1. Preparation of food medium

Wheat flour, sesame cakes, rapeseed cakes and linseed cakes were used for the culture of *A. diaperinus*. Yeast was mixed with the wheat flour at 20: 1.5 ratios to prepare control food. Sesame, rapeseed and linseed cakes were used for rearing the insect which contained no yeast.

2.2.6.3.2. Collection of eggs

Adult beetles were put in petri dishes (9 cm diameter) containing wheat flour. On the next day, eggs were collected by sieving the medium with sieves of 500 and 250-micrometer apertures (Khan and Selman, 1981). The eggs were placed in a petri dish (9.5 cm diameter) and incubated at room temperatures.

2.2.6.3.3. Transfer of larvae to the food

A fine camel hairbrush collected the larvae hatched out in 3-4 days. Newly hatched larvae were transferred to the jars (20 cm × 8 cm) containing wheat flour and oil cakes with the aid of a camel hairbrush. Three hundred larvae were used for each food and the experiment was replicated four times. Slices of potato were kept inside the jars to maintain humidity, which were changed

weekly. Larvae were checked from time to time for pupation. The newly emerged adults were set to maintain subcultures and after collection of eggs from them the experiments were set with the 1st instar larvae.

2.2.6.4. Preparation of doses with the crude extracts for the surface film test

A general concentration for each of the extracts was selected as 200mg/2ml as the stock dose for surface film application to make other successive doses by serial dilution to give 3.416-, 1.573-, 0.786-, 0.393-, 0.197- and 0.098 mg/cm^2 for *T. castaneum* and *A. diaperinus*; and 50-, 25-, 12.5-, 6.25-, 3.125- and 1.562 μg/cm^2 doses were used against *C. maculatus* adults. The volume of extract was not increased because of its sticky nature, where the insects after application just glued and living so far in that state.

2.2.6.5. Application of doses in the surface film test

To conduct surface film activity test 60 mm petri dishes were taken for all doses and their replications. One ml of each of the doses were poured into the lower part of the petri dish and allowed them to dry out. Being volatile the solvent was evaporated out within a few minutes. Ten insects were released in each of the treated petri dish. A control experiment by applying the only solvent into the petri dish was also set at the same time under the same condition.

2.2.6.6. Observation of mortality in the surface film test

Completing the all the arrangements treated petri dishes were placed in a secured place at room temperature. The whole experiment was observed from time to time and mortality was observed by every 24 h and the data was recorded. A simple microscope was used to check each and every beetle by tracing natural movement of its organs. In some cases hot needle was taken closer to the bodies (without movement) to confirm death. Attention was also paid to recovery of the insects if occurred.

2.2.6.7. Statistical analysis of data

The recorded mortality of the adults were corrected by the 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 = Control mortality (%), sometimes called natural mortality (%).

Then this percentage 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}).

2.3. Isolation, purification and characterization of the bioactive compounds

2.3.1. Chromatography on TLC plates

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. Ten mg/ml of the sample in the solvent of extract offered 100µg/spot while spotted 10 µl for each of the samples. The chromatograms then developed within a conventional chamber (Camag) with the following solvent systems:

$CHCl_3$ extract	Pet. ether: EtOAc ()1:1
MeOH extract	$CHCl_3$:MeOH:H_2O (65:35:5)

All chromatograms were observed under UV at 254 and 366 nm and marked with a pencil.

2.3.2. Godin reagent (Godin 1954)

The reagent is the mixture of the equal volume of 1% ethanolic solution of vanillin and 3% aqueous solution of parchloric acid. After spraying the reagent on the dried TLC 10% ethanolic solution of H_2SO_4 is also spayed before drying the plate at 100°C to reveal the spots of the compounds.

2.3.3. Column Chromatography

For the open column chromatography Si60 (63-200 mesh) and silica gel Si60 (230-400 mesh) (Merck) and glass column and of different size (25×1.5 cm, 25×2 cm, etc.) were used. The cotton pad used at the base of the column were soaked in acetone and dried before application. Different solvent systems were used as the eluent. The elution rate was 1 ml/min. Due to lack of a fraction collector the fractions were collected manually.

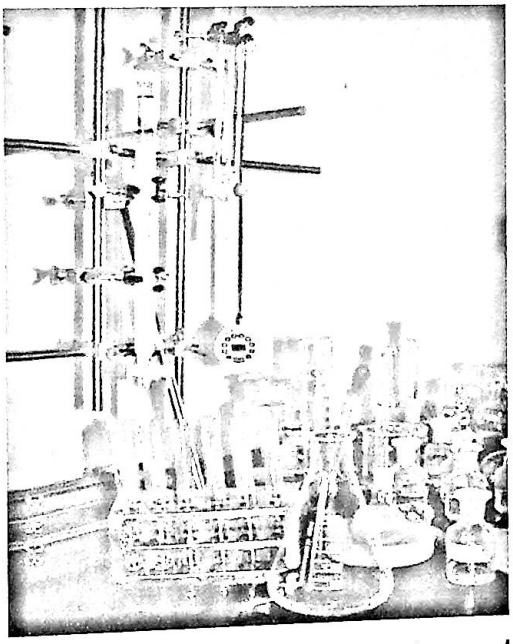


Plate 8. Open column chromatography.

2.3.4. Gel filtration

Open columns are used with Sephadex LH-20 (Pharmacia) for the chromatography of exclusion. The separation of the methanolic extracts done with MeOH using as the eluent and other lipophilic extracts also with MeOH with a little amount (not more than 50%) of $CHCl_3$. The eluent allowed about 0.5 ml/min.

2.4. Isolation of bio-active principles

Air-dried leaves, stem bark, stem wood, root bark and root wood of *G. pentaphylla* were pulverized in a hand grinder and extracted at room temperature successively with $CHCl_3$ and MeOH giving a 72 hours reflux for each of the items separately. Extracts were filtered and concentrated and were collected in vials.

Silica-gel GF_{254} coated Al sheets (Merck) were used for the selection of the solvent systems for better separation. All samples were freshly prepared prior to the bioassay. The volume spotted on the TLC was 10µl, corresponding to 100µg of extract. Test samples were prepared in favour of each of the extract type with a concentration of 10 mg/ml where $CHCl_3$ extracts were dissolved in $CHCl_3$ and the MeOH extract in MeOH. To trace bioactivity of the crude extracts, as well as, the fractions disc diffusion method was used for both bacteria and fungi used as test agents in this investigation.

2.4.1. Selection of extracts for fractionation

For fractionation of the extracts with a view to isolate biologically active compounds all the extracts were subjected to biological assay. Repetition of the same assay all along the successive fractions is required until the purification of the target compound(s), and a suitable bioassay technique was selected in this regard. A number plant pathogenic fungi, *F. vasinfactum, A. fumigatus A. flavus, Mucor* sp. and *C. albicans* were taken in this regard. Test samples were made using all the extracts of leaves, stem bark, stem wood, root bark and root wood of *G. pentaphylla* with a concentration of 10 mg/ml to give a concentration through which it was possible to diversified doses from 10 µg/ml. Paper discs treated with 10, 50, 100 and 200 µg of these samples were used to detect biological activity of the extracts.

Potato dextrose agar (PDA) medium was used to perform these antifungal activity tests through disc diffusion method. 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 root-bark and the root wood.

2.4.2. Selection of slurry (solvent system) for respective extracts

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.

2.4.3. 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 desired 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.

2.4.4. Detection of the compound on TLC by Godin revelation

Visual detection: The developed chromatogram was examined visually to detect the presence of colored compound(s).

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. [Not available in the laboratory].

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).

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.

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

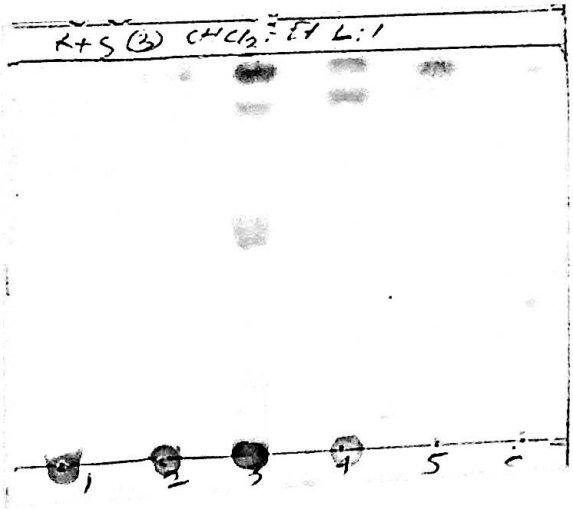


Plate 9: Revelation of compound spots by Godin reagent spray

2.4.5. Isolation of the bioactive 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 2.5 × 32 cm for 1g of the root extract. Elution time was adjusted to yield 1 ml/min. It gave 120 tubes, which were then spotted on TLC to run and reveal the compounds by reagent spray. Six fractions were made for tubes 1-36, 37-43, 44-54, 55-65, 66-79 and 80-120 to give Fr. I to Fr. VI. Biological assay with fungi indicated Fr. III for the presence of bioactive components there in and that was then subjected to fractionation. Selecting a solvent system by TLC, a slurry of chloroform and ethyl acetate ($CHCl_3$-EtOAc 12:1) was applied on a glass column of 2 × 25 cm was packed with silica gel (70-230 mesh) (Sigma). The elution was kept similar to that of the previous one. This fractionation yielded 106 tubes and TLC was made for all of them to get six sub fractions for tubes 1-21, 22-40, 41-57, 58-72, 73-87 and 88-106 to give Sfr. I to Sfr. VI. Biological assay with the test fungi indicated tubes 41-57 for the presence of bioactive components and that was then subjected to fractionation. Again selecting a solvent system by TLC, a slurry of chloroform and ethyl acetate ($CHCl_3$-EtOAc 6:1) was applied on a glass column of 1.5 × 25 cm was packed with silica gel (230-400 mesh) (Sigma). The elution was kept similar to that of the previous one. This fractionation yielded 106 tubes and TLC was made for all of them to get four sub fractions of the sub fraction for tubes 1-11, 12-14, 15-19 and 20-23 to give Ssfr. I to Ssfr. IV. Biological assay with the test fungi indicated tubes 12-14 for the pure compound GP1 that was about 12 mg in amount.

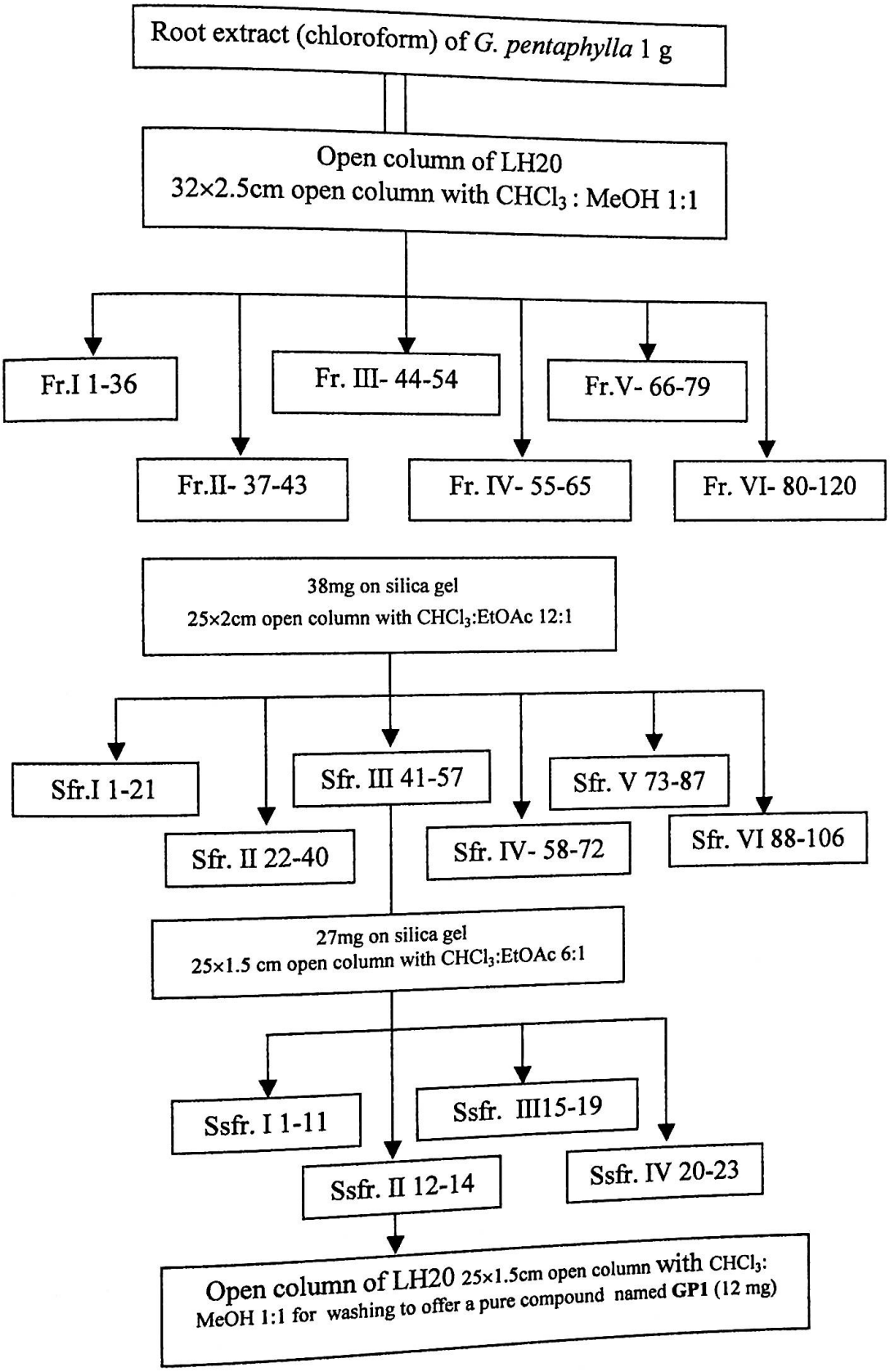


Fig. 6: Isolation pathway of compound GP1

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 2 × 25 cm for 500 mg of the root extract. Elution time was adjusted to yield 1 ml/min. It gave 58 tubes, which were then spotted on TLC to run and reveal the compounds by reagent spray. Three fractions were made for tubes 1-35, 36-48 and 49-58 to give Fr. I to Fr. III. Biological assay with fungi indicated Fr. III for the presence of bioactive components there in and that was then subjected to fractionation. Selecting a solvent system by TLC, a slurry of chloroform and ethyl acetate (nHexane-EtOAc 5:1) was applied on a glass column of 1.5 × 25 cm was packed with silica gel (70-230 mesh) (Sigma). The elution was kept similar to that of the previous one. This fractionation yielded 24 tubes and TLC was made for all of them to get three sub fractions for tubes 1-8, 9-14 and 15-24 to give Sfr. I to Sfr. III. Biological assay with fungi indicated Fr. III for the presence of bioactive components there in and that was then subjected to fractionation. Selecting a solvent system by TLC, a slurry of chloroform and ethyl acetate ($CHCl_3$-EtOAc 6:1) was applied on a glass column of 1.5 × 25 cm was packed with silica gel (230-400 mesh) (Sigma). The elution was kept similar to that of the previous one. This fractionation yielded 24 tubes and TLC was made for all of them to get three sub fractions for tubes 1-5, 6-9 and 10-11 to give Ssfr. I to Ssfr. III. Further bioassay assay with the test fungi indicated tubes 6-9 for the pure compound GP2 which was about 14 mg in amount.

2.5. Bioassay experiments with the bioactive compounds of *G. pentaphylla*

Biological activity of the purified compounds were assessed through antimicrobial activity tests. Bioassay with the purified compounds has been a major target in this investigation. Due to insufficient amount of the purified compounds it was impossible to carry out all the biological assays mentioned above, however, both the purified compounds GP1 and GP2 were subjected to antibacterial activity test.

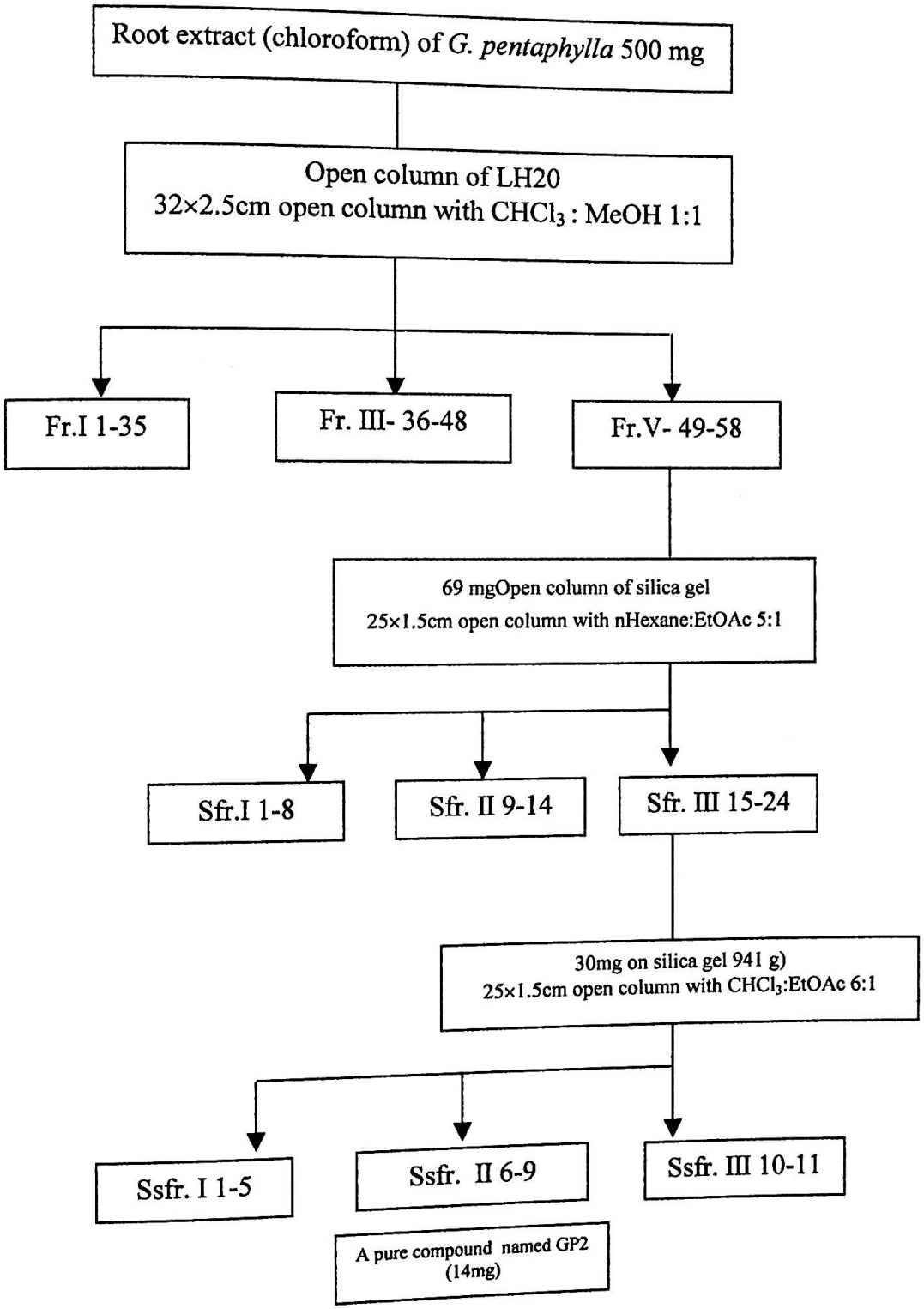
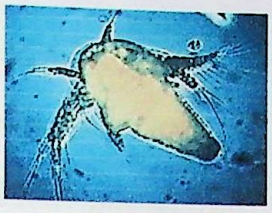
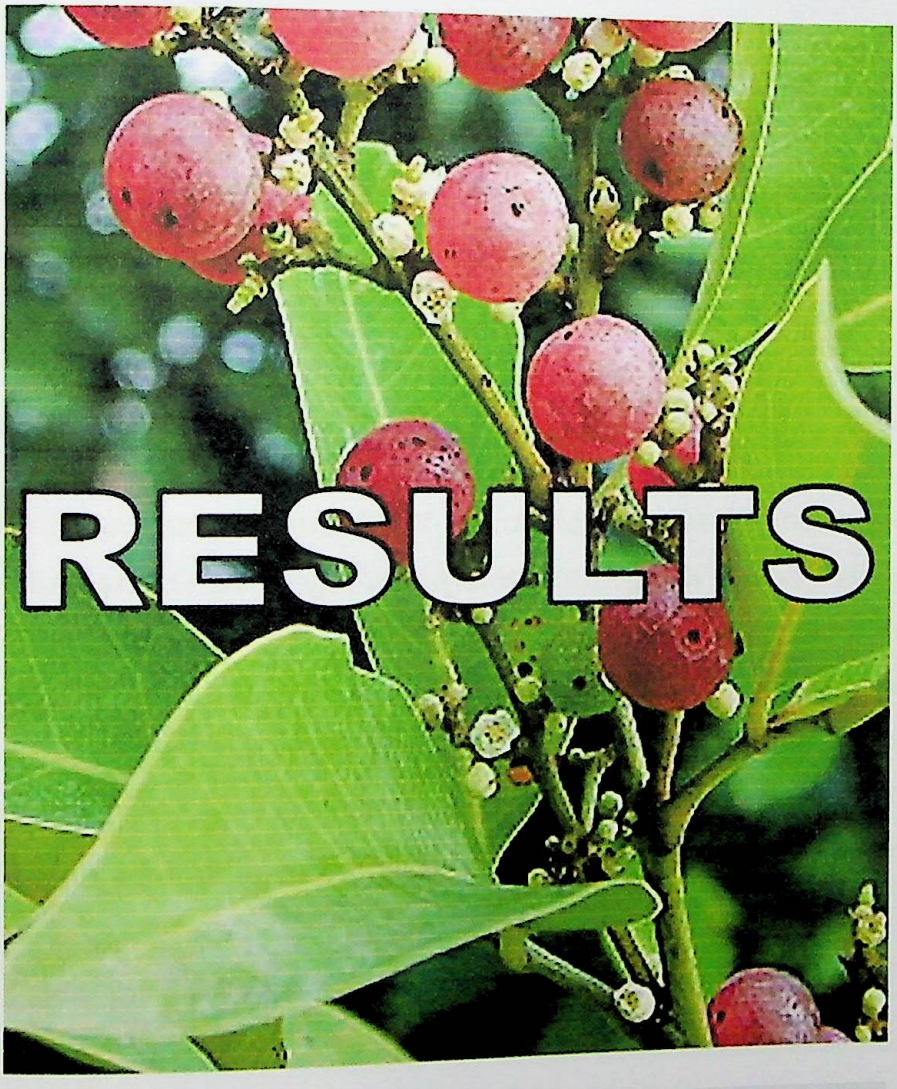
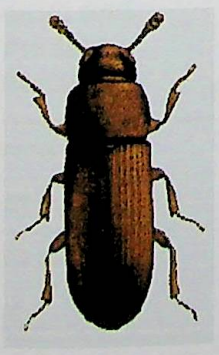
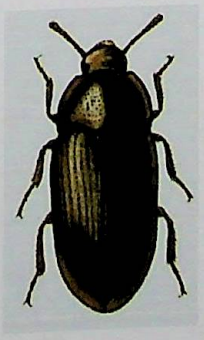


Fig. 7: Isolation pathway of compound GP2




RESULTS





3. Results

3.1. Antimicrobial activity of *G. pentaphylla* extracts

3.1.1. *In vitro* antibacterial activity of *G. pentaphylla* extracts

All the crude extracts (chloroform) of the collected *G. pentaphylla* samples (leaf, stem bark, stem wood and roots) were subjected to screen against a number of Gram positive (*S. aureus, B. megaterium, B. subtilis, S. lutea* and *S.-β-haemolyticus*) and Gram negative bacteria (*S. typhi, S. dysenteriae, S. sonnei, S. boydii, E. coli* and *P. aeruginosa*). In this antibacterial screening the crude extracts and pure compounds were used at concentrations of 200 µg/disc and a control experiment was set along with ciprofloxacine (30 µg/disc). Considering the size of the clear-zones in millimeter the efficacy of the extracts against the test bacteria was recorded.

The results of antibacterial activity of the crude extracts against a number of Gram positive and Gram negative organism are given in the Table 7a and Table 7b (Appendix Tables I-IV). From the recorded inhibition zones it is obvious that *G. pentaphylla* stem bark and leaf extracts are promisingly effective against both Gram positive and Gram negative bacteria mentioned above. However, no activity was traced against *S. dysenteriae* and *E. coli,* and also for the control dose or the only solvent (chloroform) application.

Table 7a: Antibacterial activity of the chloroform extracts of *G. pentaphylla* and the standard Ciprofloxacin

Sl.	Type of extract and the test standard	Inhibition zone (mm)				
		Gram positive				
		S. aureus	*B. megaterium*	*B. subtilis*	*S. lutea*	*S.- β - haemolyticus*
1	Leaves	-	17	16	16	14
2	Stem bark	14	15	-	15	-
3	Stem wood	-	7	14	-	12
4	Root	9	9	-	-	-
	Ciprofloxacin	31	28	33	30	31

Table 7b: Antibacterial activity of the chloroform extracts of *G. pentaphylla* and the standard Ciprofloxacin.

Sl.	Type of extract and the test standard	Inhibition zone (mm)					
		Gram negative					
		S. typhi	*S. dysenteriae*	*S. sonnei*	*S. boydii*	*E. coli*	*P. aeruginosa*
1	Leaves	16	-	20	12	-	7
2	Stem bark	11	-	15	10	-	10
3	Stem wood	-	-	-	10	-	12
4	Root	-	-	10	8	-	7
	Ciprofloxacin	35	31	33	34	33	31

Among the 11 bacteria, eight- *B. megaterium, B. subtilis, S. lutea, S.- β-haemolyticus, S. typhi, S. sonnei, S. boydii* and *P. aeruginosa* (Gram-positive – 4 and Gram-negative – 4) were responsive to the leaf extract with inhibition zones 17-, 16-, 16-, 14-, 16-, 20-, 12- and 7 mm respectively at 200 µg/disc application, while the inhibition zones for the standard Ciprofloxacin 30 µg/disc were 28-, 33- 30-, 31-, 35-, 33-, 34- and 31 mm for the above mentioned test agent respectively. Seven bacteria- *S. aureus, B. megaterium, S. lutea, S. typhi, S. sonnei, S. boydii* and *P. aeruginosa* (Gram-positive – 3 and Gram-negative – 4) were responsive to the stem bark extract with inhibition zones 14-, 15-, 15-, 11-, 15-, 10- and 10 mm respectively at 200 µg/disc application, while the inhibition zones for the standard Ciprofloxacin 30 µg/disc were 31-, 28-, 30-, 35-, 33-, 34-, and 31 mm for the same test agents respectively. Five of them- *B. megaterium, B. subtilis, S.- β-haemolyticus, S. boydii* and *P. aeruginosa* (Gram-positive – 3 and Gram-negative – 2) were responsive to the stem wood extract with inhibition zones 7-, 14-, 12-, 10- and 12 mm respectively at 200 µg/disc application, while the inhibition zones for the standard Ciprofloxacin 30 µg/disc were 28-, 33-, 31-, 34- and 31 mm for the above mentioned test agents respectively; and five others- *S. aureus, B. megaterium, S. sonnei, S. boydii* and *P. aeruginosa* (Gram-positive – 2 and Gram-negative – 3) were responsive to the root extract with inhibition zones 9-, 9-, 10-, 8- and 7 mm respectively at 200

µg/disc application, while the inhibition zones for the standard Ciprofloxacin 30 µg/disc were 31-, 28-, 33-, 34- and 31 mm for the above mentioned test agents respectively.

3.1.2. *In vitro* antifungal activity of *G. pentaphylla* extracts

The antifungal activity (diameter of the zone of inhibition in mm) of crude extract (chloroform) at a concentration of 500 µg/disc against five pathogenic fungi, *F. vasinfectum, A. fumigatus, A. flavus, Mucor* sp. and *C. albicans* are given in the Table 8 (Appendix Table V-VIII) From the recorded inhibition zones it is obvious that *G. pentaphylla* stem bark extract is more effective against *A. fumigatus* and *Mucor* sp. However, weak activity was traced against *F. vasinfectum, S. flavus* and *C. albicans.* In comparison to all other extracts the highest activity was represented by the root extract.

Table 8: Antifungal activity of the chloroform extracts of *G. pentaphylla* and the standard Nystatin

Sl. #	Type of extract and the standard	Inhibition zone									
		F. asinfectum		*A. umigatus*		*A. flavus*		*Mucor* sp.		*C. albicans*	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
1	Leaves	-	-	20	12	-	-	20	12	-	-
2	Stem bark	-	-	19	12	7	-	17	13	12	9
3	Stem wood	7	-	12	-	10	-	17	13	10	-
4	Root	7	-	15	9	-	-	12	9	-	-
Standard Nystatin		29		32		28		30		31	

Among the 5 fungi *A. fumigatus and Mucor* sp. were responsive to the leaf extract with the inhibition zones 20 mm for both of them at 500 µg/disc application for 24 h of exposure, however after 48 h also the *A. fumigatus and Mucor* sp. both were responsive to the same with 12 mm of inhibition zone; while the inhibition zones for the standard Nystatin 50 µg/disc were 32- and

30 mm for the above mentioned test fungi. Four fungi- *A. fumigatus, A. flavus, Mucor* sp. and *C. albicans* were responsive to the stem bark extract with inhibition zones 19-, 7-, 17- and 12mm at 500 µg/disc application for 24 h of exposure, however after 48 h only the *A. fumigatus, Mucor* sp. and *C. albicans* were responsive to the same with 12-, 13- and 9 mm of inhibition zones; while the inhibition zones for the standard Nystatin 50 µg/disc were 32-, 28- 30- and 31mm for the above mentioned test fungi respectively. Among the 5 fungi all were responsive to the stem wood extract with inhibition zones 7-, 12-, 10, 17- and 10 mm at 500 µg/disc application for 24 h of exposure, however after 48 h only the *Mucor* sp. remained responsive to the same with 13 mm of inhibition zone; while the inhibition zones for the standard Nystatin 50 µg/disc was 29-, 32-, 28-, 30- and 31 mm for the above mentioned test fungi. Among the five fungi *F. vasinfectum, A. fumigatus* and *Mucor* sp. were found responsive to the root extract with inhibition zones 7-, 15- and 12 mm at 500 µg/disc application for 24 h of exposure, however after 48 h *A. fumigatus and Mucor* sp. were responsive to the same, while both with 9 mm of inhibition zones; and the inhibition zones for the standard Nystatin 50 µg/disc were 29-, 32- and 30 mm for the above mentioned test fungi respectively.

3.2. Cytotoxic effects of *G. pentaphylla* extracts

The $CHCl_3$ extract of different parts of *G. pentaphylla* were subjected to apply against the brine shrimp (*A. salina*) nauplii for the detection of their biological activity. Several doses were selected by a pilot (*Ad Hoc*) experiment and a final experiment was set with 3 replications along with the control doses. The result was then subjected to probit analysis and the LC_{50} values for 24 h of exposure were shown in Table 9 (Appendix Tables IX-XII).

For leaves the LC_{50} was established as 28.579 ppm and the regression equation was $Y = -0.901 + 4.053X$, while the 95% confidence limits were 24.136 to 33.840 ppm for 24 h of exposure. For stem bark the LC_{50} was 28.659 ppm and the regression equation was $Y = 1.639 + 2.306\ X$, while the 95% confidence limits were 19.831 to 41.419 ppm for the 24 h of exposure.

For stem wood the LC_{50} was 57.213 ppm and the regression equation was Y = -1.436 + 3.662X, while the 95% confidence limits were 47.535 to 68.863 ppm for the 24 h of exposure. For root the LC_{50} was 84.111 ppm and the regression equation was Y = 1.007 + 2.074X, while the 95% confidence limits were 64.836 to 109.118 ppm for 24 h of exposure.

Table 9: Dose mortality effect of *G. pentaphylla* extracts against *A. salina* nauplii after 24 hours of exposure.

Plant	LC_{50} value (ppm)	95% confidence limits		Regression equations	χ^2 value (df)
		Upper	Lower		
Leaves	28.579	33.840	24.136	Y = -0.9008684 + 4.05267 X	4.056 (2)
Stem bark	28.659	41.419	19.831	Y = 1.638829 + 2.306482 X	1.402 (1)
Stem wood	57.213	68.863	47.535	Y = -1.436301 + 3.662196 X	5.870 (2)
Root	84.111	109.118	64.836	Y = 1.006926 + 2.074481 X	3.013 (3)

3.3. Larvicidal activity of *G. pentaphylla* extracts

3.3.1. Dose mortality effect of *G. pentaphylla* extracts on *T. castaneum* larvae

Chloroform extracts of the leaves, stem bark, stem wood and roots of *G. pentaphylla* were subjected to apply against the larvae of *T. castaneum* for the detection of their biological activity (including lethality, prolongation of larval instars, causing deformity in body, abnormality in any of the biological parameters). In the pilot experiment no mentionable activity was found in case of leaves, stem bark and stem wood extracts, and thus the experiment was continued with the root extract only. Several doses (50-, 25-, 12.5-, 6.25-, 3.125- and 1.562 mg/g, V/V) were selected by a pilot (*Ad Hoc*) experiment and a final experiment was set with 3 replications and with the necessary control doses. The observations were made by 7 days interval since this test insect normally changes its larval instars at every 3rd day, however, it was prolonged after released them in the treated food medium and thus the observation time was considered for once a week. Mortality of the larvae and abnormality in changing instars, as well as differences in size were observed,

however, the mortality indicates weak activity and widely heterogeneity (Table 10) so that probit analysis was not possible to find LD_{50} values. In the developmental stages, in some cases, the state of larvae was continuous until the end of the 5th week, while at end of the 3rd week number of short living pupae have been appeared who survived for less than a week after their appearance as pupae. Heterogeneity in the development of larvae was traced (Plate 10). Some of the pupae became black or dark brown in color, and also an abnormal white pupa was also found. The larvae that survived for long in the larval state were in shrunk form and were not easily moving. Number of dead individuals increased drastically in the 4th week.

Table 10: Effect of root extract on *T. castaneum* larvae in a treated food medium.

Dose (mg/g)	1st observation		2nd observation		3rd observation		4th observation		5th observation	
	# death	current state	#death	current state	# death	current state	# death	current state	# death	current state
50		10 L		10 L		9 L 1 P	6	2 A 2 L		3 A 1 P
25	1	9 L		9 L	2	5 L 2 P	5	2 L	2	
12.5		10 L	2	8 L		7 L 1 P	5	3 L		1 L 2 P
6.25		10 L		10 L	3	6 L 1 P	3	2 L 2 P	1/P	2 L
3.125		10 L	1	9 L		6 L 3 P	7	2 L		1 P/B 1 P
1.562	1	9 L		9 L		6 L 3 P	6	1 L 2 P	3/P	1 P
Control	2	8 L	1	7 L		6 L 1 P	1	7 P/B 1 P		5 P/B 3 P

(L = Larva(e), P = Pupa(e), A = Adult(s), B = Black or dark brown, W = white)

(Note: This is a gross result on the overall treated individuals and unfortunately photographs were not maintained, while individuals from a single set of experiment are shown in Plate 10).

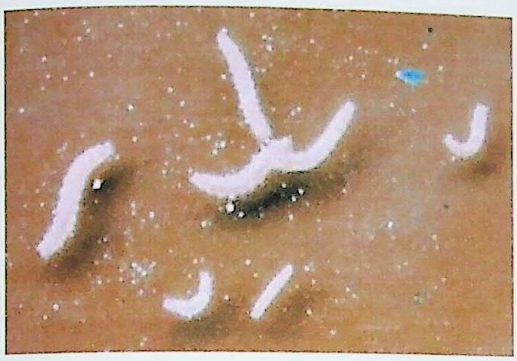


Fig. 1

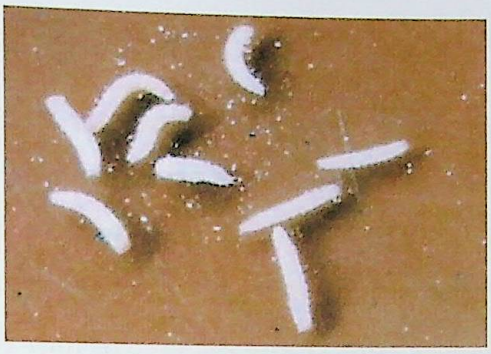


Fig. 2

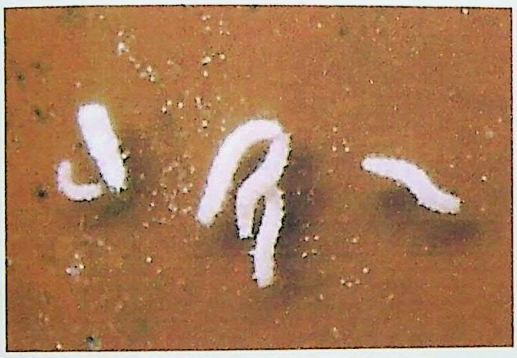


Fig. 3

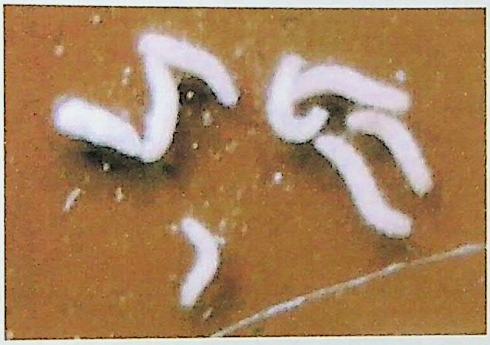


Fig. 4

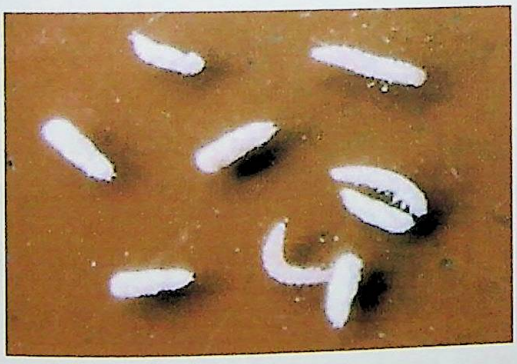


Fig. 5

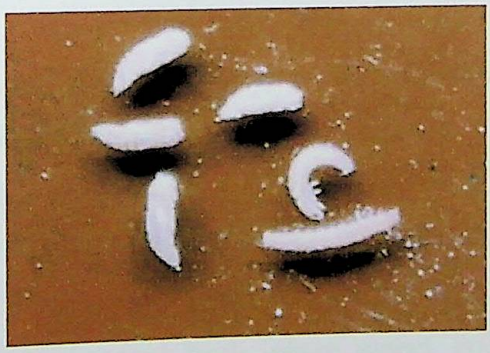


Fig. 6

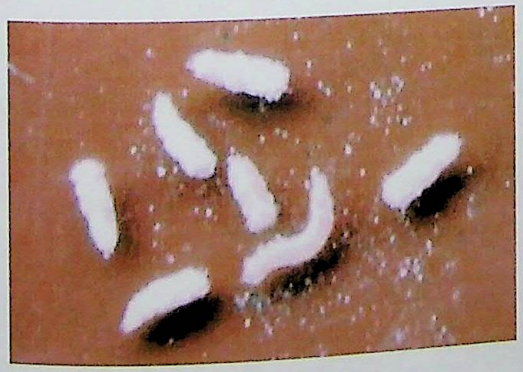


Fig. 7 (Control)

Fig. 1. Larvae treated with 50 mg/g
Fig. 2. Larvae & pupae treated with 25 mg/g
Fig. 3. Larvae & pupae treated with 12.5 mg/g
Fig. 4. Larvae & pupae treated with 6.25 mg/g
Fig. 5. Larvae & pupae treated with 3.125 mg/g
Fig. 6. Larvae & pupae treated with 1.562 mg/g
Fig. 7. Larvae in the control

Plate 10: Photographs of *T. castaneum* larvae during 3rd week of exposure.

3.3.2. Dose mortality effect of *G. pentaphylla* extracts on the 3rd instar larvae of *M. domestica*

All the $CHCl_3$ extracts of the different parts of *G. pentaphylla* were subjected to apply against the 3rd instar larvae of *M. domestica* for the detection of their biological activity. Several doses (51.282-, 25.641-, 12.821-, 6.410-, 3.205 and 1.603 mg/g, V/V) were selected by a pilot (*Ad Hoc*) experiment and a final experiment was set with 3 replications and with the necessary control doses. To find activity this was the standard dose-range for evaluating activity against *M. domestica* established here by doing dose-mortality tests on other biologically active plant extracts, however, no activity was found in case of *M. domestica* larvae.

3.4. Repellent activity of *G. pentaphylla* extracts against *T. castaneum* adults

Chloroform extracts of leaves, stem bark, stem wood, root bark and root wood of the title species *G. pentaphylla* showed repellant activity against adult beetles of *T. castaneum* even for a concentration range of 78.634-, 39.317-, 19.659-, 9.829-, 4.915- and 2.457 $\mu g/cm^2$. 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 11 (Appendix Tables XIII). The F values have been established through ANOVA with the arcsin transformed data were 60.983, 14.177, 19.437, 15.429 and 1.082 for the analysis between doses and 2.52, 1.806, 1.314, 3.468 and 1.272 for the analysis between time interval for leaf, stem bark, stem wood, root bark and root wood extracts respectively. Except the root extract strong repellent activity was found, while repellency due to differences between doses were highly significant ($P<0.001$).

The result shows that the chloroform extracts promisingly played a role as repellents against the adult beetles of *T. castaneum* and the intensity was with remarkable difference between the doses. The P values were established as 2.03E-11, 5.41E-6, 4.62E-7, 2.48E-6 and 0.4002 for the analysis between doses and 0.0733, 0.1673, 0.2989, 0.0262 and 0.3139 for the analysis between time interval for the leaf, stem bark, stem wood, root bark and root

wood extracts respectively, however it has promising role also on time of exposure and thus the intensity of repellent activity could be arranged in a descending order of leaves>stem wood>root bark>stem bark>root wood.

Table 11: ANOVA results for repellency of *T. castaneum* adults by *G. pentaphylla* extracts

Test materials	Source of variation	SS	df	MS	F	P-value
Leaves	Between doses	40904.49	5	8580.897	60.983	2.03E-11
	Between time interval	1419.201	4	354.8002	2.52	0.073346
Stem wood	Between doses	24779.38	5	4955.876	14.177	5.41E-6
	Between time interval	1339.773	4	334.9431	1.806	0.167365
Stem bark	Between doses	26948.51	5	5389.703	19.437	4.62E-7
	Between time interval	2745.829	4	686.4572	1.314	0.29891
Root bark	Between doses	20117.29	5	4023.457	15.429	2.48E-6
	Between time interval	3617.81	4	904.4524	3.468	0.026234
Root wood	Between doses	2576.381	5	515.2762	1.082	0.40020
	Between time interval	2423.457	4	605.8643	1.272	0.313922

3.5 Insecticidal activity of *G. pentaphylla* extracts

3.5.1. Effect of *G. pentaphylla* extract against *T. castaneum* adults by residual film assay

The $CHCl_3$ extracts of the roots of *G. pentaphylla* were tested against adult beetles of *T. castaneum* for concentrations of 3.146, 1.573, 0.786-, 0.393-, 0.197 and 0.098 mg/cm^2 and a control (only solvent) with three observations after 24-, 48- and 72h of exposure and no mortality was recorded. Application of the extract more in quantity was rather impossible because the surface of the petri dish became sticky with a thick layer of the extract.

3.5.2. Effect of *G. pentaphylla* extract against *C. maculatus* adults by residual film assay

The $CHCl_3$ extracts of roots of *G. pentaphylla* were tested against *C. maculatus* adults, while the doses were 786-, 393-, 197-, 98-, 49- and 25 µg/cm^2 with three replications and a control. Observation of mortality was made after 24-, 48 and 72 hours of exposure; and the results have been presented in the Table 12 (Appendix Table XV).

The LD_{50} value was 1425.036 µg/cm^2 for 24 hours of exposure and the regression equation was Y = 3.921 + 0.342 X with chi-squared values 2.40 for 4 degrees of freedom 95% confidence limits are 90.16734 to 22521.76. The LD_{50} value was 213.523 µg/cm^2 for 48 hours of exposure and the regression equation was Y = 3.809 + 0.511 X with chi-squared values 1.54 for 4 degrees of freedom 95% confidence limits are 88.21113 to 516.8505. The LD_{50} value was 91.782 µg/cm^2 for 72 hours of exposure and the regression equation was Y = 3.503 + 0.763 X with chi-squared values 2.13 for 4 degrees of freedom 95% confidence limits are 50.31486 to 167.4234. It is of course mentionable that by applying the above-mentioned doses other test materials, viz. leaf, stem bark and stem wood extracts of *G. pentaphylla* didn't show any remarkable activity against the test insect *C. maculatus* larvae.

Table 12: Dose mortality effect of the root extract of *G. pentaphylla* against *C. maculatus* adults

Exposure (h)	LC_{50} value (µg/cm^2)	95% confidence limits		Regression equations	χ^2 value (df)
		Upper	Lower		
24	1425.036	22521.76	90.16734	Y = 3.921 + 0.342 X	2.40 (4)
48	213.523	516.8505	88.21113	Y = 3.809 + 0.511 X	1.54 (4)
72	91.782	167.4234	50.31486	Y = 3.503 + 0.763 X	2.13 (4)

3.5.3. Effect of *G. pentaphylla* extract against *A. diaperinus* adults by residual film assay

The $CHCl_3$ extracts of the roots of *G. pentaphylla* were tested against adult beetles of *A. diaperinus* for concentrations of 3.146, 1.573, 0.786-, 0.393-, 0.197 and 0.098 mg/cm^2 and a control (only solvent) with three observations after 24-, 48- and 72h of exposure similar to that of *T. castaneum* and no mortality was recorded as well. Application of the extract more in quantity was rather impossible because the surface of the petri dish became sticky with a thick layer of the extract.

3.6. Summary of biological activity of *G. pentaphylla* extracts

Table 13: Biological activity of the $CHCl_3$ extracts of *G. pentaphylla* at a glance

Test types	Activity traced	Leaves	Stem bark	Stem wood	Root (bark+ wood)	Root bark	Root wood
Antimicrobial	Antibacterial activity	√	√	√	√	-	-
	Antifungal activity	√	√	√	√	-	-
Cytotoxicity	*A. salina*	√	√	√	√	-	-
Larvicidal and other activities	*M. domestica*	×	×	×	√*	-	-
	T. castaneum	×	×	×	√*	-	-
Repellency	*T. castaneum*	√	√	√	-	√	×
	T. castaneum	-	-	-	×	-	-
Insecticidal	*C. maculatus*	-	-	-	√	-	-
	A. diaperinus	-	-	-	×	-	-

'√'= activity found, '×'= activity not found, '-'= not tested, '√*'= activity other than mortality

3.7. Physical remarks and characterization of bioactive compounds

3.7.1. Physical remarks of the isolated pure compounds

The isolated compounds and there physical stature have been presented in Table 14. Two compounds from the roots were isolated and characterized. Through literature search it has been found that the compounds were previously isolated as glycozoline and glycoborinine. Glycozoline was crystal needles and it takes deep-blue in color on the TLC after Godin reagent spray and the R*f* was 0.475 with the same solvent system that applied to isolate the compound. Glycoborinine was an amorphous off-white powder and was red after Godin reagent spray, and the R*f* was 0.525 with benzene -ethyl acetate 1:1.

Table 14: Compounds purified from roots of *G. pentaphylla.*

Extract	Compound	Retention factor (R*f*)	Physical identity of the compounds /Godin reagent spray
$CHCl_3$	GP1	0.475	Crystal needles/Deep-blue
	GP2	0.525	Amorphous off-white/Reddish

3.7.2. Characterization of the purified compounds

3.7.2.1. Characterization of the purified compounds GP1

Compound GP1 was isolated from *Glycosmis pentaphylla*. The molecular ion peak (M/z=211.0965) of high resolution EIMS spectrum suggested that the molecular formula should be C14H15NO (calcd. M/z=211.0997). Thus the index of hydrogen deficiency (unsaturation number?) is 9. Two singlet peaks were observed at 2.51 (3H) and 3.91 (3H) ppm in 1H NMR spectrum. These were assigned to a methyl group attached on an aromatic ring, and one on a nitrogen or oxygen atom, respectively, from their chemical shifts. The broad peak at 7.78 ppm was considered to be an alcoholic or amine proton, which is chemically exchangeable. The aromatic protons were observed at 7.02, 7.20, 7.27, 7.29, 7.50, and 7.81 ppm. Twelve peaks in 13C NMR spectrum were observed at 104.2, 111.5, 112.4, 116.0, 121.3, 124.7, 124.8, 128.3, 129.5, 135.9, 139.7, and 154.9 ppm. These peaks suest that this compound will be a benzofuran or carbazole type compound. HSQC (proton-carbon correlation spectra) and HMBC (proton-carbon long range correlation spectra) were measured. NOE correlation between the proton at 7.50 and 7.81 ppm was observed. This result shows that compound 1 has a carbazole type structure. The chemical shifts of 1H and 13C for Glycozoline, which was reported by Chakravarty *et al.,* (1954), gave a complete agreement with our NMR data (Fig. 6 -11).

3.6.2.2 Characterization of the purified compound GP2

Compound GP2 was isolated from *Glycosmis pentaphylla*. The molecular ion peak (M/z=279.1246) of high resolution EIMS spectrum suggested that the molecular formula should be C18H17NO (calcd. M/z=279.1259). Thus the index of hydrogen deficiency is 11. Two singlet peaks were observed at 2.31 (3H) and 1.42 (6H) ppm in 1H NMR spectrum. These were assigned to one methyl group attached on an aromatic ring, and two equivalent methyl groups, respectively, from their chemical shifts. Two proton signals at 5.81 (1H) and

7.22 (1H) ppm, which were attributed the protons on double bond carbon, were observed. The aromatic protons were observed at 6.69, 6.78, 7.05, and 7.75 ppm. Fourteen peaks in 13C NMR spectrum were observed at 96.9, 110.9, 114.0, 115.6, 117.2, 118.0, 120.0, 121.5, 124.3, 131.8, 135.6, 136.6, 142.7, and 147.0 ppm. HSQC (proton-carbon correlation spectra) and HMBC (proton-carbon long range correlation spectra) were measured. This result shows that compound 4 has a carbazole type structure as shown as above. The chemical shifts of 1H and 13C for Glycoborinine, which was reported by Chakravarty *et al.*, (1954), gave a complete agreement with our NMR data (Fig. 12-17).

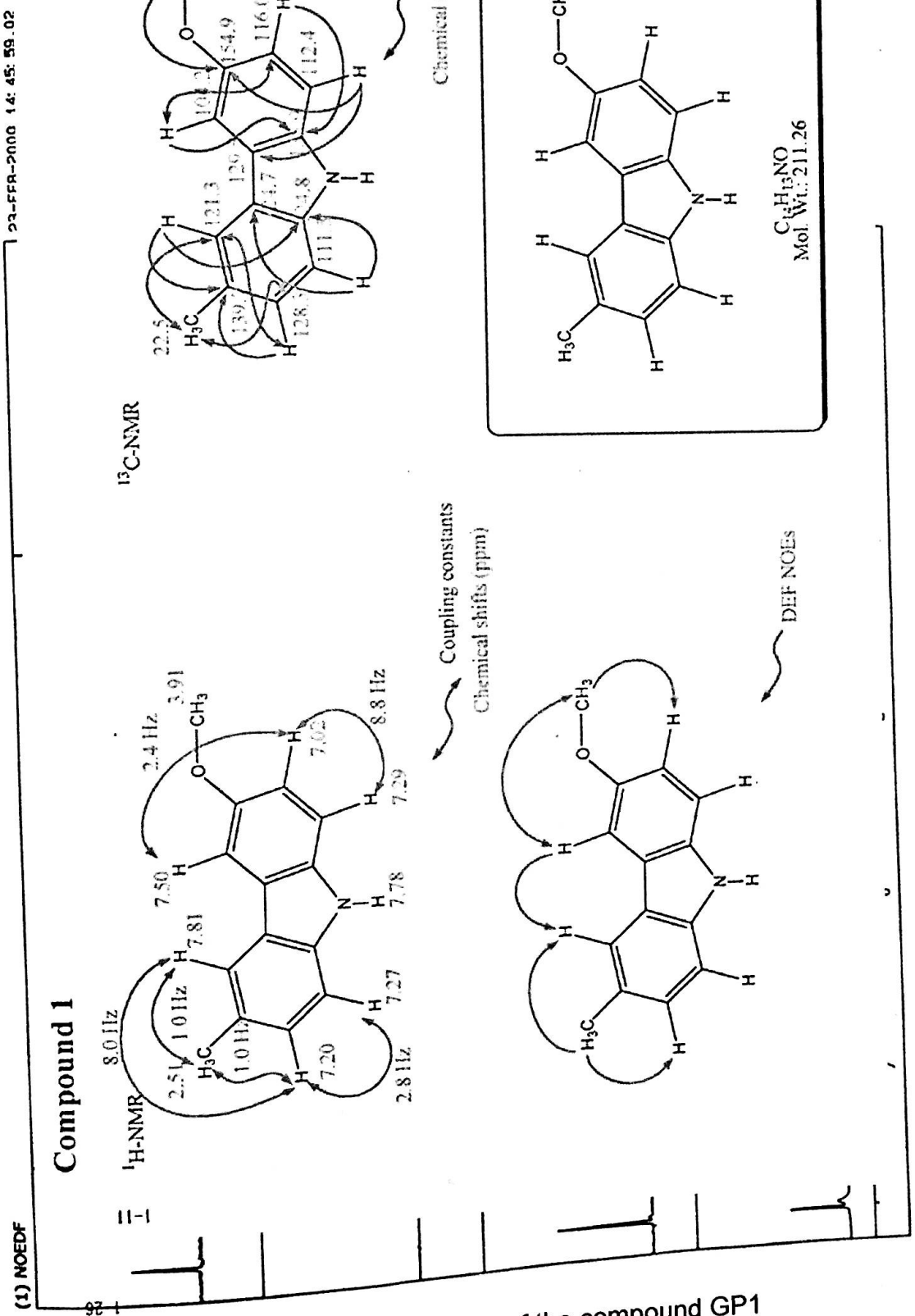


Fig. 6: Proposed structure of the compound GP1

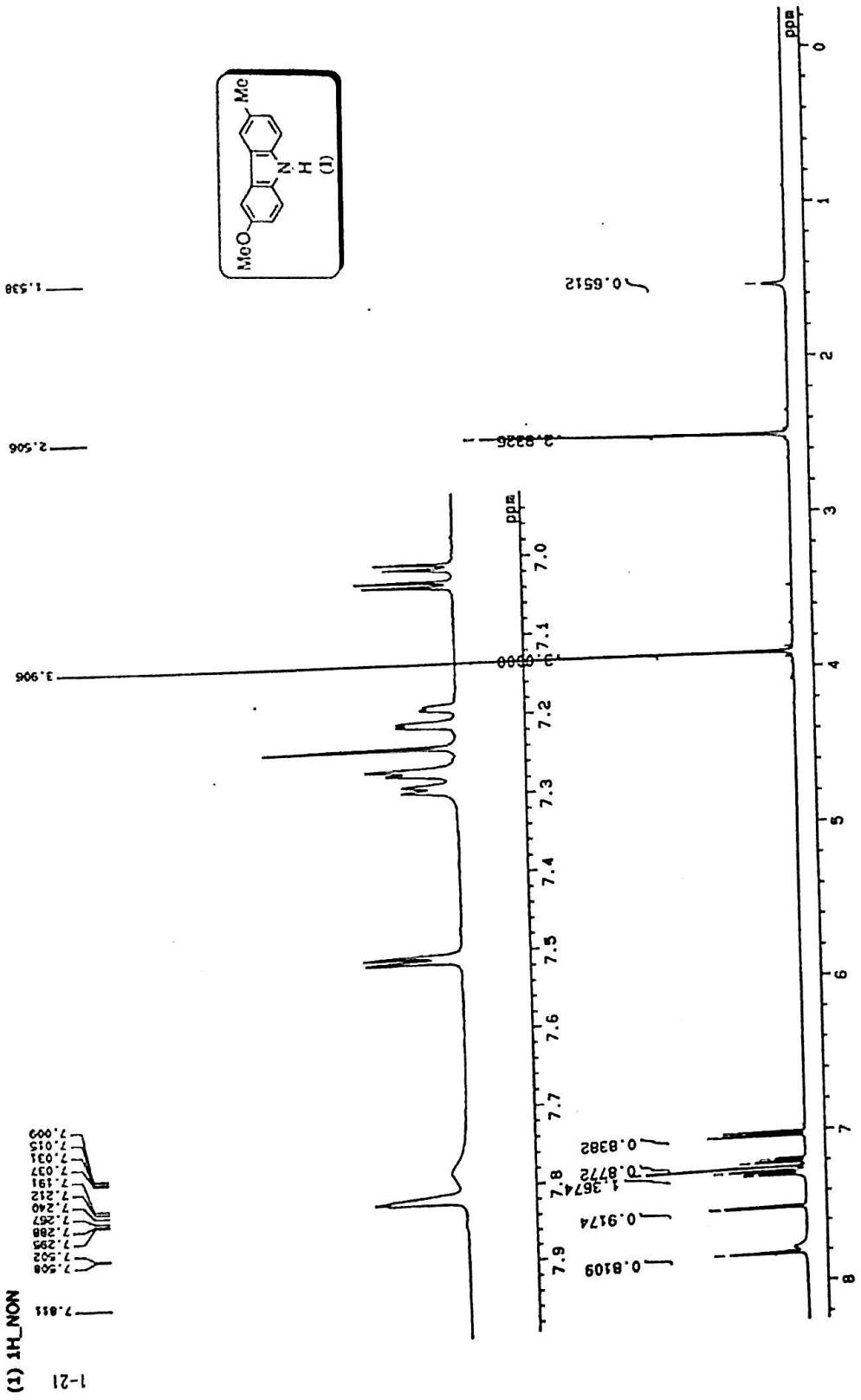


Fig. 7: ^{1}H NMR structure of the compound GP1

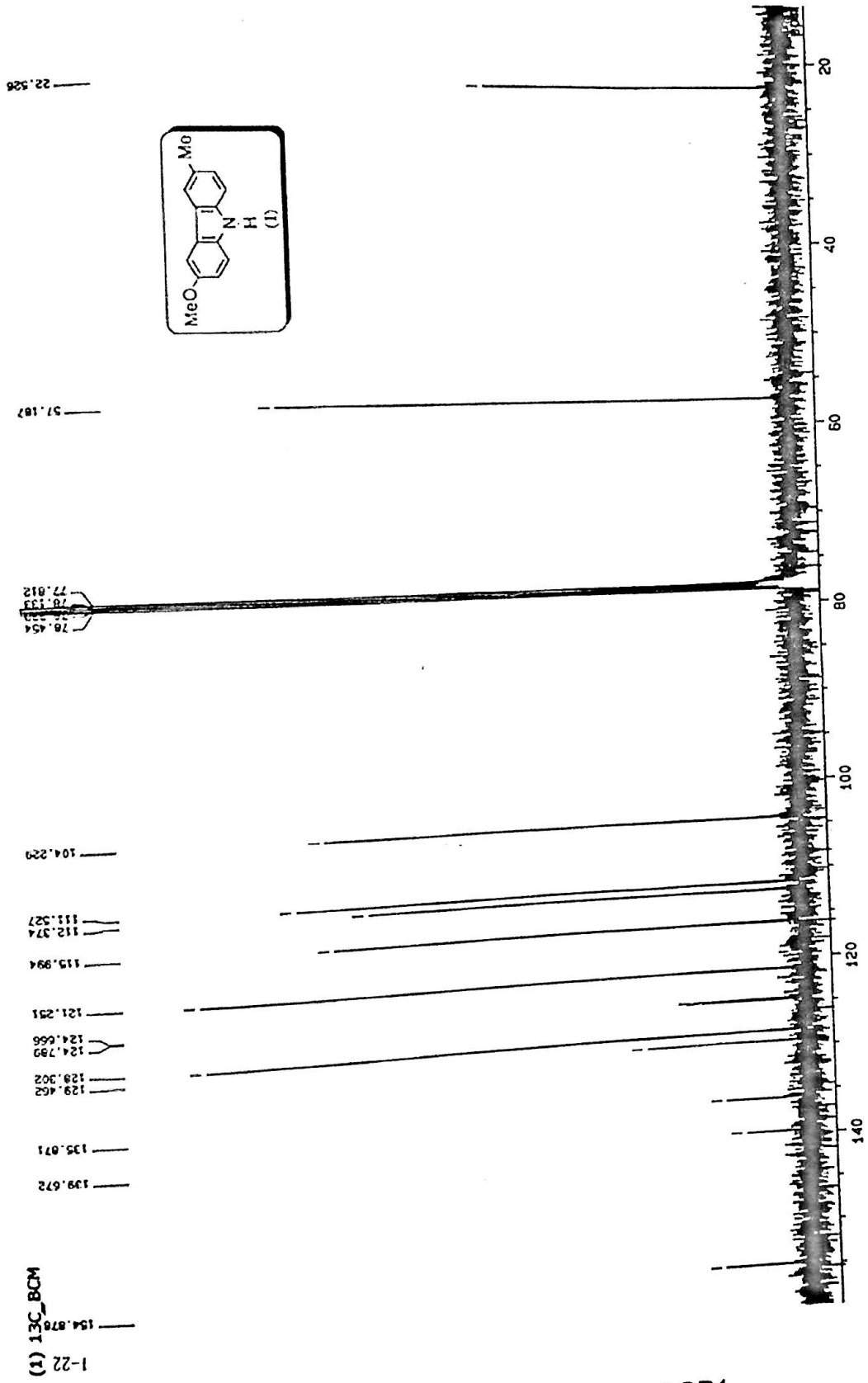


Fig. 8: ^{13}C NMR structure of the compound GP1

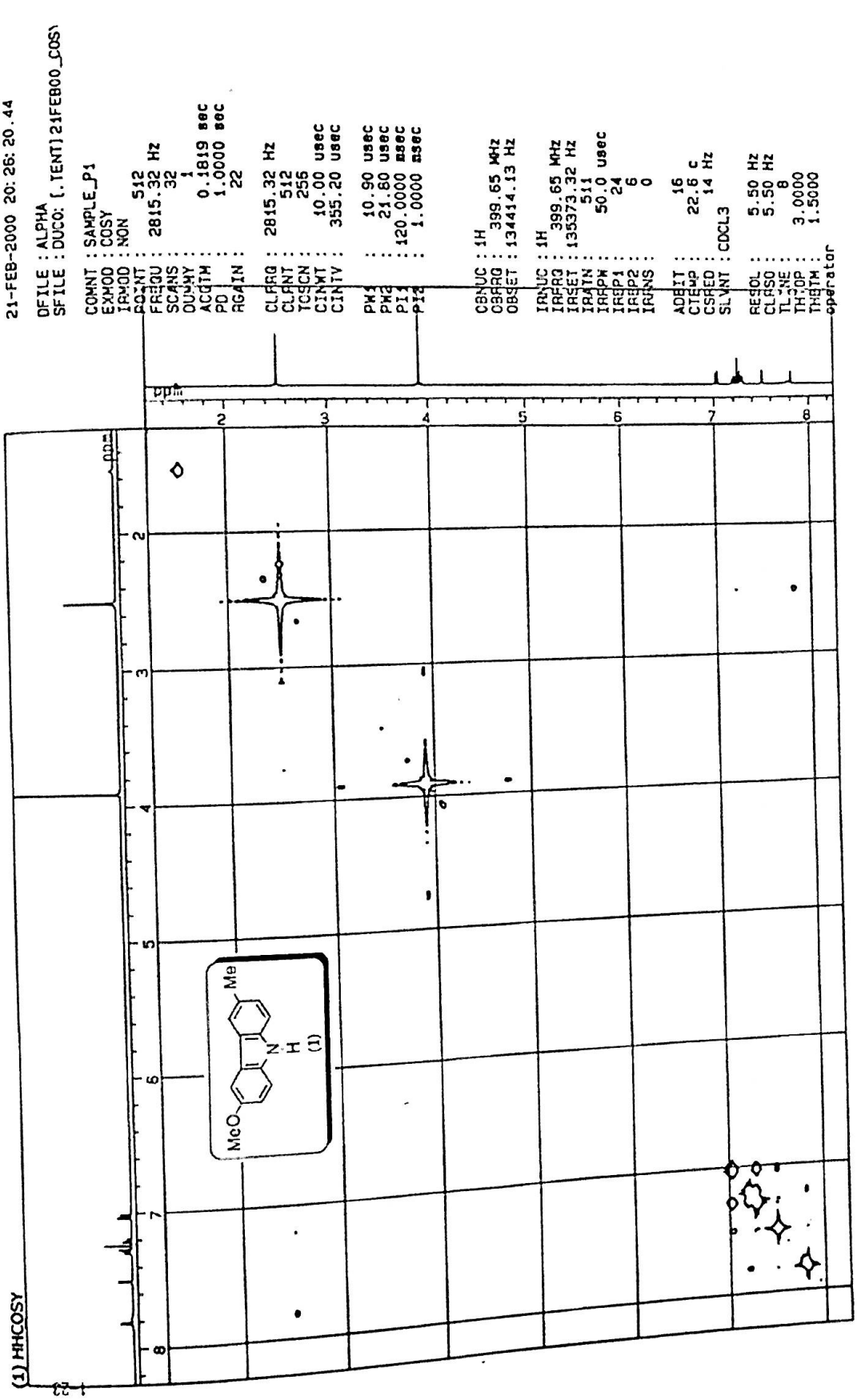


Fig. 9: HH COSY of the compound GP1

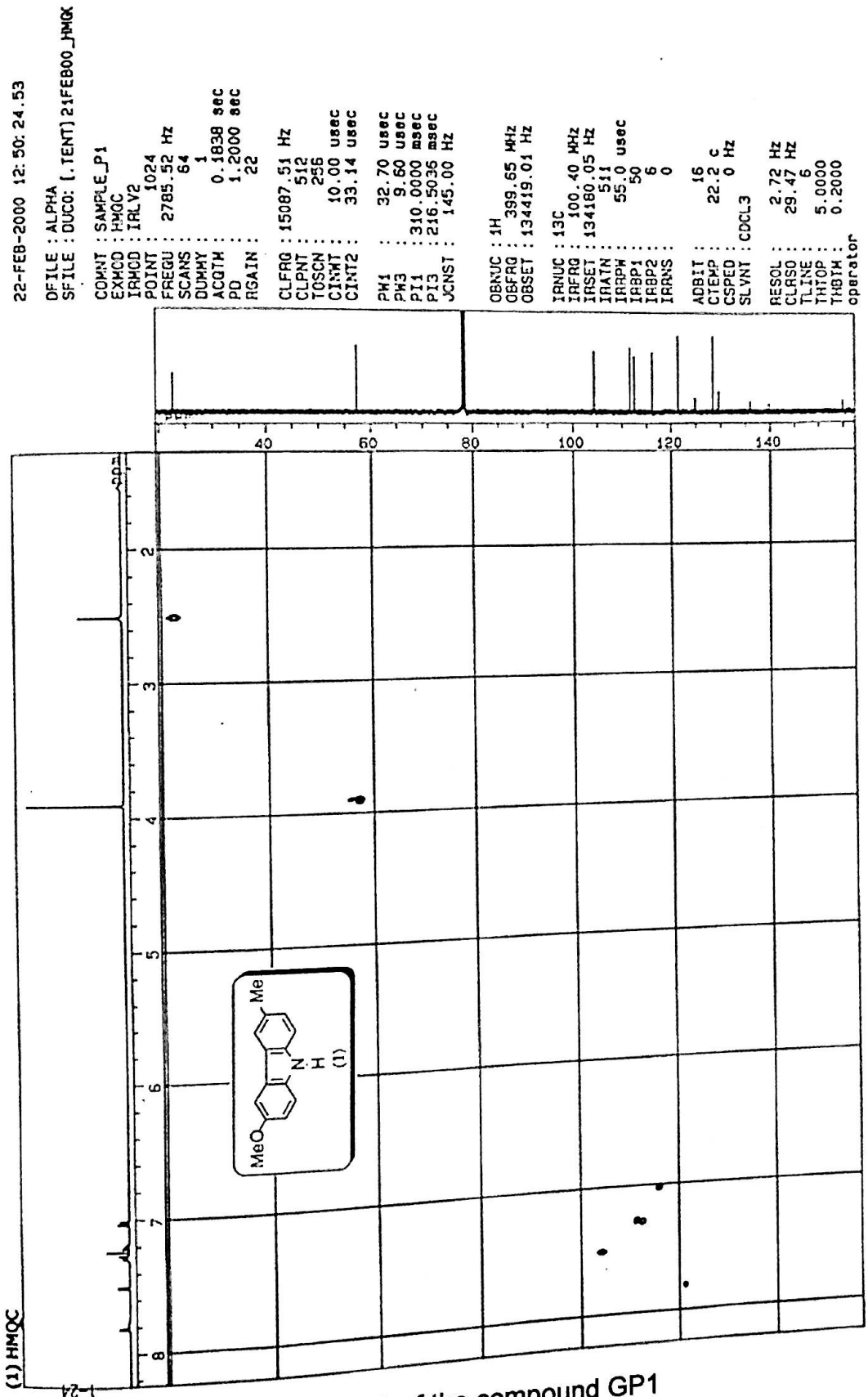


Fig. 10: HSQC of the compound GP1

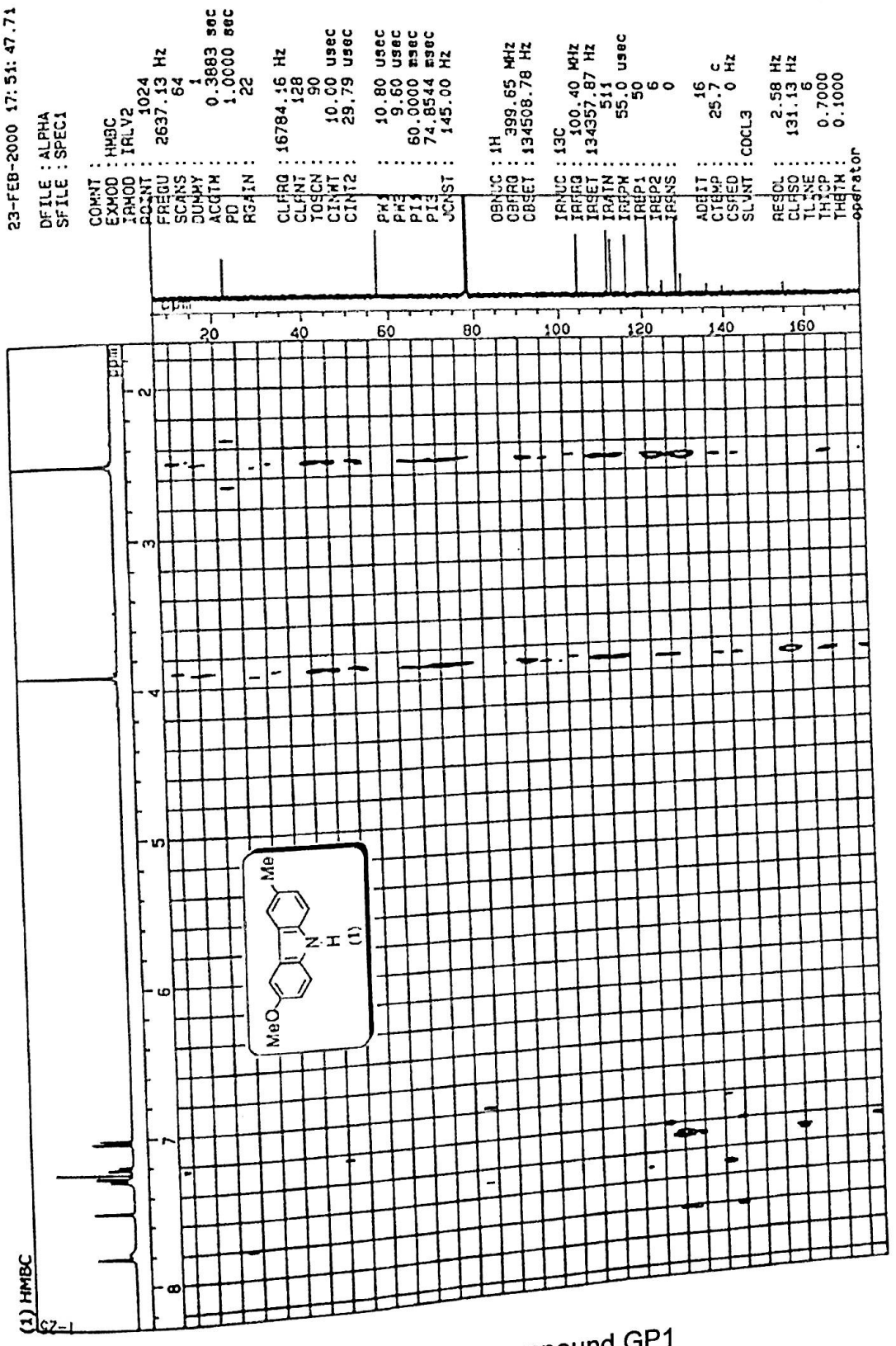


Fig. 11: HMBC of the compound GP1

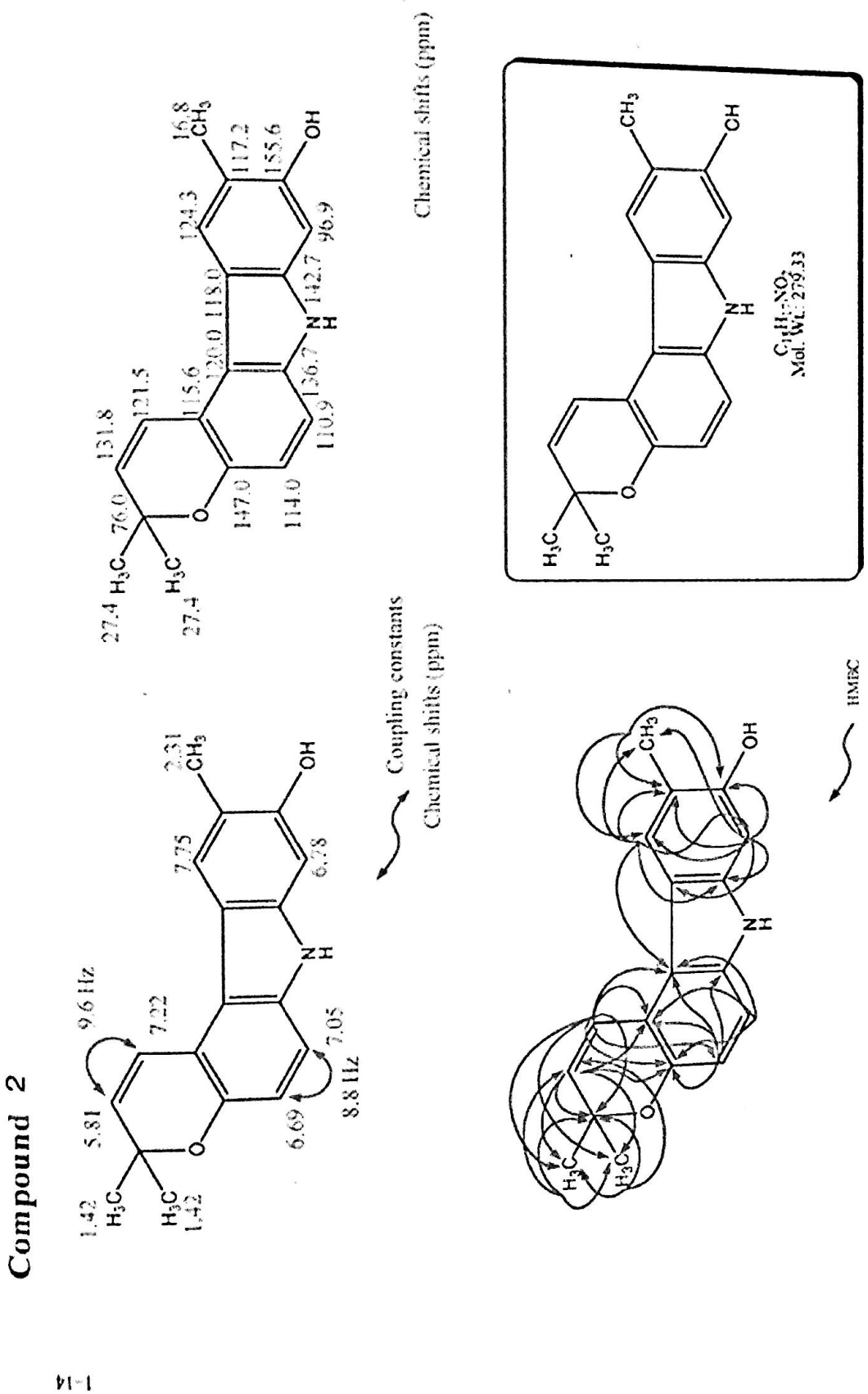


Fig. 12: Proposed structure of the compound GP2

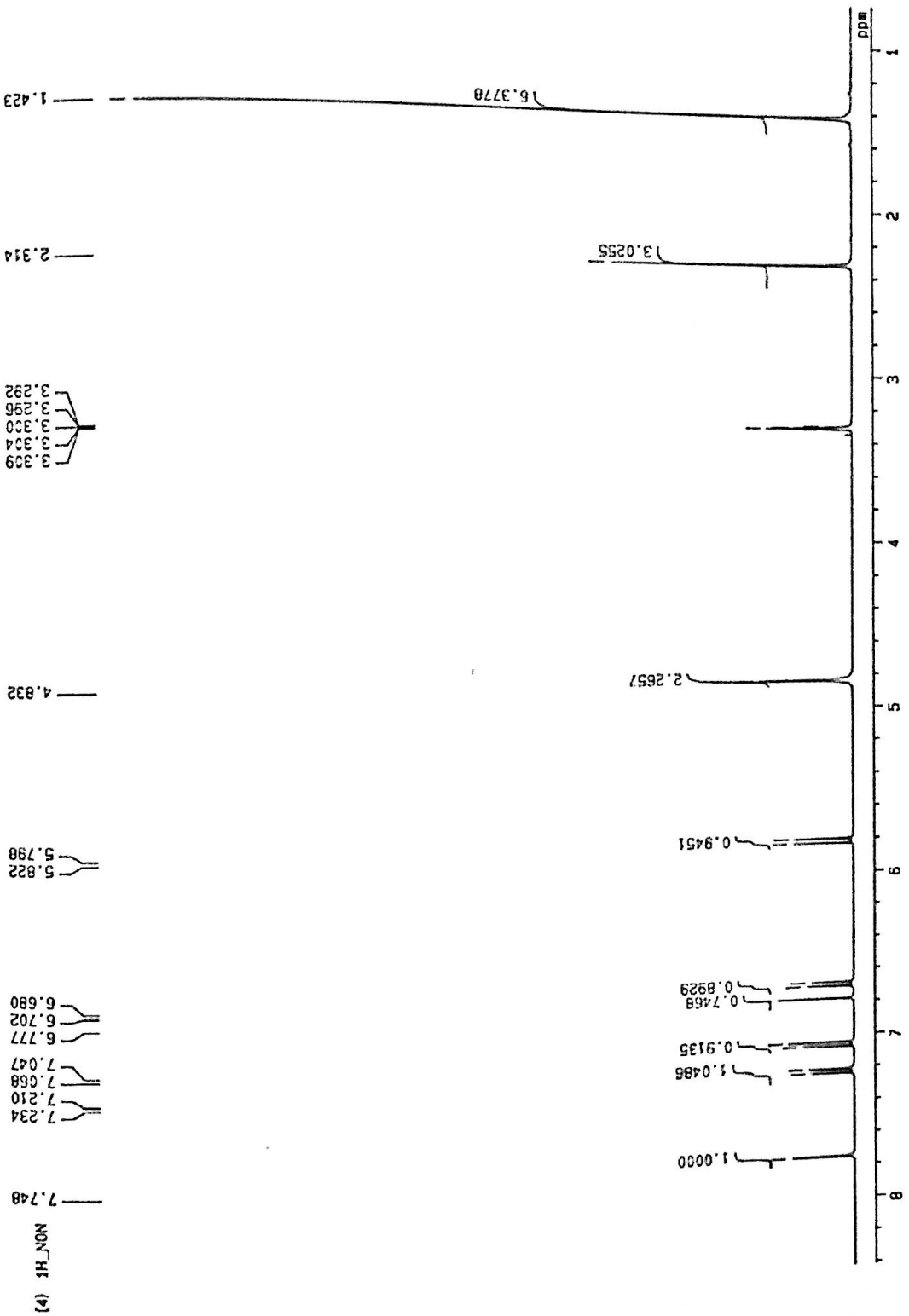


Fig. 13: ^{1}H NMR structure of the compound GP2

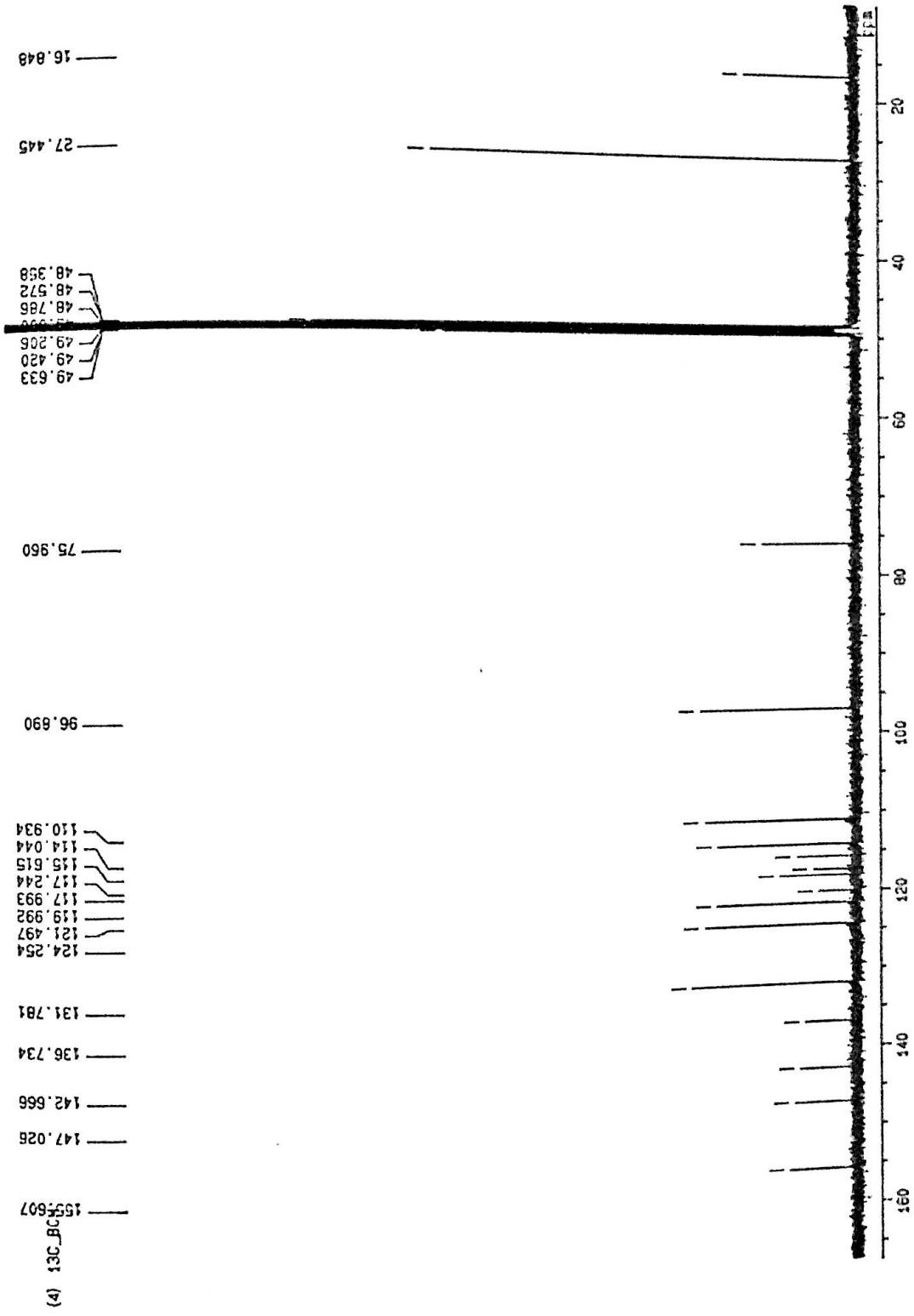


Fig. 14: ^{13}C NMR structure of the compound GP2

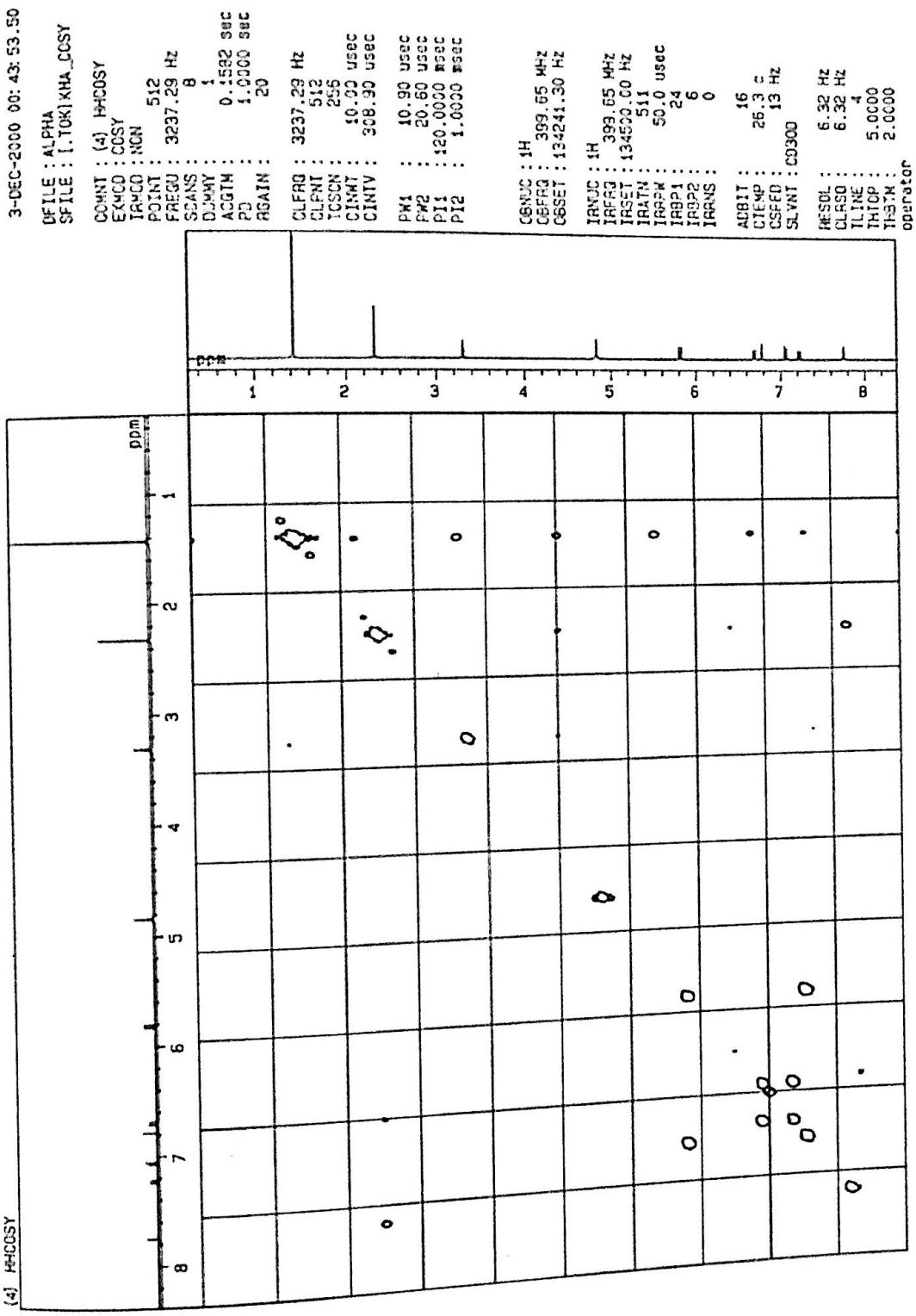


Fig. 15: HH COSY of the compound GP2

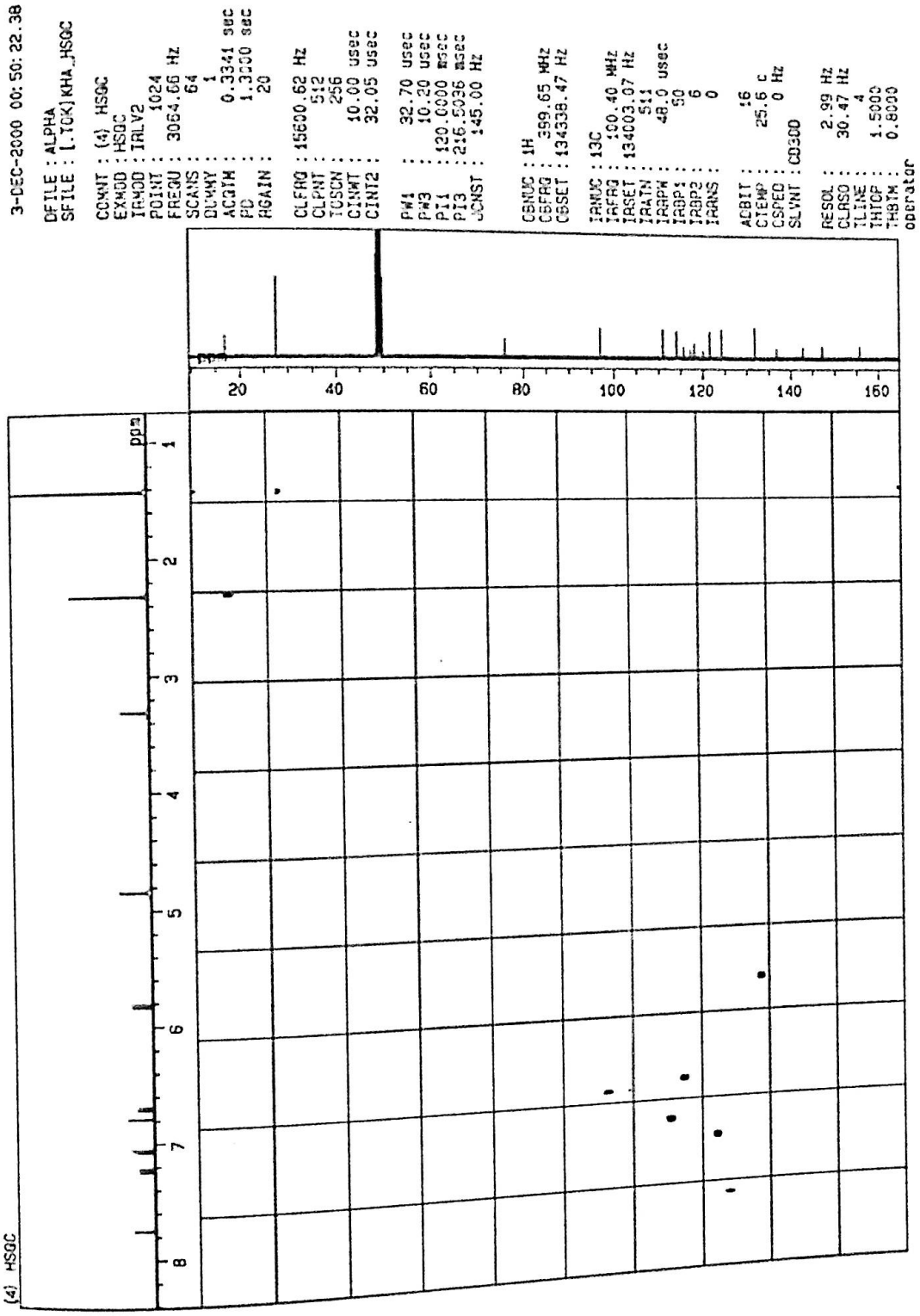


Fig. 16: HSQC of the compound GP2

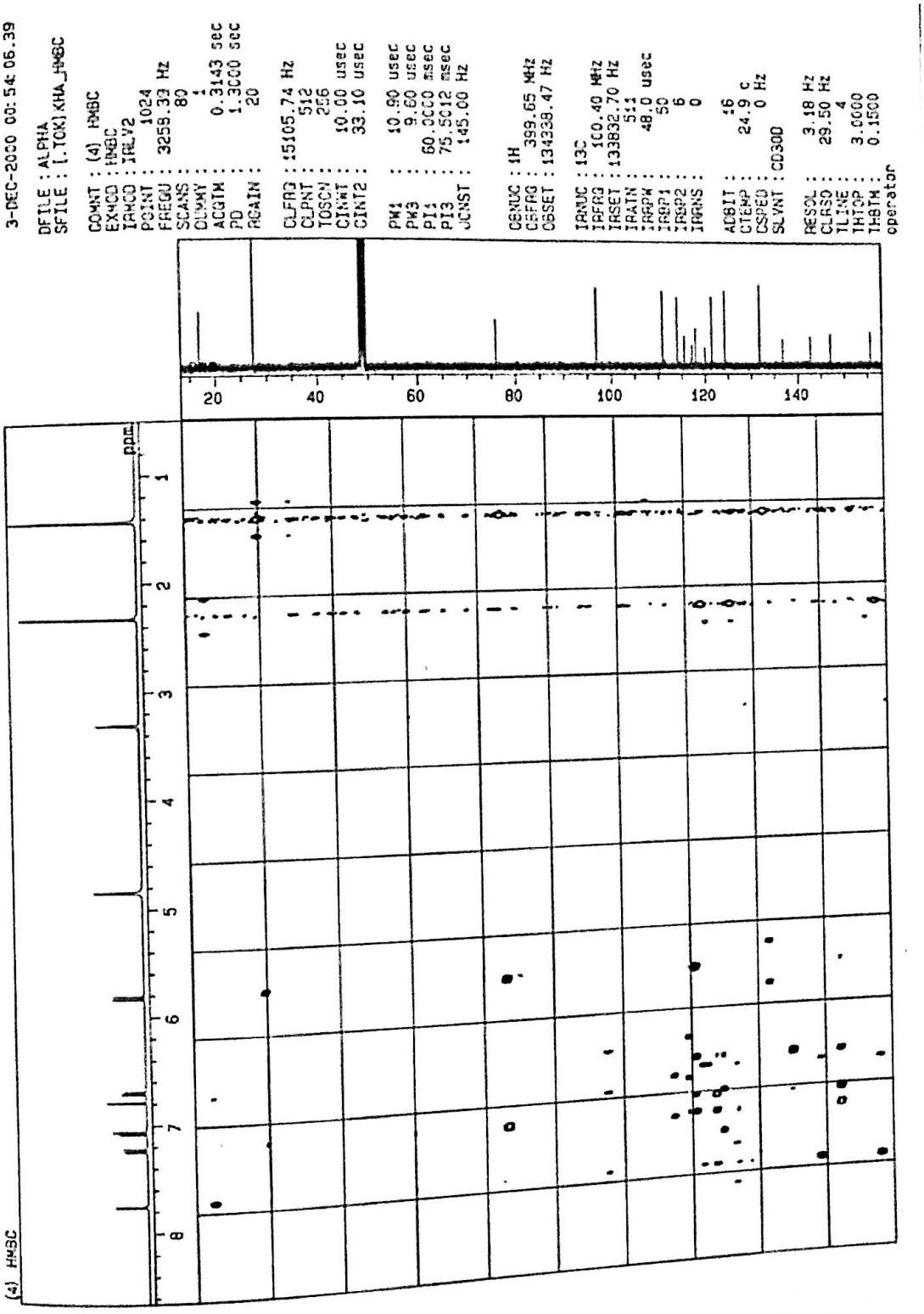


Fig. 17: HMBC of the compound GP2

3.7. Biological activity of the purified compounds

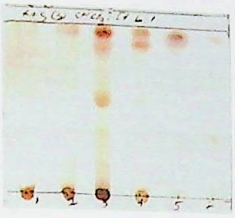
Both the purified compounds of *G. pentaphylla* roots were active against Gram positive and Gram negative bacteria, while the result is presented in Table 14a and Table 14b. The compound GP1 (Glycozoline) offered inhibition zones of 10-, 22-, 25-, 11-, 9-, 9-, 10-, 15-, 19-, 12- and 27 mm respectively and the compound GP2 (Glycoborinine) offered inhibition zones of 9-, 13-, 22-, 11-, 10-, 11-, 15-, 12-, 14-, 12- and 25 mm respectively for *S. aureus, B. megaterium, B. subtilis, S. lutea* and *S.-β-haemolyticus*) and Gram negative bacteria (*S. typhi, S. dysenteriae, S. sonnei, S. boydii, E. coli* and *P. aeruginosa* respectively.

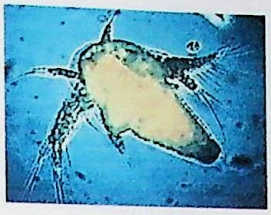
Table 14a: Antibacterial activity of glycozoline and glycoborinie isolated from the roots of *G. pentaphylla*

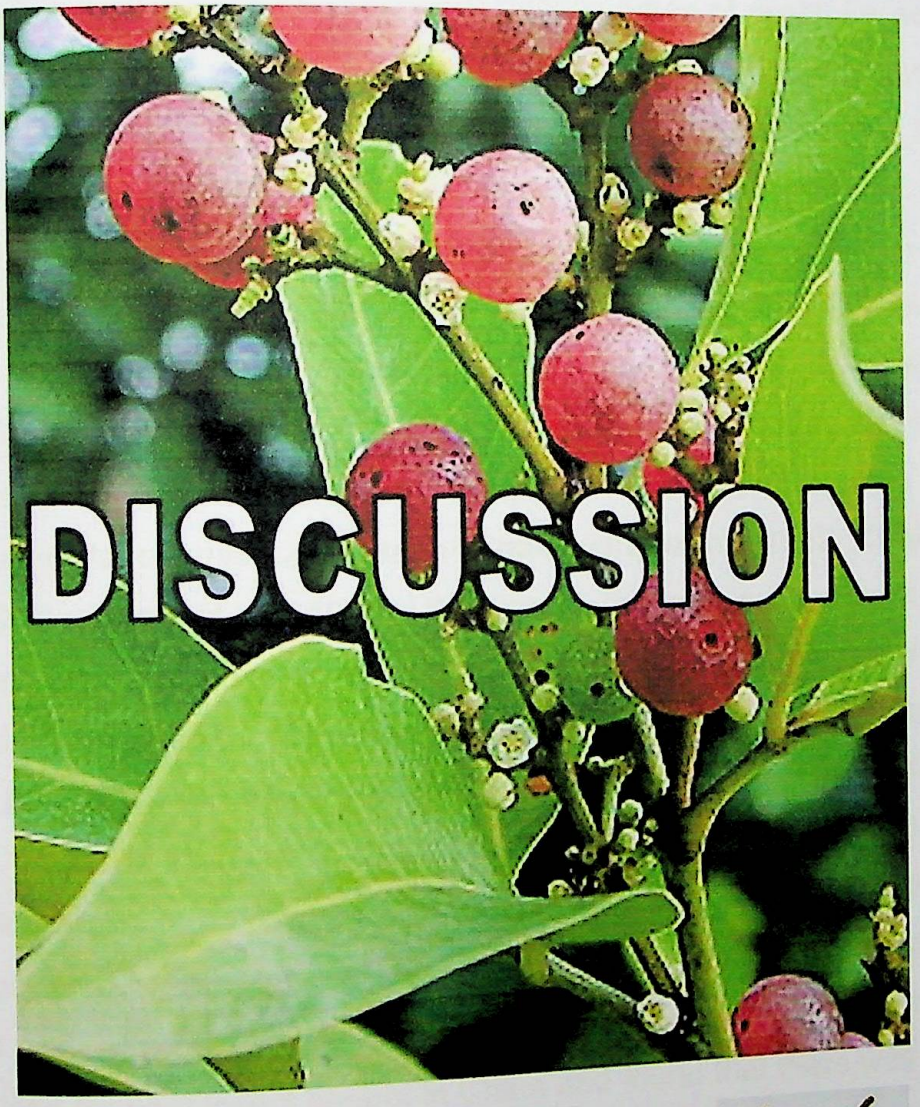
Sl.	Name of root compounds	Inhibition zone (mm)				
		Gram positive				
		S. aureus	*B. magatorium*	*B. subtilis*	*S. lutea*	*S.- β - haemolyticus*
1	GP1	10	22	25	11	9
2	GP2	9	13	22	11	10
Standard Ciprofloxacin		31	28	33	30	31

Table 14b: Antibacterial activity of glycozoline and glycoborinie isolated from the roots of *G. pentaphylla*

Sl.	Name of root compounds	Inhibition zone (mm)					
		Gram negative					
		S. typhi	*S. dysenteriae*	*S. sonnei*	*S. boydii*	*E. coli*	*P. aeruginosa*
1	GP1	9	10	15	19	12	27
2	GP2	11	15	12	14	12	25
Standard Ciprofloxacin		35	31	33	34	33	31



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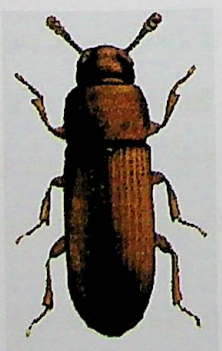


4. Discussion

Having information through search of literature the plant *G. pentaphylla* was found promising and was taken into consideration. A thorough investigation was carried out to justify its potentials in the field of crop protection going through analyses of its efficacy against various types of pests, i.e. terrestrial insects (both adult and larval forms), aquatic animal, as well as, pathogenic bacteria (both Gram positive and Gram negative) and pathogenic fungi for insecticidal, larvicidal, cytotoxic, repellent and antimicrobial activities. Shapiro (1991) mentioned that the roots of the family Rutaceae contain several classes of natural products that exhibit insecticidal or other biological activity, among those coumarines, alkaloids, amides, flavonoids, limonoids, and terpenoids are well known. The Genus has provided various alkaloids belonging to furoquinoline, acridone, quinazolone and carbazole groups, which are of taxonomic significance (Chakravarty *et al.*, 1953; Chakraborty, 1969; Chakraborty *et al.*, 1974). Species in the genus *Glycosmis* (Stone, 1985) contain a wide variety of compounds with potential biological activity, that include terpenoids (Chakravarty *et al.*, 1996), amides (Greger *et al.*, 1992; 1993a,b,; 1994, 1996; Hofer *et al.*, 1995a, 1998), imides (Hofer *et al.*, 1995b), alkaloids (Wu *et al.*, 1983; Wurz *et al.*, 1993; Ono *et al.*, 1995), coumarins (Rahmani *et al.*, 1998), and flavonoids Compounds exhibiting antifungal and insecticidal activities (Greger *et al.*, 1996) have already been isolated from several *Glycosmis* species. Chinese researchers reviewed the plants used for pest insect control in China and found that there was a strong connection between medicinal and pesticidal plants (Jembere *et al.*, 1995). So, being a medicinal plant and originally being confined to a certain area of Asia and Australia this plant deserves attention to be investigated especially for its bioactive potentials.

In the antimicrobial activity test both the antibacterial and antifungal tests offered promising outputs and the results were supported by the results achieved by the previous workers (Greger, 1993) dealt with this plant. In the

disc diffusion method the leaf extract gave the inhibition zones 17-, 16-, 16-, 14-, 16-, 20-, 12- and 7 mm for *B. megaterium, B. subtilis, S. lutea, S. - β-haemolyticus, S. typhi, S. sonnei, S. boydii* and *P. aeruginosa* respectively, while in comparison the inhibition zones for the standard were 28-, 33- 30-, 31-, 35-, 33-, 34- and 31 mm respectively. For the stem bark extract the inhibition zones were 14-, 15-, 15-, 11-, 15-, 10- and 10 mm for *S. aureus, B. megaterium, S. lutea, S. typhi, S. sonnei, S. boydii* and *P. aeruginosa* respectively, while the same for the standard were 31-, 28-, 30-, 35-, 33-, 34-, and 31 mm for the same test agents respectively. For the stem wood extract the inhibition zones were 7-, 14-, 12-, 10- and 12 mm respectively for *B. megaterium, B. subtilis, S.- β-haemolyticus, S. boydii* and *P. aeruginosa,* while the inhibition zones for the standard were 28-, 33-, 31-, 34- and 31 mm; and for the root extract the inhibition zones were 9-, 9-, 10-, 8- and 7 mm for *S. aureu, B. megaterium, S. sonnei, S. boydii* and *P. aeruginosa* respectively, while the inhibition zones for the standard were 31-, 28, 33-, 34- and 31 mm for the above mentioned test agents respectively. Many previous works support this output. Arborinine, an acridone alkaloid obtained from *G. pentaphylla*, exhibited significant inhibition of crown gall tumors produced by *Agrobacterium tumefaciens* in a potato disc bioassay.

In the antifungal activity tests the leaf extract (chloroform) of *G. pentaphylla* offered promising activity, while the inhibition zones were 20 mm for *A. fumigatus and Mucor* sp. after 24 h of exposure, however both of them remained responsive after 48 h with 12 mm of the inhibition zones; while the inhibition zones for the standard were 32- and 30 mm for the above mentioned test fungi respectively. For the stem bark extract the inhibition zones 19-, 7-, 17- and 12 mm for *A. fumigatus, A. flavus, Mucor* sp. and *C. albicans* after 24 h of exposure, however after 48 h only the *A. fumigatus, Mucor* sp. and *C. albicans* remained responsive to the same with 12-, 13- and 9 mm of inhibition zones; while the inhibition zones for the standard were 32, 28-, 30-and 31mm for the above mentioned test fungi. The stem wood extract offered inhibition zones 7-, 12-, 10-, 17- and 10 mm after 24 h of exposure

against *F. vasinfectum, A. fumigatus, A. flavus, Mucor* sp. and *C. albicans,* among them only the *Mucor* sp. remained responsive to the same with 13 mm of the inhibition zone after 48 h of exposure; while the inhibition zones for the standard were 29-, 32-, 28-, 30- and 31 mm for the above mentioned test fungi. For the root extract the inhibition zones were 7-, 15- and 12 mm for *F. vasinfectum, A. fumigatus* and *Mucor sp.* after 24 h of exposure, however after 48 h the *A. fumigatus and Mucor* sp. remained responsive to the same with 9 mm of the inhibition zones; while the inhibition zones for the standard were 29-, 32-, 31- and 30 mm for the above mentioned test fungi respectively.

In support of the antimicrobial activity test carried out in this investigation several previous works have been found. The leaves of this plant are good antidotes for eczema and other skin diseases. Moreover, the folk use of its leaves, stem-bark or twigs to heal up wounds is still prevalent in Bangladesh. Of course, the information indicates presence of antifungal properties in this plant. Three amides, illukumbin B, methylillukumbin B and methylillukumbin A, have been isolated from *Glycosmis* spp. that show different antifungal activities in bioautographic tests and appear to be accumulated mainly in individuals collected from more humid and elevated habitats (Greger, 1993). Antifungal compounds were identified as flindersine and desmethoxyanthophylline and their structures were determined by spectroscopic methods and comparison with reported data. The antifungal activities of *Glycosmis calcicola* and *G. rupestris* extracts have been traced successfully. Insecticidal and antifungal activities have been attributed to various bioactive amides from *Glycosmis* sp. and, specifically, to dehydrothalebanin B in preliminary tests (Greger *et al.*, 1996).

It is essential to indicate here that the crude extracts showed cytotoxic activity while tested on the brine shrimp nauplii, *A. salina.* The LC_{50} values established were 28.579-, 28.659-, 57.213- and 84.111ppm for leaf, stem bark, stem wood and root (root bark and root wood were not separated) extracts respectively; while the efficacy could be arranged in a descending order

Leaf>stem bark> stem wood> root. Findings of Muthukrishnan and his group (Muthukrishnan *et al.,* 1999) resembles with this result, while the addition of 10±4 to 10±5 M the quinazolone of arborine isolated from the ethyl acetate fraction of *G. pentaphylla* leaf extract to water resulted in 83 to 100% mortality of *C. quinquefasciatus* larvae.

Since only the root extract was found active in the *Ad hoc* experiment it was subjected to dose-mortality assay on *T. castaneum* larvae. All through the experiment almost weak mortality was traced, however, no useful data was able to read and subject to analysis. Whereas, moulting was delayed and thus prolongation of the instars took place. It is may be because of the reaction of the extracts with the ecdysone (the moulting hormone) to disrupt the normal rhythm of metamorphosis and development. As the tests on the adults carried out prior to the experiments done on the larvae the observation made continuous instar after instar until the emergence of the adults in the control to see what happens to its growth and development, and a growth inhibiting activity was found so far. Larvicidal assay on *M. domestica* larvae didn't offer any mortality too, while prolongation of the developmental period was traced so far.

Many previous works support findings of the present work. Exposure to partially purified leaf extract of *G. pentaphylla* in the medium extended the larval duration, inflicted very high larval mortality and induced developmental deformities in larvae of mosquitoes, *C. quinquefasciatus, Anopheles stephensi,* and *Aedes aegypti* (Pushpalatha and Muthukrishnan, 1995). Oral administration of *G. pentaphylla* leaf extract to the penultimate and final instar larvae of the castor semilooper, *Achaea janata*, decreased the weight gain and inhibited larval±pupal and pupal±adult metamorphosis, respectively (Muthukrishnan and Ananthagowri, 1994). Ethyl acetate extracts of *G. pentaphylla* leaves significantly reduced hatchability of *C. quinquefasciatus* eggs, prolonged developmental duration of mosquito larvae, reduced the

number of adults emerged and induced malformation during development and metamorphosis at sublethal concentrations (2±50 ppm) (Naik *et al.*, 1979).

However, repellents, fumigants, feeding deterrents, growth inhibitors and insecticides of natural origin are rational alternatives to synthetic insecticides *G. pentaphylla* draws a special attention for its low lethal and other environment-friendly activities. Of course, this investigation reveals its special type of potentials that the chloroform extracts of the different parts of this plant shows repellent activity. The F values have been established through ANOVA with the arcsin transformed data were 60.983, 14.177, 19.437, 15.429 and 1.082 for the analysis between doses and 2.52, 1.806, 1.314, 3.468 and 1.272 for the analysis between time interval for leaf, stem bark, stem wood, root bark and root wood extracts respectively. Except the root extract strong repellent activity was found, while repellency due to differences between doses were highly significant ($P<0.001$). The P values were established as 2.03E-11, 5.41E-6, 4.62E-7, 2.48E-6 and 0.4002 for the analysis between doses and 0.0733, 0.1673, 0.2989, 0.0262 and 0.3139 for the analysis between time interval for the leaf, stem bark, stem wood, root bark and root wood extracts respectively, and thus the intensity of repellent activity could be arranged in a descending order of leaf>stem wood>root bark>stem bark. This finding has been supported by Chopra *et al.* (1956) who mentioned that the leaves of this plant are used to keep insects away from sweets and other edible items are taken by natives in India and Australia.

Anyway, the results indicate that the test plant possess a very special type of potentiality other than killing of organisms in the bioassays. The results revealed that this plant opens no mentionable insecticidal value since very less or no mortality was recorded in case of the treatments carried out against the housefly, *M. domestica* larvae; the rust red flour beetle, *T. castaneum* adults and the lesser mealworm, *Alphitobius diaperinus* adults; while a very weak activity was traced against the *T. castaneum* larvae and against the cowpea weevil *C. maculatus* adults. The LD_{50} values for root extract against

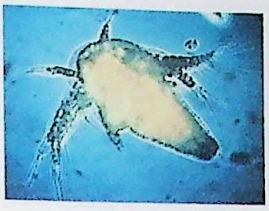
C. maculatus were 1425.036-, 213.523- and 91.782 µg/cm^2 for 24, 48 and 78 hours of exposure. It is now possible to have background to take into consideration the claims made by Shapiro (1991), Greger *et al.* (1996), Pushpalatha, 1997 and Muthukrishnan *et al.* (1999) that *G. pentaphylla* have insecticidal potentials, may on the other test insects. It is also necessary to mention here that, there are some misinformation printed in the ancient texts. For example, the wood ground with water and administered internally as an antidote for snake-bite, however, later on it was found not to act as an antidote in snake-bite (Kirtikar and Basu, 1935). Furthermore, It is possible not to have any promising insecticidal potential, since the name of this plant has been enlisted in the *Sturtevant's Edible Plants of the World*. While it is very common in the region of the world where the plant grows children used to eat its ripe fruits. This Asiatic tree is noted for the delicious flavor of its sweet fruit. Of course, it is an age-old practice in Bangladesh and in West Bengal of India to use branches of this shrub as toothbrush on account of their fibrous nature and slightly astringent, bitter taste. Their constant use not only makes the teeth clean but also keeps them strong (Kirtikar and Basu, 1935).

From the above mentioned comments regarding the findings of the present work that supported by the previous workers it is possible to come to a conclusion that that this plant contains components with very low-mammalian toxicity, but with promising potentials to control insect pests by making changes in the rhythm of their growth and development, there by reducing the number of the pest populations; and this is rather more environment friendly. Being very low in mammalian toxicity it is safe to use for the protection of the stored products. This opinion is being supported by the findings of many previous workers who claimed growth-inhibiting activity against the larvae of *Diaprepes abbreviatus* has been found in both live and milled roots from seedlings of two citrus relatives, *G. pentaphylla* or orangeberry (Shapiro *et al.*, 1997; Bowman *et al.*, 1999; Lapointe and Shapiro, 1999) and *Murraya koenigii* (Lapointe and Shapiro, 1999). A screening of foliage against the

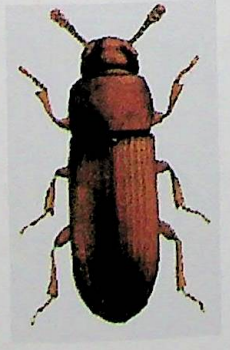
citrus leafminer, *Phyllocnistis citrella*, demonstrated activity in both *M. koenigii* and *G. pentaphylla* (Jacas *et al.*, 1997). There are many other supporting findings in this regard. Manipulation of endocrine control of development, growth, metamorphosis and reproduction is one of the mechanisms of action of these secondary plant substances (Richter and Koolman, 1991; Couillaud, F. and Peypelut, 1995). A few plant species, such as, *Calophyllum inophyllum* Linn. (Clusiaceae), *G. pentaphylla* (Retz.) DC. (Rutaceae),*Rhinacanthus nasutus* Kurz. (Acanthaceae), *Solanum surratense* Burm. (Solanaceae) and *S. trilobatum* Linn. (Solanaceae) had been screened for their insecticidal activity in the laboratories (Pushpalatha, 1997; Muthukrishnan *et al.*, 1999).

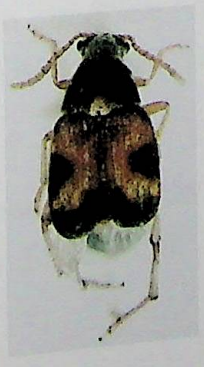
However, according to the facilities available there in the Crop Protection Laboratory and other accessible laboratories of the Institute of Biological Sciences, Department of Biochemistry and Molecular Biology, Bangladesh Council of Scientific and Industrial Research, Rajshahi, sophistication of this work was not possible. The main target of this research revolves no doubt in tracing out and isolate bioactive potentials of the test plant, and it offered two pure compounds GP1 (12 mg) and GP2 (14mg) which were glycozoline and glycoborinine respectively. Due to insufficiency in amount it was impossible to go through all the biological assays (used in this investigation) for these two pure compounds, while only antibacterial test was possible to carry out. These two compounds found active against the test bacteria used in this investigation.

The findings of the work along with the findings of the previous researchers triggered a hope for further progress in research with this promising plant *G. pentaphylla* towards a molecular level investigation of its pesticidal potentials for an environment friendly protection of crops and stored products.



LITERATURE CITED





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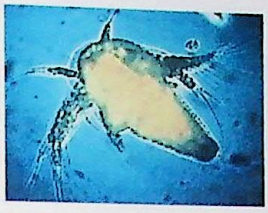

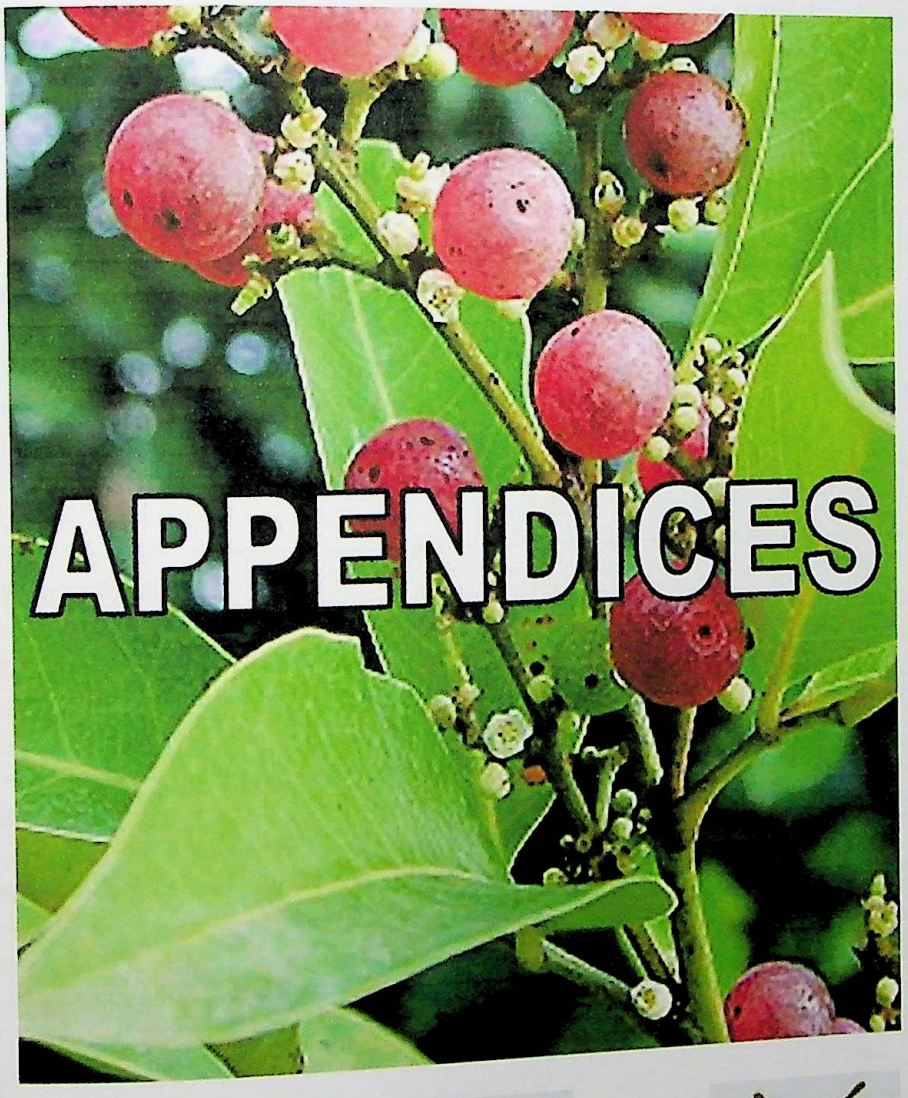
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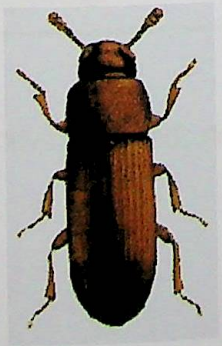

Appendix Table II: Antibacterial activity of the stem bark extract of *G. pentaphylla* and the standard Ciprofloxacin.

Test organisms	Diameter of zone of inhibition (in mm)	
	200 µg/disc	Ciprofloxacin 30 µg/disc
Gram positive bacteria		
S. aureus	14	31
B. megaterium	15	28
B. subtilis	-	33
S. lutea	15	30
S. - β -haemolyticus	-	31
Gram negative bacteria		
S. typhi	11	35
S. dysenteriae	-	31
S. sonnei	15	33
S. boydii	10	34
E. coli	-	33
P. aeruginosa	10	31

(-) No activity traced; numerical value presents diameter of clear zones in mm.

Appendix Table III: Antibacterial activity of the stem wood extract of *G. pentaphylla* and the standard Ciprofloxacin.

Test organisms	Diameter of zone of inhibition (in mm)	
	200 µg/disc	Ciprofloxacin 30 µg/disc
Gram positive bacteria		
S. aureus	-	31
B. megaterium	7	28
B. subtilis	14	33
S. lutea	-	30
S.- β –haemolyticus	12	31
Gram negative bacteria		
S. typhi	-	35
S. dysenteriae	-	31
S. sonnei	-	33
S. boydii	10	34
E. coli	-	33
P. aeruginosa	12	31

(-) No activity traced; Numerical value presents diameter of clear zones in mm

Appendix Table IV: Antibacterial activity of the root extract of *G. pentaphylla* and the standard Ciprofloxacin.

Test organisms	Diameter of zone of inhibition (in mm)	
	200 µg/disc	Ciprofloxacin 30 µg/disc
Gram positive bacteria		
S. aureus	9	31
B. megaterium	9	28
B. subtilis	-	33
S. lutea	-	30
S.- β -haemolyticus	-	31
Gram negative bacteria		
S. typhi	-	35
S. dysenteriae	-	31
S. sonnei	10	33
S. boydii	8	34
E. coli	-	33
P. aeruginosa	7	31

(-) No activity traced; Numerical value presents diameter of clear zones in mm

Appendix Table V: Antifungal activity of the leaf extract of *G. pentaphylla* and the standard Nystatin

Test fungi	Diameter of zone of inhibition (in mm)		
	Chloroform extract (500µg/disc)		Nystatin 50µg/disc
	After 24 h	After 48	
F. vasinfectum	-	-	29
A. fumigatus	20	12	32
A. flavus	-	-	28
Mucor sp.	20	12	30
C. albicans	-	-	31

(-) No activity traced; Numerical value presents diameter of clear zones in mm

Appendix Table VI: Antifungal activity of the stem bark extract of *G. pentaphylla* and the standard Nystatin

Test fungi	Diameter of zone of inhibition (in mm)		
	Chloroform extract (500µg/disc)		Nystatin 50µg/disc
	After 24 h	After 48	
F. vasinfectum	-	-	29
A. fumigatus	19	12	32
A. flavus	7	-	28
Mucor sp.	17	13	30
C. albicans	12	9	31

(-) No activity traced; Numerical value presents diameter of clear zones in mm

Appendix Table VII: Antifungal activity of the stem wood extract of *G. pentaphylla* and the standard Nystatin

Test fungi	Diameter of zone of inhibition (in mm)		
	Chloroform extract (500µg/disc)		Nystatin 50µg/disc
	After 24 h	After 48	
F. vasinfectum	7	-	29
A. fumigatus	12	-	32
A. flavus	10	-	28
Mucor sp.	17	13	30
C. albicans	10	-	31

(-) No activity traced; Numerical value presents diameter of clear zones in mm

Appendix Table VIII: Antifungal activity of the root extract of *G. pentaphylla* and the standard Nystatin

Test fungi	Diameter of zone of inhibition (in mm)		
	Chloroform extract (500µg/disc)		Nystatin 50µg/disc
	After 24 h	After 48	
F. vasinfectum	7	-	29
A. fumigatus	15	9	32
A. flavus	-	-	28
Mucor sp.	12	9	31
C. albicans	-	-	30

(-) No activity traced; Numerical value presents diameter of clear zones in mm

Appendix Table IX: Probit mortality of *A. salina* by $CHCl_3$ extract of leaves of *G. pentaphylla* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr%	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.000	2.000	30	29	96.667	95	6.64	7.111	6.335	3.30	7.204
50.000	1.699	30	28	93.333	91	6.34	5.927	6.288	14.13	5.984
25.000	1.398	30	17	56.667	41	4.77	4.742	4.766	18.48	4.764
12.500	1.097	30	9	30.000	5	3.36	3.557	3.365	8.07	3.545

Results:
Y = -.9008684 + 4.052676 X
Chi-squared is 4.056469 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.456042
LD_{50} is 28.5787 µg/insect
95% confidence limits are 24.13588 to 33.83931 µg/insect

Appendix Table X: Probit mortality of *A. salina* by $CHCl_3$ extract of stem bark of *G. pentaphylla* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr%	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
100.000	2.000	30	28	93.333	87	6.13	6.267	6.077	11.10	6.252
50.000	1.699	30	27	90.000	80	5.85	5.577	5.780	17.43	5.557
25.000	1.398	30	21	70.000	40	4.75	4.887	4.760	18.81	4.863

Results:
Y = 1.638829 + 2.306482 X
Chi-squared is 1.402584 with 1 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.457272
LD_{50} is 28.65971 µg/insect
95% confidence limits are 19.8311 to 41.41873 µg/insect

Appendix Table XI: Probit mortality of *A. salina* by $CHCl_3$ extract of stem-wood of *G. pentaphylla* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr%	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
250.000	2.398	30	29	96.667	95	6.64	7.266	6.190	2.76	7.345
125.000	2.097	30	29	96.667	95	6.64	6.200	6.500	12.15	6.243
62.500	1.796	30	23	76.667	63	5.33	5.134	5.315	19.02	5.140
31.250	1.495	30	13	43.333	11	3.77	4.067	3.791	13.17	4.038

Results:
Y = -1.436301 + 3.662196 X
Chi-squared is 5.870407 with 2 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.757498
LD_{50} is 57.21342 µg/insect
95% confidence limits are 47.53493 to 68.86255 µg/insect

Appendix Table XII: Probit mortality of *A. salina* by $CHCl_3$ extract of roots of *G. pentaphylla* after 24 h of exposure.

Dose	Log dose	# Ud	# Kl	% Kill	Cr%	E Pro	Ex Pro	Wk Pro	Weight	Final Pro
250.000	2.398	30	25	83.333	76	5.71	5.964	5.718	14.13	5.981
125.000	2.097	30	24	80.000	71	5.55	5.331	5.526	18.48	5.357
62.500	1.796	30	19	63.333	48	4.95	4.698	4.956	18.03	4.732
31.250	1.495	30	12	40.000	14	3.92	4.065	3.914	13.17	4.108
15.625	1.194	30	10	33.333	5	3.36	3.432	3.360	7.14	3.483

Results:
Y = 1.006926 + 2.074481 X
Chi-squared is 3.013783 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.924855
LD_{50} is 84.11138 µg/insect
95% confidence limits are 64.83567 to 109.1177 µg/insect

Appendix Table XIII: Repellency of *T. castaneum* adults by chloroform extract of stem bark, root, root bark, Leaf & stem wood of *G. pentaphylla*.

Type of Extracts	Dose ($\mu g/cm^2$)	Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin transformations data				
		1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
Leaf	78.634	9.33	9.33	9.00	9.66	9.66	86.6	86.6	80.0	93.2	93.2	68.53	68.53	63.44	74.88	74.88
	39.317	9.00	9.66	7.33	7.33	7.00	80.0	93.2	46.6	46.6	40.0	63.44	74.88	43.05	43.05	39.23
	19.659	6.00	6.66	7.00	7.33	6.66	20.0	33.2	40.0	46.6	33.2	26.56	35.18	39.23	43.05	33.18
	9.829	8.00	7.00	7.00	6.33	6.00	60.0	40.0	40.0	26.6	20.0	50.77	39.23	39.23	31.05	26.56
	4.915	5.33	3.66	4.33	3.66	3.66	6.6	-26.8	-13.4	-26.8	-26.8	14.89	-31.18	-21.47	-31.18	-31.18
	2.457	4.66	3.66	4.00	3.00	1.33	-6.8	-26.8	-20.0	-40.0	-73.4	-15.12	-31.18	-26.56	-39.23	-58.95
Stem bark	78.634	7.00	7.00	4.33	4.00	7.66	40.0	40.0	-13.4	-20.0	53.2	39.23	39.23	-21.47	-26.56	46.83
	39.317	8.33	8.00	4.33	7.66	6.33	66.6	60.0	-13.4	53.2	26.6	54.70	50.77	-21.47	46.83	31.05
	19.659	7.00	7.00	6.66	5.66	6.66	40.0	40.0	33.2	13.2	33.2	39.23	39.23	35.18	21.30	35.18
	9.829	3.66	3.00	3.33	4.00	4.66	-26.8	-40.0	-33.4	-20.0	-6.8	-31.18	-39.23	-35.30	-26.56	-15.12
	4.915	4.00	4.66	4.00	3.00	2.66	-20.0	-6.8	-20.0	-26.8	-46.8	-26.56	-15.12	-26.56	-31.18	-43.17
	2.457	2.66	3.00	3.00	3.66	4.00	-46.8	-40.0	-40.0	-26.8	-20.0	-43.17	-39.23	-39.23	-31.18	-26.56
Wood	78.634	7.66	7.33	6.66	7.66	7.33	53.2	46.6	33.2	53.2	46.6	46.83	43.05	35.18	46.83	43.05
	39.317	5.33	3.66	4.33	3.66	3.66	6.6	-26.8	-13.4	-26.8	-26.8	14.89	-31.18	-21.47	-31.18	-31.18
	19.659	4.66	5.33	5.66	4.00	1.66	-6.8	6.6	13.2	-20.0	-66.8	-15.12	14.89	21.30	-26.56	-54.82
	9.829	7.00	7.33	7.00	6.00	6.33	40.0	46.6	40.0	20.0	26.6	39.23	43.05	39.23	26.56	31.05
	4.915	3.66	3.33	4.00	3.33	4.33	-26.8	-33.4	-20.0	-33.4	-13.4	-31.18	-35.30	-26.56	-35.30	-21.47
	2.457	5.00	4.00	3.66	4.33	4.33	00.0	-20.0	-26.8	-13.4	-13.4	00	-26.56	-31.18	-21.47	-21.47
Root bark	78.634	10.0	10.0	10.0	10.0	10.0	100	100	100	100	100	-	-	-	-	-
	39.317	9.00	9.00	8.66	5.66	5.66	80.0	80.0	73.2	13.2	13.2	63.44	63.44	58.82	21.30	21.30
	19.659	9.00	8.33	7.00	7.33	7.00	80.0	66.6	40.0	46.6	40.0	63.44	54.70	39.23	43.05	39.23
	9.829	6.00	5.33	5.33	4.66	4.33	20.0	6.6	6.6	-6.8	-13.4	26.56	14.89	14.89	-15.12	-21.47
	4.915	7.00	7.33	6.00	4.66	4.33	40.0	46.6	20.0	-6.8	-13.4	39.23	43.05	26.56	-15.12	-21.47
	2.457	3.66	3.33	4.00	3.33	5.00	-26.8	-33.4	-20.0	-33.4	00	-31.18	-35.30	-26.56	-35.30	-
Root wood	78.634	8.33	9.66	9.33	10.00	9.66	66.6	93.2	86.6	100.0	93.2	54.70	74.88	68.53	-	74.88
	39.317	9.00	9.66	9.66	9.66	9.33	80.0	93.2	93.2	93.2	86.6	63.44	74.88	74.88	74.88	68.53
	19.659	8.00	9.66	9.66	9.66	10.00	60.0	93.2	93.2	100.0	100.0	50.77	74.88	74.88	-	-
	9.829	7.00	7.66	8.66	8.66	8.66	40.0	53.2	73.2	80.0	73.2	39.23	46.83	58.82	63.44	58.82
	4.915	8.33	7.33	9.00	9.00	9.00	66.6	46.6	80.0	66.6	80.0	54.70	43.05	63.44	54.70	63.44
	2.457	6.33	6.66	8.66	8.66	8.33	26.6	33.2	73.2	53.2	66.6	31.05	35.18	58.82	46.89	54.70

Appendix Table XIV: ANOVA results of repellency by chloroform extract of leaf, stem bark, stem wood, root bark and root wood of *G. pentaphylla* against *T. castaneum* adults.

Leaves					
Source of variation	SS	df	MS	F	P-value
Between doses	42904.49	5	8580.897	60.98325***	2.03E-11
Between hourly observation	1419.201	4	354.8002	2.521516	0.073346
Error	2814.182	20	140.7091		
Total	47137.87	29			
Stem bark					
Source of variation	SS	df	MS	F	P-value
Between doses	26948.51	5	5389.703	14.17715***	5.41E-06
Between hourly observation	2745.829	4	686.4572	1.805666	0.167365
Error	7603.368	20	380.1684		
Total	37297.71	29			
Stem wood					
Source of variation	SS	df	MS	F	P-value
Between doses	24779.38	5	4955.876	19.43674***	4.62E-07
Between hourly observation	1339.773	4	334.9431	1.313633	0.298914
Error	5099.493	20	254.9747		
Total	31218.64	29			
Root bark					
Source of variation	SS	df	MS	F	P-value
Between doses	20117.29	5	4023.457	15.42936***	2.48E-06
Between hourly observation	3617.81	4	904.4524	3.46844*	0.026234
Error	5215.327	20	260.7663		
Total	28950.42	29			
Root					
Source of variation	SS	df	MS	F	P-value
Between doses	2576.381	5	515.2762	1.081782	0.400282
Between hourly observation	2423.457	4	605.8643	1.271965	0.313922
Error	9526.433	20	476.3217		
Total	14526.27	29			

*** = P<0.001, * = P<0.05

Appendix Table XV: Probit mortality of *C. maculatus* by $CHCl_3$ extract of roots of *G. pentaphylla* after 24 h of exposure.

Dose µg/cm^2	Log dose	# Used	# Kill	% Kill	Corr %	Emp Probit	Expt Probit	Work Probit	Weight	Final Probit
786	2.895	30	15	50.000	50	5.00	4.903	4.990	19.02	4.912
393	2.594	30	14	46.667	47	4.92	4.802	4.942	18.81	4.809
197	2.294	30	8	26.667	27	4.39	4.702	4.402	18.48	4.706
98	1.991	30	10	33.333	33	4.56	4.601	4.551	18.03	4.602
49	1.690	30	10	33.333	33	4.56	4.500	4.570	16.74	4.499
25	1.398	30	9	30.000	30	4.48	4.402	4.480	16.74	4.399

Results:
Y = 3.921 + 0.342 X
Chi-squared is 2.40 with 4 degrees of freedom
No significant heterogeneity
LOG LD-50 IS 3.153826
LD_{50} is 1425.036 µg/cm^2
95% confidence limits are 90.16734 to 22521.76

Appendix Table XVI: Probit mortality of *C. maculatus* by $CHCl_3$ extract of roots of *G. pentaphylla* after 48 h of exposure.

Dose µg/cm^2	Log dose	# Used	# Kill	% Kill	Corr %	Emp Probit	Expt Probit	Work Probit	Weight	Final Probit
786	2.895	30	20	66.667	66	5.41	5.277	5.436	18.81	5.289
393	2.594	30	17	56.667	55	5.13	5.129	5.115	19.02	5.135
197	2.294	30	13	43.333	41	4.77	4.981	4.765	19.02	4.982
98	1.991	30	13	43.333	41	4.77	4.831	4.786	18.81	4.827
49	1.690	30	13	43.333	41	4.77	4.683	4.767	18.03	4.673
25	1.398	30	11	36.667	34	4.59	4.539	4.572	17.43	4.524

Results:
Y = 3.809 + 0.511 X
Chi-squared is 1.54 with 4 degrees of freedom
No significant heterogeneity
LOG LD-50 IS 2.329444
LD_{50} is 213.523 µg/cm^2
95% confidence limits are 88.21113 to 516.8505

Appendix Table XVII: Probit mortality of *C. maculatus* by $CHCl_3$ extract of roots of *G. pentaphylla* after 72 h of exposure.

Dose ng/cm^2	Log dose	# Used	#Kill	% Kill	Corr%	Emp Probit	Expt Probit	Work Probit	Weight	Final Probit
786	2.895	30	23	76.667	76	5.71	5.723	5.702	15.96	5.712
393	2.594	30	23	76.667	76	5.71	5.492	5.672	18.03	5.482
197	2.294	30	16	53.333	52	5.05	5.262	5.072	18.81	5.253
98	1.991	30	14	46.667	45	4.87	5.029	4.875	19.11	5.022
49	1.690	30	15	50.000	48	4.95	4.799	4.948	18.48	4.792
25	1.398	30	11	36.667	34	4.59	4.574	4.572	17.43	4.569

Results:
Y = 3.503 + 0.763 X
Chi-squared is 2.13 with 4 degrees of freedom
No significant heterogeneity
Log ld-50 is 1.962756
LD_{50} is 91.782 µg/cm^2
95% confidence limits are 50.31486 to 167.4234