PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION
OF *Vitex peduncularis* Wall.

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BY
MD. EYAZUL HAQUE
STUDENT NO: 0412033112F
REGISTRATION NO: 0412033112
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DEPARTMENT OF CHEMISTRY
BANGLADESH UNIVERSITY OF ENGINEERING AND TECHNOLOGY (BUET)
DHAKA-1000, BANGLADESH
DECEMBER, 2016
DEDICATED
TO
MY PARENTS

Md. Mofizuddin Mollah
and
Nazma khatun
DECLARATION

This thesis work has been done by the candidate himself and does not contain any material extracted from elsewhere or from a work published by anybody else. The work for this thesis has not been presented elsewhere by the author for any degree or diploma.

______________________________
Md. Eyazul Haque
M. Phil Student
Roll No. 04120331124
Department of Chemistry
BUET, Dhaka-1000
Bangladesh.
Bangladesh University of Engineering and Technology (BUET)
Dhaka-1000, Bangladesh
Department of Chemistry

THESIS ACCEPTANCE LETTER

This thesis titled “PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION OF Vitex peduncularis Wall.” submitted by Md. Eyazul Haque, Roll No. 0412033112F, Session- April, 2012 has been accepted as satisfactory in partial fulfillment of the requirements for the degree of Master of Philosophy (M. Phil) in Chemistry on December 10, 2016.

Board of Examiners

1. Prof. Dr. Shakila Rahman
   Department of Chemistry, BUET
   Dhaka, Bangladesh
   (Supervisor)

2. Dr. Ismet Ara Jahan
   Principal Scientific Officer
   Chemical Research Division
   BCSIR, Dhaka, Bangladesh
   (Co-supervisor)

3. Dr. Md. Rafique Ullah
   Head, Department of Chemistry
   BUET, Dhaka, Bangladesh

4. Prof. Dr. Md. Abdur Rashid
   Department of Chemistry, BUET
   Dhaka, Bangladesh

5. Prof. Dr. S. M. Mizanur Rahman
   Department of Chemistry
   University of Dhaka
   Dhaka, Bangladesh

Chairman

Member

Member (Ex-officio)

Member
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Author
Md. Eyazul Haque
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Abstract

Verbenaceae plants such as *Vitex peduncularis* is enriched with important biologically active compounds. From the profound interest of these compounds and their useful biological activities, the bark of this plant was extracted with methanol. Then the methanol extract was partitioned with *n*-hexane, dichloromethane, ethyl acetate and 1-butanol respectively. The dichloromethane and ethyl acetate fractions were subjected to various chromatographic techniques to isolate the pure compounds for characterization and determination of their structures. Four compounds were purified and the structures were determined. Among them, the compound-1 to compound-3 was isolated from the dichloromethane part and compound-4 was isolated from the ethyl acetate part.

2α, 3β-dihydroxy olean-12-en-27, 28-dioic acid

2α, 3β, 23-trihydroxy olean-12-en-27,28-dioic acid

5-butyl-6-ethyl-3,4,5,10-tetrahydroisochromen-1-one

Methyl 3,4-dihydroxybenzoate
The above structures of the compounds (1-4) were elucidated by various spectroscopic methods (UV, IR, $^1$H-NMR, $^{13}$C-NMR, DEPT-135, COSY-45 and HMBC etc.) According to the reported literature, all of these compounds were found to be isolated for the first time from the plant.

The ethyl acetate part of methanol extract was found to have high antioxidant activity whereas, the n-hexane part of methanol extract showed low antioxidant activity.

Thus the isolation, characterization and investigation of potent antioxidant activity of the crude extracts might generate much synthetic and biological interest in this class of the natural products.
CHAPTER ONE

INTRODUCTION
1.1 General

From the beginning of civilization man has accumulated an amount of knowledge of drugs derived from various plants. Plants have formed the basis for traditional medicine systems which have been used for thousands of years in many countries. These plant-based systems continue to play an important role in health care and it has been estimated by the World Health Organization that majority percent of the world’s inhabitants rely mainly on traditional medicines for their primary health care. Illness, physical discomfort, injuries, wounds and fear of death had forced early man to use any natural substance for relieving the pain and suffering caused by these abnormal conditions and for preserving health against diseases and death. Primitive man started to distinguish nutritional and pharmacologically active plants for their survival. By their experience, this knowledge of herbal remedies was transferred from one generation to another at first orally and later in written from a papyri, backed clay tablets, parchments, manuscripts, pharmacopoeias and other works. Therefore, medicinal plants have been in use for the eradication and human suffering since ancient time. Plant products also play an important role in the health care systems of the remaining percent of the population, mainly residing in developed countries.

As far as record goes, it appears that Babylonians (about 3000 years B.C.) were aware of a large number of medicinal plants and their properties. Some of them are still in use for the same purpose like henbane (Hyoscyamus Spp.), Opium (Papaver somniferum), Castor oil (Ricinus communis), Aloevera (Aloe spp.) etc.

The Chinese have an effective and unique system of medicine. The earliest known Chinese pharmacopoeia, The Pen Tsao, described over 300 medicinal plants and their uses. Although various of several ancient pharmacopoeia still exist today, the main surviving text book is on herbalism written in the 16th century by the physician Li Shih-Cheu describing almost 200 herbs and 10,000 herbal remedies. Today Chinese herbalism is very much an orthodox form of therapy and preventive treatment in China and it is increasingly practiced in the west. Chinese herbalism can be used for a wide range of ailments, including asthma, skin diseases, menstrual problems, digestive disturbances, migraine and is effective when used on its own or in conjunction with another therapy such as acupuncture.

The material media of the great Greek physician Hippocrates (460-370 B.C.) consists of some 300 to 400 medicinal plants which included opium, mint rosemary, sage and verena. In the middle age, the great Greek pharmacist-physician Galen (131-200 A.D.) used a large number of medicinal plants in preparing his recipes.
The Arabian Muslim physicians like Al-Razi and Ibn Sina (9th to 14th century A.D.) brought about a revolution in the medicine by bringing new drugs of plant and mineral origin into general use. Enriching the original Greek system of medicine by introducing these new materials and knowledge they laid down the foundation stone of modern western medicine. The medicinal use of plants in the Indian subcontinent is the Rig Veda (4500 – 1600 BC), which noted that Indo-Aryans used the Soma plant (*Amanita muscaria*) as a medicinal agent. The Vedas made many references to healing plants including sarpagondha (*Rauvolfia serpentine*), while a comprehensive Indian Herbal, the Charaka Samhita, cities more than 500 medicinal plants.

Since disease, decay and death have always co-existed with life, the study of diseases and their treatment must also have been contemporaneous with the dawn of the human intellect. It is apparent that whatever progress science might have made in the field of medicine over the years, plants still remain the primary source of supply of many important drugs used in modern medicine. Indeed, the potential of obtaining new drugs from plant sources is so great that thousands of substances of plant origin are now being studied for activity against such formidable foes as heart diseases, cancer, diabetes and AIDS. This type of study is sure to bring fruitful results, because of the fact that the plant kingdom represents a virtually untapped reservoir of new chemical compounds and it has been estimated that only 5-15% of the approximately 2,50,000-5,00,000 species of higher plants of which more than 80,000 are medicinal has been investigated pharmacologically. Thus there are considerable chances of finding new natural compounds with pharmacological activities, useful for the development of new drugs. Scientists are now working together to find out new drug for incurable diseases. Taxonomist, Chemist, Biochemist, Pharmacologist and Pharmacist are working under collaborative program for making a plant product(s) into a commercial drug.

### 1.2 Medicinal importance of plant materials

Plants not only provide man with food, shelter, and medicine, but also the sustaining oxygen. From ancient time to modern age the human has been successfully used plants and plant products as effective therapeutic tools for fighting against diseases and various health hazards.

Although with the advent of synthetic drugs the use and procurement of plant derived drugs have declined to a large extent, a large number of drugs of modern medicine are obtained from plant sources. According to some generous estimates, almost 80% of the present day medicines are directly or indirectly obtained from plants.
As therapeutic use of plants continued with the progress of civilization and development of human knowledge, scientists endeavored to isolate different chemical constituents from plants, put them to biological and pharmacological tests and thus have been able to identify and isolate therapeutically active compounds, which have been used to prepare modern medicines. In course of time their synthetic analogues have also been prepared. In this way, the discovery of vinblastine and vincristine were done from *Catharanthus roseus*, which is, used in the treatment of cancer. Vinblastine and vincristine are also used in the treatment of Hodgkin’s disease and childhood leukemia, respectively. Both are considered antimitotic drugs because they inhibit cell division. They act by binding to tubulin and preventing it from polymerizing into microtubules. Both treatments are reported to be highly effective.

Paclitaxel, derived from the yew tree, is the first anticancer drug discovered that stabilized microtubules and thus promotes their polymerization. Since its discovery paclitaxel has become a blockbuster drug used in the treatment of lung, ovarian, and breast cancer and Kaposi’s sarcoma.

Calanolide A is a reverse-transcriptase inhibitor isolated from the Malaysian rainforest tree, *Calophyllum Langerum* by the US NCI. It has exhibited synergistic anti-HIV activity in
combination with nucleoside reverse-transcriptase inhibitor, including AZT, ddI and ddC. Medicem pharmaceuticals, Inc., and the state of Sarawak, Malaysia have begun clinical development of Calanolide A as a potential treatment for AIDS and HIV infections.

Ancient Chinese medical texts written 2000 years ago describe the herb *Artemisia annua* as a remedy for malaria. From this indication, in the late 1960s Chinese researchers initiated evaluation of various extracts of this herb. Bioassay guided isolation yielded the new antimalarial compound artemisinin which is effective in treating chloroquine resistant cases and other severe cases without major toxicity.

Artemether, a synthetic analogue of artemisinin has been developed in the People’s Republic of China. Two recent clinical studies suggested that artemether is as effective as quinine in the treatment of severe malaria.

For several centuries, elderly people in some parts of Mainland China have brewed tea from the leaves of the club moss (*Huperzia serrata*) for improvement of their memory. In the early 1980s, Chinese scientist isolated huperzine from the plant Club moss as a potent, reversible and selective inhibitor of acetylcholinesterase. A total synthesis has been developed due to very low levels in nature, and the product is found to be a promising candidate for the treatment of cholinergic related neurodegenerative disorders such as Alzheimer’s disease (AD). In a prospective, multicenter, double-blind trial with 103 patients, huperzine A was found to be safe and superior to placebo and induced improvement in memory cognition and behavior in about 58% of patients with AD.
Galanthamine is a long acting, centrally active competitive cholinesterase inhibitor; a natural product isolated from *Galanthus nivalis* in the 1950s. Galanthamine under the name of Nivalein is marketed in Austria for AD and in Germany for other indication such as facial neuralgia.\(^{11}\)

In the antidiabetes area, the past decade has witnessed the market introduction of several \(\alpha\)-glucosidase inhibitors derived from natural products. Acarbose, a complex oligosaccharide one of them, was isolated from *Actinoplanes* sp. At Bayer from a search for \(\alpha\)-glucosidase enzyme inhibitors. By inhibiting \(\alpha\)-glucosidase, acarbose decreases the release of glucose from ingested carbohydrate and slows the increase of food-induced blood glucose levels. Acarbose is now approved in Germany, Japan, the US and other countries and has been used as adjuvant therapy in diabetes. Forskolin (Colforsin) is a diterpene natural product isolated from the Indian plant *Coleus forskohlii* at Hoechst's research labs in India and have blood pressure lowering and cardioactive properties. Later Forskolin was found as a potent adenylate cyclase activator. Colforsin daprate (NHK-477) is a semisynthetic product of forskolin derivative and was then brought into phase III clinical trials in Japan for treatment of cardiac insufficiency and phase II trials for treatment of asthma.

Triptolide (12) is an active component isolated from the Chinese plant *Tripterygium wilfordii*, a plant traditionally used for the treatment of rheumatoid arthritis. Variety of formulations were developed in Mainland China and are shown to be effective in the
treatment of inflammatory and autoimmune diseases. Triptolide was demonstrated to significantly inhibit arthritis in animal modal and have potent cytotoxicity.

The Chinese tree *Ginkgo biloba* has been used therapeutically for thousands of years. More recently, extracts of the leaves have become available in many European countries as over-the-counter products for the treatment of cerebral vascular insufficiency and tinnitus. Ginkgolides, a class of unique diterpene cage like molecules were isolated from the leaves of *Ginkgo biloba* and represent a group of highly selective platelet activity factor (PAF) receptor antagonists. Among them, Ginkgolide B (BN-52021, 13) has been advanced to phase III clinical trials for the treatment of septic shock in patients with severe sepsis caused by Gram positive bacterial infections and also good result in inflammatory and autoimmune disorders.\(^\text{12}\).

![Structure-13: Ginkgolide B](image1.png) ![Structure -14: Gomisin A](image2.png)

Gomisin A (14) is a lignin derivative isolated from the dry fruit of *Schisondra chinensis*, a traditional Chinese medicine used for the treatment of liver intoxication. Gomisin A was found to be hepatoprotectant and protect liver damage in various animal models.

![Structure-15: Dextromethorphan](image3.png) ![Structure -16: Quinine](image4.png)

Morphine was first isolated by Serturner in 1806 followed by Codeine in 1832 by Robiquet and then the non-morphine alkaloid papaverine by Merck in 1848 from the seeds of *Poppy*. Dextromethorphan is a semisythetic product of morphine used in most cough syrup today.
Two active anti-tumor agents etoposide and teniposide was isolated from the root of various species of the genus *Podophyllum*. These plants possess a long history of medicinal use by early American and Asian cultures, including the treatment of skin cancer and wart.

Camptothecin was isolated from the Chinese ornamental tree *Camptotheca acuminata* by Wani and Wall. It was advanced to clinical trial by NCI in the 1970s but was dropped because of severe bladder toxicity. But toptican is a modified camptothecin was approved for use in the USA in 1996. The discovery of quinine was done from *Cinchona* bark, which is, used in the treatment of malaria by French scientist Caventon and Pelletier. Some of the most effective cancer treatments to date are natural products or compounds derived from natural products. The history of natural products as anticancer compounds began in 1947 with podophyllotoxin (19) being isolated from *Podophyllum peltatum*. Podophyllotoxin (19), which is too toxic for use as an anticancer agent, is used in the topical treatment of genital warts. Etoposide (18) and teniposide (18), which are modifications of
an analog, 4′-demethylepipodophyllotoxin (20), are used clinically to treat cancer.\textsuperscript{17} Podophyllotoxin (19) acts by preventing the polymerization of tubulin into microtubules. However, the 4′-demethylepipodophyllotoxin analogs do not act via the same mechanism. Instead they inhibit topoisomerase II, preventing the cleavage and resealing of DNA strands.\textsuperscript{18,19}

Uses of folk or traditional medicine represent the way of shortcut discovery of modern medicine. An inventory of medicinal plants compiled by WHO on the basis of literature from 91 countries including the classical text on Ayurvedic and Unani medicine list 21000 species of “medicinal plants”. According to WHO, around 80% of the world’s 5.76 billion populations in the developing world rely on herbal remedies for their basic health care need. The use of medicinal plants as a source for relief from illness can be traced back over five millennia from written documents of the early civilizations in China, India and near east but it is doubtless an art as old as mankind. Even today, plants are the almost exclusive source of drugs for the majority of the world’s population. In industrialized countries, medicinal plant research has had its ups and downs during the last decades.

The list of modern medicine derived from medicinal plants is very long now. Some of them are as follows:

\textbf{Table 1.1: Important drugs/chemicals from plant source and their actions/chemical uses.}

<table>
<thead>
<tr>
<th>Drug/Chemical</th>
<th>Action/Clinical use</th>
<th>Plant source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrographolide</td>
<td>Antispasmodic, antifertility and anthelmistic</td>
<td>\textit{Andrographis peniculata}</td>
</tr>
<tr>
<td>Acarbose</td>
<td>Anti diabetics</td>
<td>\textit{Actinoplanes sp.}</td>
</tr>
<tr>
<td>Betulinic acid</td>
<td>Anticancerous</td>
<td>\textit{Betula alba}</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>Anticancerous</td>
<td>\textit{Camptotheca acuminata}</td>
</tr>
<tr>
<td>Chymopapain</td>
<td>Proteolytic, mucolytic</td>
<td>\textit{Carica papaya}</td>
</tr>
<tr>
<td>Cissampeline</td>
<td>Skeletal muscle relaxant</td>
<td>\textit{Cissampelos pareira}</td>
</tr>
<tr>
<td>Colchicine amide</td>
<td>Antitumor agent</td>
<td>\textit{Colchicum autumnale}</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Antitumor agent, anti-gout</td>
<td>\textit{Colchicum autumnale}</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Choleretic</td>
<td>\textit{Curcuma longa}</td>
</tr>
<tr>
<td>Cynarin</td>
<td>Choleretic</td>
<td>\textit{Cynara scolymus}</td>
</tr>
<tr>
<td>Danthron</td>
<td>Laxative</td>
<td>\textit{Cassia species}</td>
</tr>
<tr>
<td>L-Dopa</td>
<td>Anti-parkinsonism</td>
<td>\textit{Mucuna sp}</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Antitumor agent</td>
<td>\textit{Podophyllum peltatum}</td>
</tr>
<tr>
<td>Glaucarubin</td>
<td>Amoebicide</td>
<td>\textit{Simarouba glauca}</td>
</tr>
</tbody>
</table>
1.3 Status of medicinal plants in Bangladesh

The number of medicinal herbs included in the Materia Medica of traditional medicine in this subcontinent at present stands at about 2000. About 450 to 500 of such medicinal herbs have so far been enlisted as growing or available in Bangladesh. Almost 80% of rural population is dependent on medicinal plants for their primary health care. Herbal medicine is widely used in Bangladesh in traditional healthcare system such as Ayurvedic, Unani, Hekimi and other form of folk treatments.
1.4 Description of the Family Verbenaceae

The Verbenaceae family is known as the verbena family or vervain family, of mainly tropical flowering plants. It contains trees, shrubs, and herbs notable for heads, spikes, or clusters of small flowers, many of which have an aromatic smell. Recent phylogenetic studies have shown that numerous genera traditionally classified in Verbenaceae belong instead in Lamiaceae. The new narrowly circumscribed Verbenaceae family includes some 35 genera and 1,200 species. The mangrove genus Avicennia, sometimes placed in the Verbenaceae or in its own family, Avicenniaceae, has rather confidently been placed in the Acanthaceae. *Vitex peduncularis* Wall belongs to Verbenaceae family. It has about 250 species and are native throughout the tropics and subtropics, with a few species in Eurasia. The leaf lamina is dorsiventral, or bifacial, Stomata mainly confined to one surface (abaxial), or on both surfaces; anomocytic (mostly), or diacytic, or paracytic. Hairs present (with numerous kinds represented in the family); eglandular and glandular. Cystoliths present. The mesophyll is with sclerenchymatous idioblasts, or without sclerenchymatous idioblasts; containing crystals. The crystals mostly solitary-prismatic and minor leaf veins without phloem transfer cells (6 genera).

1.5 Description of *Vitex peduncularis*

![Fig-1.1: Vitex peduncularis Plant](image)

Fig-1.1: *Vitex peduncularis* Plant
1.5.1 Scientific classification

Kingdom : Plantae
Phylum : Magnoliophyta
Class : Magnoliota
Order : Lamiales
Family : Verbenaceae
Genus : Vitex
Species: Vitex peduncularis Wall.

Binomial name: Vitex peduncularis

Other names: Boruna, Goda; Horina (Chittagong); Awal (Sylhet).

1.6 General Description and Chemical Investigation of Vitex peduncularis

Vitex peduncularis is a big sized tree grown in Bangladesh, India, Myanmar, Nepal, Cambodia, Laos, Thailand and Vietnam. Branch lets, sparsely pubescent when young, glabrescent. A medium-sized to large deciduous tree, 6-12 m high, Leaves 3-foliate, rarely 4, petiole up to 10 cm long; leaflets lanceolate or narrow elliptic, 5-15 cm long, entire, long acuminate, base cuneate. Flowers are yellowish, in axillary many-flowered, panicled cymes, 15-20 cm long, long-peduncled. Fruit is a drupe, globular, size of a black pepper, black when ripe.

Constituents: Flavonoids, triterpenoid, vitexin, pachypodol, ursolic acid, α-hydroxyursolic acid, agnuside and iridoid are the main phytochemical constituents of Vitex peduncularis. According to the literature survey the reported compounds are as follows:

Structure -21: Vitexin

Structure -22: Isovitexin
Actions: According to Suksamran and Bheemsankarrao, leaves of Vitex peduncularis contain compounds like peduncularaside, iridoid anguside, vitexin, triterpenoids and flavonoids which act as anti-inflammatory properties.\textsuperscript{28, 29} It is also known to promote cardiovascular health by improving blood and nutrient flow to the heart muscles. Though the traditional system of medicine has a long history of use but they lack the scientific documentation, particularly in light of modern scientific knowledge.\textsuperscript{30}

1.7 Medicinal Importance of Vitex peduncularis

Nowadays, research on medicinal plants has attracted a lot of attention globally. A number of evidence has been accumulated to demonstrate promising potential of medicinal plants used in various traditional, complementary, and alternative systems. In recent years, a medicinal plant, Vitex peduncularis, and its major active phytochemicals have been extensively studied for several pharmacological activities. To understand the mechanism of action, researches have to be carried out at molecular levels. The present review aims at compiling consequential compendium of pharmacological benefits of health on this plant and its major flavonoid constituent that have been tested in various experimental models using modern scientific methodologies. An herb is a plant or plant part used for its scent, flavor, or therapeutic properties, and medicinal products made of them are frequently taken to improve health as dietary supplements. Herbs are usually eaten for a long time in combinations, in relatively large, unmeasured quantities under folklore remedies. Therefore, the real challenge lies not in proving whether herbs have health benefits, but in defining what these benefits are and developing the methods to expose them by scientific means.\textsuperscript{31}

The genus Vitex belonging verbenaceae family includes more than 270 species, predominantly shrubs and trees and is restricted to tropical and subtropical regions. The
plants of this genus has a plethora of ethanopharmacological uses and has been used to treat a range of human ailments related to insects, bacteria, fungi, snakes and poisonous spiders and diseases associated with menstruation and gynecological problems. Traditionally, the boiled bark extract of *Vitex peduncularis* is used as a drink to treat joint ache.

*In vitro* and *in vivo* studies have shown that chemicals isolated from the *Vitex* plant have potential anti-inflammatory\(^\text{32}\), antibacterial\(^\text{31}\), antifungal\(^\text{34,35}\) and analgesic\(^\text{32,36,37}\) activities. Leaves and barks of *Vitex peduncularis* are used for diabetes, malaria and jaundice\(^\text{32}\). The bark is used for making an external application for pains in the chest\(^\text{33}\). Chakmas takes the bark extract orally at least three times a day for the cure of jaundice. Root juice is given to stop excessive menstrual bleeding. Leaves and barks are used for diabetes and malaria in Khagrachari hill area. Extraction of the dried leaves yielded pachypodol, ursolic acid, 2α-hydroxyursolic acid, vitexin and peduncularcin. In Orissa, a decoction of the leaves is taken as tea during the cold season. An infusion of leaves administered intramuscularly or orally to rabbits increases the osmotic resistance of cells and inhibits haemolysis produced by saponin, cobra venom, bile salts, or saline solution.\(^\text{38}\)

### 1.8 Morphology of Vitex genus

*V. agnus-castus*, commonly known as chaste tree or sage tree, is a beautiful little deciduous tree or large shrub with a showy summertime flower display. *V. agnus-castus* is a sprawling plant that grows 3-6 m and about as wide. The leaves are 7.6-10 cm in diameter and are palmately compound with 5-7 fingerlike leaflets. The foliage is aromatic and is typically grey-green to dark green above and lighter on the undersides. Branched flower clusters are produced on new wood in late spring and early summer and bloom sporadically until early fall. It is also fragrant and attracts pollinating bees and hummingbirds. Flowers are followed by a fleshy fruit that contains four seeds that are sometimes used as seasoning, similar to black pepper (monk's pepper is another of these species common names). Flower color ranges from violet to blue to deep purple.\(^\text{39}\)

*V. negundo* are large and erect aromatic shrubs, which grow to a height of 2-5 m. The leaves have five leaflets in a palmately arrangement, an acute terminal leaflets (16-32 mm) with petiolate having 1-1.3 cm long, lanceolate, 4-10 cm long, hairy beneath and pointed at both ends. The bluish purple flowers are numerous. The fruit is succulent, black when ripe, rounded and about 4 mm in diameter.\(^\text{40}\)

*V. rotundifolia* is a sprawling shrub 6-8 feet in diameter and 6 inches to 2 feet tall. The round leaves are gray-green to silvery and 1-2 inches long. The foliage has a spicy
fragrance. The 1 inch flowers are bluish purple and are produced in small clusters at the ends of the branches throughout the year.41

*V. trifolia* is a fast-growing shrub, is popular for its variegated foliage and pretty blue flowers and grows to a height of 10-12 feet. The trifoliate evergreen leaves are gray-green with white marginal variegation. These soft leave have grayish pubescence on their underside and smell pungent when crushed. Attractive blue or lavender flowers with white spots appear in terminal clusters during the summertime.42

### 1.9 Pharmacological Potential of *Vitex* genus

The available literature reveals that among 36 species of *Vitex*, only 16 species, i.e., *V. agnus-castus*, *V. negundo*, *V. rotundifolia*, *V. trifolia*, *V. gardneriana*, *V. ferrugenia*, *V. cannabifolia*, *V. doniana*, *V. polygama*, *V. leucoxylon*, *V. pinnata*, *V. scabra*, *V. mollis*, *V. altissima*, *V. glabra*, *V. megapotamica*, *V. quinata* have been evaluated for their pharmacological activities. This study gave us a clue that bicyclic terpenes isolated from *V. agnus-castus* fruits are used for the treatment of movement disorders.43 Flavonoids and diterpenoids, isolated from ethyl acetate extract of *V. agnus-castus* fruits, have been reported to exhibit antioxidant activity and n-hexane extract did not show any effect.44 Essential oils, isolated from *V. agnus-castus*, showed a significant antibacterial activity.45 Caffeic and chlorogenic acids, extracted from leaves and fruits of *V. agnus-castus*, exhibited potent antioxidant activity.46 Ethanolic extracts of *V. agnus-castus* fruits, exhibited estrogenic activity at two dose levels 0.6 and 1.2 g/kg per body wt. (b.w.) when studied by the vaginal smear and uterine weight methods for normal and ovariectomized female rats.47 Cell culture experiments showed that flavonoid apigenin, isolated from *V. agnus-castus* exhibited estrogenic activity.48

The chloroform extracts of *V. negundo* (40 mg/kg/body wt. and 135 mg/kg), exhibited broad cytotoxicity in a human cancer cell line panel.49 Two pentacyclic triterpenoids, isolated from *V. negundo* leaves have been reported to exhibit antifeedant activity against the larvae of an agricultural pest, the castor semilooper (*Achoea janata*), and also possess antibacterial activity against *Bacillus subtilis* and *Escherichia coli*, when tested by the paper disk method.50,51 It has been reported that flavone glycoside, isolated from ethanolic extract of *V. negundo* leaves, exhibits significant antifungal activity against *Trichophyton mentagrophytes* and *Cryptococcus neoformans* at Minimum inhibitory concentration MIC 6.25 μg/mL.52 Lignans, isolated from *V. negundo* exhibited potent inhibitory activity against lipoxygenase enzyme, while moderate activity against butyryl-cholinesterase.53 This study showed that lignans
isolated from *V. negundo* roots, were found to be active against α-chymotrypsin (Ki values 31.75-47.11 μM). Tris (2,4-di-tert-butylphenyl) phosphate (TDTBPP) was isolated from the leaves of *V. negundo* and the acute anti-inflammatory activity of TDTBPP was assessed by carrageenan-induced rat paw edema. TDTBPP reduced the raw paw edema volume significantly at the tested doses of 50 mg/kg and 70 mg/kg body weight. Cataract was induced by single subcutaneous injection of sodium selenite (4 mg/kg body weight) and methanolic extract of leaves of *V. negundo* (1 mg/Kg bodyweight) was administered i.p., (intraperitoneal) It showed modulated selenite induced cataractogenesis in rat pups by preventing loss of chaperone property. The anti-hyperglycemic effect of iridoid glucoside from the leaves of *V. negundo* (50 mg/kg b.w) was comparable with glibenclamide. It also possesses the significant productive effect on glycoprotein metabolism in addition to its antidiabetic effect.

It has been reported that methanol extract of *V. rotundifolia* fruits, exhibits antioxidative activity with the references to α-tocopherol and Butylated hydroxyanisole BHA using ferric thiocyanate method. Diterpenoid (ferruginol), isolated from the fruit of *V. rotundifolia* also exhibited a stronger antioxidative activity. Casticin, a flavonoid isolated from fruits of *V. rotundifolia* exhibited considerable growth inhibitory activity against human lung cancer cells (PC-12) and human colon cancer cells (HCT116) using the 3-(4,5-dimethythiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) MTT assay. These results suggested that rotundifuran isolated from the fruit of *V. rotundifolia*, may be used as a potential chemopreventive and chemotherapeutic agent. Polymethoxyflavonoids, isolated from the fruit of *V. rotundifolia*, exhibited antiproliferative activity in human myeloid leukemia HL-60 cells. *V. rotundifolia* fruit extract inhibits cholesterol acyltransferase, thus can be used in prevention and treatment of cardiovascular disease caused by hypercholesterolemia. It has been reported that Casticin exhibits antimitotic activity on the growth of KB cells at 0.23 μM. Polyphenol, trans-resveratrol isolated from *V. rotundifolia* exhibited highly potent tyrosinase inhibition effect, thus inferring its importance in cosmetic industries as anti aging and skin-whitening agent. The results showed that essential oil isolated from the fruits of *V. rotundifolia* exhibits estrogenic activity on proliferation of MCF-7 cells by proliferation assay method.

*V. trifolia* exhibited anticancer activity on the proliferation of mammalian cancer cells, evaluated by sulforhodamine B, which is widely used in Chinese folk medicine. The fruit extracts of *V. trifolia* exhibited antipyretic, analgesic, and anti-inflammatory
activity.\textsuperscript{69} Flavonoids, isolated from methanolic extract of \textit{V. trifolia} exhibited bacteriostasis activity.\textsuperscript{70} Vitetrifoline E, isolated from \textit{V. trifolia} leaves has been reported to exhibit tracheospasmodic activity by blocking spontaneous contraction of male guinea pig trachea induced by histamine at the doses 1.3 × 10 M.\textsuperscript{71} \textit{V. trifolia} exhibited antimalarial activity in the range of 10- 100 × 10 g/mL against \textit{Plasmodium falciparum} (K1, multidrug resistant strain) in vitro.\textsuperscript{72} Aqueous and ethanolic extracts of leaves of \textit{V. trifolia} were investigated for hepatoprotective activity against carbon tetrachloride (CCl\textsubscript{4}) induced liver damage. Results showed significant reduction in total bilirubin and serum marker enzymes, increase in total protein at dose level of 20 and 30 mg/kg/day p.o. Silymarin was used as standard at a dose of 100 mg/kg/day p.o. (oral route).\textsuperscript{73} A study reported that extracts of \textit{V. leucoxylon} exhibited hypoglycemic, anti-inflammatory, and antipyretic activity.\textsuperscript{74,75} Iridoid glucosides, isolated from the ethyl acetate extracts of \textit{V. altissima} leaves showed potent antioxidant activity by both the superoxide nitro blue tetrazolium (NBT) riboflavin photoreduction, free-radical-scavenging and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging methods.\textsuperscript{76} Ethyl acetate extract of \textit{V. altissima} leaves exhibited significant anti-inflammatory activity in rat paw edema model.\textsuperscript{77} Pinnatoside iridoid glucoside, isolated from of \textit{V. pinnata} bark, exhibited modest antifungal activity against \textit{Candida albicans}.\textsuperscript{78} Hydroalcoholic extracts of \textit{V. polygama} leaves showed potent anti-inflammatory, antinociceptive and antioxidant activity. It is also used in folk medicine to prevent kidney stone and inflammation.\textsuperscript{79} The oils from glandular trichomes of \textit{V. ferruginea} exhibited significant antifungal activity against dermatophyte strains with MIC between 0.16 and 0.64 μL/mL.\textsuperscript{80} Organic extracts of \textit{V. mollis} leaves showed insecticidal and insect growth regulatory activity on fall armyworm neonate larvae (Spodoptera frugiperda), an important insect pest of corn.\textsuperscript{81} A study suggested that aqueous extract of \textit{V. doniana} fruit exhibits antidiarrheal activity at doses of 150-650 mg/kg.\textsuperscript{82} The ethanol and distilled water extracts of \textit{V. doniana} showed DPPH scavenging activity in CCl\textsubscript{4} treated albino rats. Vitamin C was used as the standard antioxidant. The water extracts produced a significant decrease (P < 0.05) in liver malondialdehyde, while the levels of superoxide dismutase and catalase (CAT) significantly increased (P < 0.05) relative to the positive control.\textsuperscript{83} Ecdysteroids, isolated from \textit{V. scabra} stem bark exhibited very low moulting activity in the Musca bioassay.\textsuperscript{84}
It has been reported that ethyl acetate as well as n-butanol extracts of *V. meagapotamica* leaves showed the hypoglycemic effect in diabetic rats, but ethyl acetate fraction produced the maximum hypoglycemic effects at the doses of 400 and 800 mg/kg.\(^8\) Ethanol extracts of *V. glabrata* (EEVG) was evaluated for the anti-inflammatory activity using carrageenan-induced paw edema and cotton pellet induced granuloma formation in rat models. EEVG showed significant anti-inflammatory activity in rats at a dose of 400 mg/kg, p.o. and was comparable (P < 0.05) to that of diclofenac sodium (standard, 50 mg/kg, p.o.).\(^8\) EEVG was also evaluated for the antioxidant and hepatoprotective effects in a CCl\(_4\)-induced liver damage model in rats. Hepatoprotective activity was evaluated by changes in the levels of the serum enzymes, i.e., AST, ALT, ALP and total bilirubin, and further by histopathological examinations of liver tissues. Antioxidant activity was measured in terms of superoxide dismutase, glutathione, lipid peroxidation, CAT and peroxidase levels in liver homogenate.\(^8\)

A phytochemical investigation of the leaves of *V. quinata* (Lour.) guided by the MCF-7 human breast cancer cell line, led to the isolation of a new δ-truxinate derivative\(^8\) and a new phytonoic acid derivative,\(^9\) together with 12 known compounds. The structures of the new compounds were determined by spectroscopic methods as dimethyl 3,4,3',4'-tetrahydroxy-δ-truxinate\(^8\) and methyl 10R-methoxy-12-oxo-9-16E-phytodienoate,\(^9\) respectively. In a cytotoxicity assay, S-5-hydroxy-7,4’-dimethoxyflavanone\(^5\) was found to be the sole active principle, with ED values of 1.1-6.7 μM, respectively, when tested against a panel of three human cancer cells. Methyl-3,4,5-O-tricaffeoyl quinate\(^6\) showed activity in an enzyme-based Enzyme-linked immunosorbent assay (ELISA) NF-κB p65 assay, with an ED (50) value of 10.3 μM.\(^9\)

### 1.10 Clinical Activities of *Vitex* genus

In clinical trials, serotonin reuptake inhibitors, i.e. fluoxetine and the extract of *V. agnus-castus* were studied for the treatment of PMDD. In this study *V. agnus-castus* extract showed that fluoxetine was more effective for psychological symptoms while the extract diminishes the physical symptoms of premenstrual disorders.\(^9\) Dopaminergic compounds present in extract of *V. agnus castus* fruits are widely used to treat premenstrual mastodynina (mastalgia) indicated by double-blind placebo-controlled studies.\(^9\) This study suggested that at low doses *V. agnus-castus* increases milk production in lactating women and also used to modify libido.\(^9\) Water extract of *V. negundo* in combination with matra basti as 500 mg tablets showed relief from signs and symptoms of sciatica pain, weakness, numbness, and other discomforts.
along the path of the sciatic nerve often accompany low back pain, herniated disk, spinal stenosis, piriformis syndrome in clinical studies on 119 patients in the age group of 20-60 years.\textsuperscript{94}

1.10 Aim of the project
Bangladesh is a good repository of medicinal plants belonging to various families, including Verbenaceae. The Verbenaceae plants contain wide range of unique pharmacologically active compounds, having antiseptic, astringent, anti-inflammatory, antibacterial, antifungal, analgesic, stomachic, anti-rheumatic, anti-diarrhea, anticancer and anti-emetic activities.

Though a large number of Vitex species have been investigated but locally a little attention was given to this particular species. Therefore, an attempt has been taken to study the chemical constituents and biological activities of \textit{Vitex peduncularis}. These investigations may provide some interesting compounds, which may be pharmacologically active. If significant results are obtained, these can be used as remedies for the treatment of some diseases. Since this plant is available in Bangladesh and a lot of herbal health centers and herbal industries are using such related herbal plants for treatments, so if the biological activity of this plant can be studied thoroughly, this may be a cost-effective treatment. So, the objective of the study is isolation and structural elucidation of the bioactive compounds by chemical and spectroscopic methods (UV, IR, \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR, DEPT-135, COSY-45 and HMBC etc).

1.11 Present study protocol
The present study was designed to isolate pure compounds as well as to observe biological activities of the crude extracts and their different fractions. The study protocol consists of the following steps:
1. The bark of the plant was dried at room temperature and was grounded into powder.
2. Extraction of the bark powder with methanol.
3. Fractionation of the crude methanol extract by liquid-liquid partition chromatography was used n-hexane, dichloromethane, ethyl acetate, 1-butanol respectively and with water.
4. Sub-fractionation of each fraction by column chromatography (CC).
5. Isolation and purification of the pure compounds from different sub-fractions by repeated column chromatographic methods including high performance liquid chromatography (HPLC).
6. Determination of the structure of the isolated pure compounds with the help of chemical and spectroscopic methods (UV, IR, \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR, DEPT-135, COSY-45 and HMBC etc).
7. Bioassay analyses of the crude extracts (Antioxidant) were also performed.
CHAPTER TWO

EXPERIMENTAL SECTION
2.0 General methods
The following sections of this chapter are a brief description of the various method followed in extraction, fractionation & purification of the compounds in the course of experimental works.

2.1 Solvents and chemicals
Analytical or laboratory grade solvents and chemicals were used in the experiments. All solvents and reagents used in the experiments were produced from E. Merck (Germany), BDH (England). The commercial grade solvents (ethyl acetate, acetone, methanol, ethanol, n-hexane, dichloromethane and 1-butanol) were distilled before use.

2.2 Distillation of the solvents
The analytical grade solvents (ethyl acetate, acetone, methanol, ethanol, n-hexane, dichloromethane and 1-butanol) were distilled. Distilled solvents were used throughout the investigation.

![Distillation process](image)

Fig 2.1: Distillation process.

2.3 Evaporation
All evaporations were carried out under reduced pressure using a rotary evaporator at a bath temperature not more than 45°C. The residual solvent in the extract and compounds were removed under high vacuum.
2.4 Preparation of the reagents

Different types of reagent used during the research were prepared as follows.

2.4.1 Spray reagent (Developing reagent)

8g Ce(SO$_4$)$_2$•2H$_2$SO$_4$
100 mL 15% 2H$_2$SO$_4$ (aq.)

2.5 Chromatographic techniques

Three types of chromatographic techniques such as Thin Layer Chromatography (TLC), Column Chromatography (CC) and High Performance Liquid Chromatography (HPLC) were used.

2.5.1 Thin layer chromatography (TLC)

Pre-coated 0.2 mm thin coatings TLC plates on aluminum sheets were used throughout the experiment.

2.5.2 Sample application

The TLC plates were spotted with a small amount of the crude extract by using a narrow space capillary tube. The capillary one was washed with acetone before each sample applied.
2.5.3 Solvent system

The solvents of different polarity used for TLC are given below:
- n-Hexane : Dichloromethane (in different ratios)
- Dichloromethane : Methanol (in different ratios)
- Ethyl acetate : Dichloromethane (in different ratios)

2.5.4 Preparation of TLC tanks

The ascending technique in glass jars & glass tanks were used to develop TLC plates. A suitable solvent system was poured into glass jar or tank in a required amount. The tank was
then covered with a lid and kept for a certain period for allowing it to achieve saturation. A filter paper was usually introduced into the tank to promote the saturation process. The solvent level at the bottom of the tank must not be above the line of the spot where the sample solution was applied to the plate. As the solvent rises, the plate becomes moistened. When the solvent front moves almost near the end of the plate, the plate was then taken out and dried. The solvent front was not allowed to travel beyond the end of the silica-coated surface.

2.5.5 Detection of spots

For the location of the separated compounds, the plates were examined under UV light in different wavelengths, 254 and 365 nm. The plates were developed by the spray reagent ceric sulfate followed by heating in an oven at 105°C for 5 minutes.

![Fig 2.6: TLC plates under UV lamp](image)

2.5.6 The $R_f$ value

Retardation factor ($R_f$) is the ratio of the distance the compound travels and the distance the solvent front moves.

$$R_f = \frac{\text{Distance travelled by a substance}}{\text{Distance travelled by a solvent}}$$

Usually, the $R_f$ value is constant for any given compound and it corresponds to a physical property of that compound.

![Fig 2.7: Calculation of $R_f$ value](image)
2.5.7 Stationary phases of column chromatography

For normal phase column chromatography, silica gel of particle size 230-400 mesh (Merck) was used and separation was performed by gravitational flow with solvents of increasing polarity. The sample was applied into the column either as a solution or in a powdered form or by adsorbing samples by the silica gel. The eluted samples were collected in several test tubes and were monitored by TLC to make different fractions on the basis of $R_f$ values.

2.5.8 Preparation of column

A column was plugged with a small amount of cotton to prevent the adsorbent from leaking. The column was filled with the slurry of column grade silica gel with solvent. It was ensured that the “Sub Column” is free from air bubbles by recycling the solvents several times. The samples were applied at the top of the column. Elution was started with n-hexane followed by increasing polarity.
2.6 Spectroscopic Techniques

2.6.1 Infra-Red Spectroscopy (IR)
A Shimadzu IR prestige-21(FT-IR) spectrometer was used for recording infrared spectrum. Major bands \( (\nu_{\text{max}}) \) were recorded in wave number \( (\text{cm}^{-1}) \) as KBr pellets.

2.6.2 Nuclear Magnetic Resonance (NMR) Spectroscopy
\(^1\text{H} (400 \text{ MHz}) \) and \(^{13}\text{C} (100 \text{ MHz}) \) NMR spectra were recorded on a BRUKER NMR DPX-400 MHz instrument, with chemical shift data reported in ppm relative to the solvent used. The spectra were taken by using CDCl\(_3\) & CD\(_3\)OD.

2.6.3 Ultra-Violet Spectroscopy
UV absorbance for obtaining the \( \lambda \)-max of the isolated compounds was taken by Shimadzu UV-1601 UV spectrophotometer.

2.7 Investigation of *Vitex peduncularis*

2.7.1 Collection of the plant materials
The bark of *Vitex peduncularis* (Locally known as Goda) was collected from Rangamati, Bangladesh during the month of May 2014.

2.7.2 Identification of species
The taxonomy of the plant was confirmed consulting with the National Herbarium’s taxonomist (Accession number DACB-38583). A voucher specimen of this plant was deposited at Bangladesh National Herbarium, Dhaka, Bangladesh.
2.7.3 Extraction, partition and isolation of the compounds from *Vitex peduncularis*

The bark and leaf of this plant were separated and dried under in open air & then dried in oven at 37 °C. Afterwards it was powdered (~200 mesh) by a grinding machine. This bark powder (1 kg) was used throughout this investigation. The moisture content and ash content of this bark sample was 10.72% and 11.81% respectively. The bark powder of *Vitex peduncularis* was extracted three times with methanol during three weeks. The extractive was concentrated using rotary vacuum evaporator and was dried to solid mass (139 g) using fridge dryer. The dried methanol extract was then suspended in water and partitioned by separating funnel by using *n*-hexane, dichloromethane, ethyl acetate and 1-butanol successively. All the extracts and fractions were subjected to biological activities (antioxidant screening).

![Scheme-1: Extraction and Partition of the bark of *Vitex peduncularis* powder](image-url)
After extraction and partition, the phytochemical group tests for different fractions of *Vitex peduncularis* bark were done.

Table-2.1: Result of different phytochemical group tests for different fractions of *Vitex peduncularis* samples

<table>
<thead>
<tr>
<th></th>
<th>Methanol Part</th>
<th>n-Hexane Part</th>
<th>Dichloromethane Part</th>
<th>Ethyl acetate Part</th>
<th>1-Butanol Part</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Saponin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic Compound</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reducing Sugar</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Protein</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroid</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

(+) Present in low concentration, (++) Present in medium concentration, (++++) Present in high concentration, (-) Not present

2.7.4 Thin layer chromatographic (TLC) analysis of different fractions

TLC analysis of the dichloromethane and ethyl acetate soluble fractions showed several spots on TLC plate but *n*-hexane and 1-butanol fractions were not showed significant spots after running on TLC plate under UV lamp, which were further confirmed by the development with spray reagent on TLC plate for detecting the spots.

2.8 Investigation of dichloromethane fraction

2.8.1.1 Fractionation of CH$_2$Cl$_2$ soluble part (VPD) of *Vitex peduncularis* by silica gel column chromatography

a. Preparation of column

A glass column (68 cm X 13 cm; id.) was packed with column grade silica gel (110 g: 230-400 mesh, ASTM, Merck) using *n*-hexane as the equilibrating solvent. The column was then equilibrated with three column volumes of *n*-hexane.

Length of column = 68 cm

Diameter of column = 13 cm

Bed of silica = 34 cm

Bed of sample = 3 cm
b. Preparation of the sample
The CH₂Cl₂ soluble part (7.39 g) was dissolved in dichloromethane and column grade silica gel (9.81 g) was added to it. The suspension was thoroughly mixed and evaporated to dryness. The dried material was made into fine powder by a mortar and pestle.

c. Application of the sample to the column
The dried powdered sample was applied into the top of the column. The sample was then eluted with n-hexane and the polarity of the solvent was gradually increased by the addition of dichloromethane followed by methanol. The eluted fractions were collected in 45 conical flasks (Table-2.2).

Table-2.2: Fractionation of CH₂Cl₂ soluble part (VPD) by silica gel column chromatography

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Solvent System</th>
<th>Amount (cc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-Hexane</td>
<td>CH₂Cl₂</td>
</tr>
<tr>
<td>1-2</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>11-15</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>17-18</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>97</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>95</td>
</tr>
<tr>
<td>21-25</td>
<td>-</td>
<td>93</td>
</tr>
<tr>
<td>26-30</td>
<td>-</td>
<td>90</td>
</tr>
<tr>
<td>31-35</td>
<td>-</td>
<td>88</td>
</tr>
<tr>
<td>36-40</td>
<td>-</td>
<td>85</td>
</tr>
<tr>
<td>41-42</td>
<td>-</td>
<td>83</td>
</tr>
<tr>
<td>43-45</td>
<td>-</td>
<td>80</td>
</tr>
</tbody>
</table>
2.8.1.2 Isolation of the pure compound-1

Dichloromethane Extract (VPD, 7.39 g)

Silica gel column (230-400 mesh) using n-hexane, DCM and MeOH as solvent

- **VPD₁** (0.180 g)
- **VPD₂** (0.279 g)
- **VPD₃** (0.373 g)
- **VPD₄** (0.615 g)
- **VPD₅** (1.121 g)
- **VPD₆** (0.275 g)
- **VPD₇** (0.124 g)
- **VPD₈** (0.114 g)

Silica gel column (230-400 mesh) using n-hexane, DCM and MeOH as solvent

- **VPD₄₁** (25 mg)
- **VPD₄₂** (60 mg)
- **VPD₄₃** (73 mg)
- **VPD₄₄** (48 mg)
- **VPD₄₅** (57 mg)

Silica gel column (230-400 mesh) using 2% MeOH in DCM as mobile phase

- **Compound-1** (16 mg)

Scheme-2: Isolation of the pure compound-1

All the collected samples were examined by TLC and were combined according to their $R_f$ values. Eight different fractions were obtained and amount of each fraction and their TLC patterns are recorded in Table-3.3
Table-2.3: Different CH$_2$Cl$_2$ soluble fractions (VPD) combined by TLC pattern

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Solvent (ratio)</th>
<th>Amount (g)</th>
<th>TLC pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPD$_1$</td>
<td>n-Hexane 25 CH$_2$Cl$_2$ 75 Me-OH -</td>
<td>0.180</td>
<td>Four spots with tail</td>
</tr>
<tr>
<td>VPD$_2$</td>
<td>20 80 -</td>
<td>0.279</td>
<td>Separated three spots</td>
</tr>
<tr>
<td>VPD$_3$</td>
<td>15 85 -</td>
<td>0.373</td>
<td>Single spot with tailing</td>
</tr>
<tr>
<td>VPD$_4$</td>
<td>15 85 -</td>
<td>0.615</td>
<td>Single spot with tailing</td>
</tr>
<tr>
<td>VPD$_5$</td>
<td>- 100 -</td>
<td>1.121</td>
<td>Two spots with tailing</td>
</tr>
<tr>
<td>VPD$_6$</td>
<td>- 95 5</td>
<td>0.275</td>
<td>Broad tail</td>
</tr>
<tr>
<td>VPD$_7$</td>
<td>- 90 10</td>
<td>0.124</td>
<td>Broad tail</td>
</tr>
<tr>
<td>VPD$_8$</td>
<td>- 85 15</td>
<td>0.114</td>
<td>Broad tail</td>
</tr>
</tbody>
</table>

2.8.1.3 Fractionation of the fraction VPD$_4$ (0.615 g)

The fraction VPD$_4$ (0.615 g) found good and showed single spot with some tailing. It was further fractioned by silica gel column chromatography by the following ways:

a. Preparation of column

A glass column (45 cm X 10 cm; i.d.) was packed with column grade silica gel (52 g; 230-400 mesh, ASTM, Merck) using dichloromethane as the equilibrating solvent. The column was then equilibrated with three column volumes of dichloromethane.

Length of column = 45 cm
Diameter of column = 10 cm
Bed of silica = 20 cm
Bed of sample = 1 cm

b. Preparation of the sample

The fraction VPD$_4$ (0.615 g) was dissolved in minimum amount of dichloromethane and column grade silica gel (1.0 g) was added into it. The suspension was thoroughly mixed and evaporated to dryness. The dried material was made into fine powder by a mortar and pestle.

c. Application of the sample into the column

The dried powdered sample (1.615 g) was applied into the top of the column. The sample was then eluted with n-hexane and the polarity of the solvent was gradually increased by the addition of dichloromethane followed by methanol. The eluted fractions were collected in 65 conical flasks. All the eluted samples were examined by TLC. The eluted samples were
combined according to their $R_f$ values and five different fractions were obtained. Amount of each fraction and their TLC patterns are given in Table-2.4

Table-2.4: TLC pattern of different sub-fractions of fraction VPD$_4$ (0.615 g)

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Solvent (ratio)</th>
<th>Amount (g)</th>
<th>TLC pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPD$_4$1</td>
<td>n-Hexane 90 CH$_2$Cl$_2$ Me-OH</td>
<td>25</td>
<td>Long tail</td>
</tr>
<tr>
<td>VPD$_4$2</td>
<td>- 100 - -</td>
<td>60</td>
<td>Single spot with tailing</td>
</tr>
<tr>
<td>VPD$_4$3</td>
<td>- 97 3</td>
<td>73</td>
<td>Elongated two spots</td>
</tr>
<tr>
<td>VPD$_4$4</td>
<td>- 92 8</td>
<td>48</td>
<td>Two spots with tail</td>
</tr>
<tr>
<td>VPD$_4$5</td>
<td>- 90 10</td>
<td>57</td>
<td>Broad tail</td>
</tr>
</tbody>
</table>

2.8.1.4 Purification of fraction VPD$_4$2 (60 mg) by silica gel column chromatography
A small glass column was made with silica gel (5.0 g) using dichloromethane. The sample VPD$_4$2 (60 mg) was dissolved in a minimum volume of CH$_2$Cl$_2$ and it was then applied into the column. The sample was eluted by using 2% MeOH in CH$_2$Cl$_2$ as a mobile phase and a single compound (Compound-1, 16 mg) was obtained.

2.8.1.5 Characterization of the compound-1
Appearance:
$R_f$ value: 0.617 (2% MeOH in dichloromethane solvent system).
Solubility: soluble in CH$_2$Cl$_2$ solvent.
Percent yield: $1.6 \times 10^{-3}$ (in 1000 g)

2.8.1.6 Infra-red spectroscopy of compound-1
The IR spectrum (Fig-3.1.1) of compound-1 showed absorption frequencies at 3419.6 cm$^{-1}$, 2941.2 cm$^{-1}$, 1652.9 cm$^{-1}$, 1693.4 cm$^{-1}$, 1458.1 cm$^{-1}$, 1386.7 cm$^{-1}$, 1365.5 cm$^{-1}$, 1276.8 cm$^{-1}$, 1234.4 cm$^{-1}$ and 1035.7 cm$^{-1}$.

2.8.1.7 $^1$H-NMR spectroscopy of compound-1
The $^1$H-NMR (400 MHz) spectrum of Compound-1 (Fig-3.1.2, 3.1.2a and 3.1.2b) had signals at 3.41 ppm (singlet), 4.00 ppm (broad doublet), 5.27 ppm (doublet), 0.73 ppm (singlet), 0.84 ppm (singlet), 1.01 ppm (singlet), 0.96 ppm (singlet), 0.92 ppm (singlet) and 0.94 ppm (singlet).
2.8.1.8 $^{13}$C-NMR spectroscopy of compound-1

The $^{13}$C-NMR (100 MHz) spectrum of Compound-1 (Fig.3.1.3, and 3.1.3a) had 30 signals and these were at 41.65, 66.53, 78.92, 38.42, 52.55, 18.01, 36.60, 38.24, 48.10, 38.24, 22.91, 122.48, 143.67, 48.10, 27.62, 27.94, 46.49, 47.34, 45.86, 32.40, 36.70, 30.60, 28.50, 17.10, 16.35, 16.49, 178.81, 182.82, 33.07 and 23.59 ppm.

2.8.2 Fractionation of the sub-fraction VPD$_5$ (1.121 g)

The fraction VPD$_5$ (1.121 g) was further fractioned by silica gel column chromatography by the following ways:

   a. **Preparation of column**

   A glass column (45 cm X 10cm; id.) was packed with column grade silica gel (75 g: 230-400 mesh ASTM, Merck) using dichloromethane as the equilibrating solvent. The column was then equilibrated with three column volumes of dichloromethane.

   *Length of column = 45 cm*
   *Diameter of column 10 cm*
   *Bed of silica = 28 cm*
   *Bed of sample = 2 cm*

   b. **Preparation of the sample**

   The sample (1.121 g) was dissolved in dichloromethane and column grade silica gel (2.0 g) was added to it. The suspension was thoroughly mixed and evaporated to dryness. The dried material was made into fine powder by a mortar and pestle.

   c. **Application of the sample in to the column**

   The dried powdered sample was applied into the top of the column. The sample was then eluted with dichloromethane and the polarity of the solvent was gradually increased by the addition of methanol. The eluted fractions were collected in 88 conical flasks. All eluted samples were examined by TLC. The eluted samples were combined according to their $R_f$ values and seven different fractions were obtained. Amount of each fraction and their TLC patterns are recorded in Table-2.5
2.8.3 Isolation of the pure compound-2

\[
\text{VPD}_5 (1.121 \, \text{g})
\]

Silica gel column (230-400 mesh) using \(n\)-hexane, DCM and MeOH as solvent

<table>
<thead>
<tr>
<th>Fraction no. (mg)</th>
<th>Amount (mg)</th>
<th>TCL pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPD(_{5,1}) 52</td>
<td>52</td>
<td>Elongated tow sports</td>
</tr>
<tr>
<td>VPD(_{5,2}) 79</td>
<td>79</td>
<td>Three sports</td>
</tr>
<tr>
<td>VPD(_{5,3}) 36</td>
<td>36</td>
<td>Broad tail</td>
</tr>
<tr>
<td>VPD(_{5,4}) 45</td>
<td>45</td>
<td>Single sport with tail</td>
</tr>
<tr>
<td>VPD(_{5,5}) 110</td>
<td>110</td>
<td>Two sport</td>
</tr>
<tr>
<td>VPD(_{5,6}) 69</td>
<td>69</td>
<td>Single sport</td>
</tr>
<tr>
<td>VPD(_{5,7}) 102</td>
<td>102</td>
<td>Long tail</td>
</tr>
</tbody>
</table>

2.8.2.1 Purification of the sub-fraction VPD\(_{5,6}\) (69 mg) by silica gel column chromatography

A small glass column was made with silica gel (5.0 g) using dichloromethane. The sample VPD\(_{5,6}\) (69 mg) was dissolved in a minimum volume of CH\(_2\)Cl\(_2\) and it was then applied into
the column. The sample was eluted by using 6% MeOH in CH$_2$Cl$_2$ as a mobile phase and a single compound (Compound-2, 22 mg) was obtained.

2.8.2.2 Characterization of the compound-2

Appearance:
Solubility: Soluble in few drops MeOH in CH$_2$Cl$_2$ solvent.

R$_f$ value: 0.514 (6º/o MeOH in dichloromethane solvent system).

Percent yield: 2.2×10$^{-3}$ (in 1000 g)

2.8.2.3 Infra-red spectroscopy of compound-2

The IR spectrum (Fig-3.2.1) of the compound-2 showed absorption frequencies at 3419.6 cm$^{-1}$, 2929.7 cm$^{-1}$, 1652.9 cm$^{-1}$, 1695.3 cm$^{-1}$, 1458.1 cm$^{-1}$, 1379 cm$^{-1}$, 1271 cm$^{-1}$, 1234.4 cm$^{-1}$, 1147.6 cm$^{-1}$ and 1039.6 cm$^{-1}$.

2.8.2.4 $^1$H-NMR spectroscopy of compound-2

The $^1$H-NMR (400 MHz) spectrum of compound-2 (Fig-3.2.2, 3.2.2a and 3.2.2b) had signals at 3.56 ppm (singlet), 3.84 ppm (broad singlet), 5.20 ppm (doublet), 3.45 ppm (doublet), 3.37 ppm (doublet), 0.67 ppm (singlet), 0.91 ppm (doublet), 0.71 ppm (singlet), 0.80 ppm (doublet) and 0.83 ppm (singlet).

2.8.2.5 $^{13}$C-NMR spectroscopy of compound-2

The $^{13}$C-NMR (100 MHz) spectrum of Compound-2 (Fig-3.2.3, 3a, 3b, and 3c) had 30 signals and these were at 41.21, 66.21, 78.12, 39.46, 52.66, 17.71, 33.75, 39.24, 47.27, 37.78, 23.30, 143.92, 47.83, 27.84, 27.50, 47.70, 47.19, 45.84, 29.59, 36.70, 32.42, 70.69, 16.0, 16.89, 17.22, 180.88, 181.07, 32.96, and 23.53 ppm.

2.8.3 Fractionation of the sub-fraction VPD$_3$ (0.373 g)

The fraction VPD$_3$ (0.373 g) was further fractioned by silica gel column chromatography by the following ways:

a. Preparation of column

A glass column (45 cm 10cm; id.) was packed with column grade silica gel (75 g: 230-400 mesh ASTM, Merck) using n-hexane as the equilibrating solvent. The column was then equilibrated with three column volumes of n-hexane.

Length of column = 45 cm
Diameter of column 10 cm
Bed of silica = 28 cm
Bed of sample = 1.5 cm
b. **Preparation of the sample**

The sample (0.373 g) was dissolved in *n*-hexane and column grade silica gel (1 g) was added to it. The suspension was thoroughly mixed and evaporated to dryness. The dried material was made into fine powder by a mortar and pestle.

c. **Application of the sample in to the column**

The dried powdered sample was applied into the top of the column. The sample was then eluted with *n*-hexane and the polarity of the solvent was gradually increased by the addition of DCM and methanol. The eluted fractions were collected in 70 conical flasks. All eluted samples were examined by TLC. The eluted samples were combined according to their *R* <sub>f</sub> values and three different fractions were obtained. Amount of each fraction and their TLC patterns are recorded in Tab-2.6

### 2.8.3.1 Isolation of the pure compound-3

![Scheme-4: Isolation of the pure compound-3](image)

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Solvent (ratio)</th>
<th>Amount (mg)</th>
<th>TLC pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPD&lt;sub&gt;3_1&lt;/sub&gt;</td>
<td>50</td>
<td>79</td>
<td>Single sport</td>
</tr>
<tr>
<td>VPD&lt;sub&gt;3_2&lt;/sub&gt;</td>
<td>20</td>
<td>49</td>
<td>Three sports</td>
</tr>
<tr>
<td>VPD&lt;sub&gt;3_3&lt;/sub&gt;</td>
<td>-</td>
<td>40</td>
<td>Single sport with tail</td>
</tr>
</tbody>
</table>

**Table-2.6** TLC pattern of different sub-fractions of fraction VPD<sub>3</sub> (0.373 g)
2.8.3.2 Purification of the sub-fraction VPD$_{3.1}$ by silica gel column chromatography

A small glass column was made with silica gel (5.0 g) DCM. The sample VPD$_{3.1}$ (79 mg) was dissolved in a minimum volume of DCM and it was then applied into the column. The sample was eluted by using 0.5 % MeOH in CH$_2$Cl$_2$ as a mobile phase and a single compound (Compound-3, 32 mg) was obtained.

2.8.3.3 Characterization of the compound-3

**Appearance:**

$R_f$ value: 0.1 (1% acetone in n-hexane solvent system).

**Solubility:** soluble in CH$_2$Cl$_2$ solvent.

**Percent yield:** $3.2\times10^{-3}$ (in 1000 g)

2.8.3.4 Ultraviolet (UV) spectroscopy of Compound-3

The UV spectrum of the compound-3 (Fig: 3.3.1) has absorption at $\lambda_{\text{max}}$ 308 nm in methanol and calculated $\lambda_{\text{max}}$ is 313 nm.

2.8.3.5 Infrared (IR) spectroscopy of compound-3

The IR spectrum (in KBr) of the compound-3 (Fig: 3.3.2) has important absorption at 1599.78 (-C=C- ), 2959.44 and 2930.31 (-C-H str aliphatic), 2860.62 (C-H str in CH$_2$ and CH$_3$), 1726.99 (C=O), 1122.03 (C-C), 1270 (C-O str), 794.96 cm$^{-1}$ (CH$_2$ rocking).

2.8.3.6 $^1$H-NMR spectroscopy of Compound-3

The $^1$H-NMR spectrum (400 MHz, CDCl$_3$) of the compound-3 (Fig: 3.3.3, 3a and 3b) has signals at $\delta_H$ (ppm) 7.6 (1H, t), 7.4 (1H, t), 4.2 (2H, q), 1.6 (2H, q), 1.4 (2H, h), 1.3 (6H, m), 1.2(2H, s), 0.9 (3H, m) and 0.9 (3H, m).

2.8.3.7 $^{13}$C-NMR spectroscopy of Compound-3

The $^{13}$C-NMR spectrum (100 MHz) in CDCl$_3$ of the compound-3 (Fig: 3.3.4, 4a and 4b) has 15 signals at $\delta_C$ (ppm) 10.96, 14.10, 22.98, 23.78, 28.94, 29.70, 30.23, 38.64, 38.94, 68.16, 128.80, 129.03, 130.86, 132.49 and 167.73.

2.8.4.1 Investigation of ethyl acetate extract

2.8.4.2 Fractionation of ethyl acetate soluble part (VPE) of *Vitex peduncularis* by silica gel column chromatography

**a. Preparation of column**

A glass column (68 cm X 13 cm; id.) was packed with column grade silica gel (127 g: 230-400 mesh, ASTM, Merck) using $n$-hexane as the equilibrating solvent. The column was then equilibrated with three column volumes of $n$-hexane.

Length of column = 68 cm

Diameter of column = 13 cm
Bed of silica = 34 cm
Bed of sample = 3.5 cm

b. Preparation of the sample
The ethyl acetate soluble part (VPE, 10.9 g) was dissolved in \( n \)-hexane and column grade silica gel (10 g) was added to it. The suspension was thoroughly mixed and evaporated to dryness. The dried material was made into fine powder by a mortar and pestle.

c. Application of the sample to the column
The dried powdered sample was applied into the top of the column. The sample was then eluted with \( n \)-hexane and the polarity of the solvent was gradually increased by the addition of dichloromethane followed by methanol. The eluted fractions were collected in 45 conical flasks (Table-2.7).

Table-2.7: Fractionation of ethyl acetate part (VPE) by silica gel column chromatography

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Solvent System</th>
<th>Amount (cc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n )-Hexane</td>
<td>CH(_2)Cl(_2)</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>99</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>98</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>97</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>95</td>
</tr>
<tr>
<td>11-15</td>
<td>-</td>
<td>93</td>
</tr>
<tr>
<td>16-19</td>
<td>-</td>
<td>90</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>85</td>
</tr>
<tr>
<td>21-22</td>
<td>-</td>
<td>80</td>
</tr>
<tr>
<td>23-24</td>
<td>-</td>
<td>70</td>
</tr>
<tr>
<td>25-26</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>27-30</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>31-35</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>36-40</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>41-42</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>43-45</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
All the collected samples were examined by TLC and were combined according to their Rf values. Ten different fractions were obtained and amount of each fraction and their TLC patterns are recorded in Table-2.8.

**2.8.4.3 Isolation of the pure compound-4 and compound-5**

*Ethyl acetate extract VPE (10.9 g)*

Silica gel column (230-400 mesh) using *n*-hexane, DCM and MeOH as solvent

VPE<sub>1</sub> (0.11 g) → VPE<sub>2</sub> (0.91 g) → VPE<sub>3</sub> (1.21 g) → VPE<sub>4</sub> (0.50 g) → VPE<sub>5</sub> (0.62 g) → VPE<sub>6</sub> (0.42 g) → VPE<sub>7</sub> (0.21 g) → VPE<sub>5</sub> (0.71 g) → VPE<sub>9</sub> (0.51 g) → VPE<sub>10</sub> (1.32 g)

- **Filter**
- **Precipitated part**
- **Wash & dry**
- **P-HPLC**

**Compound-4**

(11 mg)

- VPE<sub>10,1</sub> (90 mg)
- VPE<sub>10,2</sub> (59 mg)
- VPE<sub>10,3</sub> (70 mg)

- Preparative HPLC

**Compound-5**

(49 mg)

Scheme-3: Isolation of the pure compound-4 and compound-5
Table-2.8: Different fractions of ethyl acetate extract (VPE) combined by TLC pattern

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Solvent (ratio)</th>
<th>Amount (g)</th>
<th>TLC pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPE₁</td>
<td>25 75 -</td>
<td>0.11</td>
<td>Four spots</td>
</tr>
<tr>
<td>VPE₂</td>
<td>20 80 -</td>
<td>0.91</td>
<td>Separated two spots</td>
</tr>
<tr>
<td>VPE₃</td>
<td>15 85 -</td>
<td>1.21</td>
<td>Elongated two spots</td>
</tr>
<tr>
<td>VPE₄</td>
<td>15 85 -</td>
<td>0.50</td>
<td>Single spot with tailing</td>
</tr>
<tr>
<td>VPE₅</td>
<td>- 100 -</td>
<td>0.62</td>
<td>Single two spots</td>
</tr>
<tr>
<td>VPE₆</td>
<td>- 95 5</td>
<td>0.42</td>
<td>Broad tail</td>
</tr>
<tr>
<td>VPE₇</td>
<td>- 95 5</td>
<td>0.21</td>
<td>two spots with tail</td>
</tr>
<tr>
<td>VPE₈</td>
<td>- 90 10</td>
<td>0.71</td>
<td>Broad tail</td>
</tr>
<tr>
<td>VPE₉</td>
<td>- 90 10</td>
<td>0.51</td>
<td>Single spot</td>
</tr>
<tr>
<td>VPE₁₀</td>
<td>- 80 20</td>
<td>1.32</td>
<td>Three spots with tail</td>
</tr>
</tbody>
</table>

2.8.4.4 Purification of fraction VPE₉ (0.51 g) by filtration method
A white solid was found in fraction VPE₉. It was filtered and washed with acetone and a pure compound (compound-4, 11 mg) was obtained.

2.8.4.5 Characterization of compound-4

**Appearance:**

**Solubility:** Soluble in Methanol.

**R_f value:** 0.34 (10% MeOH in dichloromethane solvent system).

**Percent yield:** 1.1×10^{-3} (in 1000 g)

**Physical state:** white amorphous solid

2.8.4.6 Infrared (IR) spectroscopy of Compound-4

The IR spectrum (in KBr) of the compound-4 (Fig: 3.4.1) has important transmittance at 3484.19 (OH stretching), 2921.34 & 2852.20 (C-H stretching), 1728.19 (C=O), 1597.06, 1504.36, 1453.98 (aromatic C=C), 1279.66 (C-O) and 805.88 & 762.65 cm⁻¹ (meta & para substituted benzene ring).

2.8.4.7 ^1^H-NMR spectroscopy of Compound-4

The ^1^H-NMR spectrum (400 MHz, CD₃OD) of the compound-4 (Fig: 3.4.2 and 2a) has signals at δ_{H} (ppm) 7.5 (2H, s), 6.8 (1H, d) and 3.88 (3H, s).
2.8.4.8 $^{13}$C-NMR spectroscopy of Compound-4

The $^{13}$C-NMR spectrum (100 MHz, CD$_3$OD) of the compound-4 (Fig: 3.4.3 and 3a) has signals at $\delta_C$ (ppm) 168.67, 151.27, 147.28, 123.88, 121.80, 114.45, 112.55 and 55.06.

2.8.5.1 Characterization of compound-5

Appearance:
Solubility: Soluble in Methanol.

$R_f$ value: 0.34 (10%o MeOH in dichloromethane solvent system).

Percent yield: $4.9 \times 10^{-3}$ (in 1000 g)

Physical state: green color solid

$^1$H-NMR, and $^{13}$C-NMR showed the compound-5 was not in pure state. Therefore further purification is required.
CHAPTER THREE

RESULT & DISCUSSION
3.1 Preliminary investigation of the plant material

3.1.1 Plant material
A species of the Verbenaceae family, *Vitex peduncularis* has been investigated in this work. The bark was used during this research.

3.1.2 Extraction of the plant material
The air-dried and powdered plant material (1 kg) was suspended in methanol for three weeks with shaking after every two hour for the purpose of cold extraction. Every week the extract was filtered through clean white cloth and finally with Whatman No.1 filters paper. The volume of the filtrate was concentrated with a rotary vacuum evaporator at low temperature (not more than 40°C) under reduced pressure.

3.1.3 Isolation and characterization of compounds
From the crude extracts, pure compounds were isolated applying various chromatographic techniques. The isolated pure compounds were then characterized using different spectroscopic techniques and reported literature study.

3.2 Characterization of isolated compounds from *Vitex peduncularis*

3.2.1.1 Characterization of Compound-1 as triterpenoid
The R_f value of the compound is 0.617 (DCM : Methanol = 4.9:0.1). It is soluble in dichloromethane. It was isolated as white amorphous solid (16.0 mg) from the CH_2Cl_2 soluble fraction of methanol extract of *Vitex peduncularis* using repeated column chromatography. This compound-1 showed a pink colour spot when developed with ceric sulphate spraying reagent on TLC plate indicating that the compound might be a triterpenoid type compound.

2α, 3β-dihydroxy olean-12-en-27, 28-dioic acid

Fig: 3.1 Structure of compound-1
3.2.1.2 Characterization of Compound-1 by spectroscopic methods

The structure of the compound-1 (Fig: 3.1) has been established by IR, $^1$H-NMR, $^{13}$C-NMR and DEPT-135 spectral evidences.

3.2.1.3 Infrared (IR) spectroscopy of Compound-1

The IR spectrum (Fig-3.1.1) of compound-1 showed absorption frequency at 3419.6 cm$^{-1}$ due to the $\text{—O—H}$ stretching. Sharp absorption frequencies at 2941.2 cm$^{-1}$ were due to aliphatic C—H asymmetric and symmetric stretching vibrations. Weak absorption band at 1652.9 cm$^{-1}$ indicated the non-conjugated double bond (C=C) stretching vibrations. The absorption band at 1693.4 cm$^{-1}$ indicated the C=O stretching vibrations in $\text{—COOH}$ group. Absorption band at 1458.1 cm$^{-1}$ was due to $\text{—CH}_2$— bending vibrations which is characteristic of methylene ($\text{—CH}_2$—) group. Absorption frequencies at 1386.7 cm$^{-1}$ and 1365.5 cm$^{-1}$ were due to methyl ($\text{—CH}_3$) asymmetric and symmetric bending respectively. The absorption bands at 1276.8 cm$^{-1}$ and 1234.4 cm$^{-1}$ with about the equal intensities were the characteristics peaks of geminal dimethyl groups. The absorption frequency at 1035.7 cm$^{-1}$ was due to $\text{C—O}$ stretching vibration.

3.2.1.4 $^1$H-NMR spectroscopy of Compound-1

The $^1$H-NMR spectrum of compound-1 (Fig-3.1.2, 2a and 2b) showed the presence of six methyl groups at 0.73 (s), 0.84 (s), 0.96 (s), 0.92 (s), 0.94 (s), and 1.01 (s). The one proton signal at 4.00 ppm (1H, bd, $J = 11.6$ Hz) with coupling constant of 11.6 Hz indicated the presence of an oxygenated methine proton with β-position in the cyclohexane ring adjacent to a methane proton ($\text{>CH—}$) and a quaternary carbon. The singlet at 3.41 ppm (1H, S) indicated the presence of another methane proton at α-position in the cyclohexane ring. Another one proton signal at 5.27 ppm (broad doublet) with a coupling constant 12.4 Hz indicated the presence of double bond in the ring system and it was in between a quaternary carbon and a methylene carbon where two protons were not symmetrical.

Table-3.1: $^1$H-NMR data of compound-1 compared with published data$^{95}$ of triterpenoid

<table>
<thead>
<tr>
<th>Position of carbon</th>
<th>Type of proton</th>
<th>Compound-1 (δ_H ppm)</th>
<th>Triterpenoid (δ_H ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>$\text{—CH}_2$—</td>
<td>1.6, 1.2 (2H, m)</td>
<td>1.6, 1.1</td>
</tr>
<tr>
<td>C-2</td>
<td>$\text{&gt;CH—}$</td>
<td>3.41 (1H, s)</td>
<td>3.38</td>
</tr>
<tr>
<td>C-3</td>
<td>$\text{&gt;CH—}$</td>
<td>4.0 (1H, d, $J=11.6$ Hz)</td>
<td>3.9</td>
</tr>
<tr>
<td>C-5</td>
<td>$\text{&gt;CH—}$</td>
<td>1.08 (1H, s)</td>
<td>1.0</td>
</tr>
<tr>
<td>C-6</td>
<td>$\text{—CH}_2$—</td>
<td>1.6, 1.2 (2H, m,)</td>
<td>1.5, 1.2</td>
</tr>
<tr>
<td>C-7</td>
<td>$\text{—CH}_2$—</td>
<td>1.5, 1.2 (2H, m,)</td>
<td>1.5, 1.2</td>
</tr>
<tr>
<td>C-9</td>
<td>$\text{&gt;CH—}$</td>
<td>1.46 (1H, s)</td>
<td>1.50</td>
</tr>
<tr>
<td>C-11</td>
<td>$\text{—CH}_2$—</td>
<td>2.0, 1.7 (2H, m)</td>
<td>2.0, 1.7</td>
</tr>
</tbody>
</table>
### 3.2.1.5 $^{13}$C-NMR Spectroscopy of Compound-1

The compound-1 contained 30 carbons as it contained 30 signals in the $^{13}$C-NMR spectrum (Fig-3.1.3 and 3a). The $^{13}$C-NMR data of the compound-1 was compared with those of known triterpenoids published\textsuperscript{95} and the assignment was done accordingly. Two signals at 143.67 ppm and 122.46 ppm were due to two olefinic carbons of C-13 and C-12 respectively and that was the most characteristics carbon signals of olean-12-enes series of triterpenoids. The most deshielded chemical shift at 143.67 ppm should be due to the presence of a carboxyl group at C-14. The signal at 182.82 and 178.61 ppm indicated the presence of two carboxylic groups (—COOH). A comparative literature study suggested the position of these two —COOH groups at C-27 and C-28 positions. The signals for methyl, methylene, methine and quaternary carbons present in the compound were distinguished by the $^{13}$C-NMR (Fig-3.1.3 and 3a) and DEPT-135° (Fig-3.1.4 and 4a) experiments. Six signals of methine carbons at 66.53, 78.90, 52.5, 48.12, 122.48 and 47.34 ppm were assigned to C-2, C-3, C-5, C-9, C-12 and C-18 positions respectively. Nine signals at 41.65, 18.01, 36.60, 22.91, 27.62, 27.94, 41.91, 36.70 and 30.60 were assigned to the methylene carbons C-1, C-6, C-7, C-11, C-15, C-16, C-19, C-21 and C-22 respectively. Six signals at 28.50, 17.10, 16.49, 16.35, 33.07 and 23.59 ppm were assigned to six methyl carbons at C-23, C-24, C-25, C-26, C-29 and C-30 respectively. Two signals 48.10 and 46.49 ppm were assigned for C-14 and C-17 positions respectively. Rest of the signals of the spectrum at 38.42, 38.24, 38.24, 143.67 and 32.40 ppm were accounted for the quaternary carbons at C-4, C-8, C-10, C-13 and C-20 respectively. The $^{13}$C-NMR data of the compound-1 fitted well with reported pentacyclic triterpenoids\textsuperscript{96}.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Chemical Group</th>
<th>Chemical Shift (ppm)</th>
<th>J (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-12</td>
<td>&gt;CH—</td>
<td>5.27</td>
<td>12.4</td>
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<tr>
<td>C-15</td>
<td>—CH₂—</td>
<td>1.6, 1.4</td>
<td></td>
</tr>
<tr>
<td>C-16</td>
<td>—CH₂—</td>
<td>1.6, 1.2</td>
<td></td>
</tr>
<tr>
<td>C-18</td>
<td>&gt;CH—</td>
<td>2.12</td>
<td>11.2</td>
</tr>
<tr>
<td>C-19</td>
<td>—CH₂—</td>
<td>1.5, 1.1</td>
<td></td>
</tr>
<tr>
<td>C-21</td>
<td>—CH₂—</td>
<td>1.5, 1.1</td>
<td></td>
</tr>
<tr>
<td>C-22</td>
<td>—CH₂—</td>
<td>1.9, 1.4</td>
<td></td>
</tr>
<tr>
<td>C-23</td>
<td>—CH₃</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>C-24</td>
<td>—CH₃</td>
<td>0.84</td>
<td></td>
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<tr>
<td>C-25</td>
<td>—CH₃</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>C-26</td>
<td>—CH₃</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>C-29</td>
<td>—CH₃</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>C-30</td>
<td>—CH₃</td>
<td>0.94</td>
<td></td>
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</table>
Table-3.2: $^{13}$C-NMR data of compound-1 compared with published data$^{95, 96}$ of triterpenoid

<table>
<thead>
<tr>
<th>Position of carbon</th>
<th>Type of carbon</th>
<th>Compound-1 (δ$_c$ ppm)</th>
<th>Triterpenoid (δ$_c$ ppm)</th>
<th>Chemical shift (δ$_H$ ppm)</th>
<th>Coupling constant (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>—CH$_3$—</td>
<td>41.6</td>
<td>38.7</td>
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<tr>
<td>C-2</td>
<td>&gt;CH—</td>
<td>66.5</td>
<td>68.1</td>
<td>3.41 (2H, s)</td>
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</tr>
<tr>
<td>C-3</td>
<td>&gt;CH—</td>
<td>78.9</td>
<td>78.0</td>
<td>4.00</td>
<td>(1H, d), 11.6Hz</td>
</tr>
<tr>
<td>C-4</td>
<td>&gt;C&lt;</td>
<td>38.4</td>
<td>39.8</td>
<td></td>
<td></td>
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<tr>
<td>C-5</td>
<td>&gt;CH—</td>
<td>52.5</td>
<td>55.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-6</td>
<td>—CH$_2$—</td>
<td>18.0</td>
<td>18.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-7</td>
<td>—CH$_2$—</td>
<td>36.6</td>
<td>33.8</td>
<td></td>
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</tr>
<tr>
<td>C-8</td>
<td>&gt;C&lt;</td>
<td>38.2</td>
<td>39.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-9</td>
<td>&gt;CH—</td>
<td>48.1</td>
<td>48.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-10</td>
<td>&gt;C&lt;</td>
<td>38.2</td>
<td>38.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-11</td>
<td>—CH$_2$—</td>
<td>22.9</td>
<td>23.7</td>
<td></td>
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</tr>
<tr>
<td>C-12</td>
<td>&gt;CH—</td>
<td>122.4</td>
<td>121.7</td>
<td>5.27</td>
<td>(1H, d), 12.4 Hz</td>
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<tr>
<td>C-13</td>
<td>&gt;C&lt;</td>
<td>143.6</td>
<td>145.0</td>
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<tr>
<td>C-14</td>
<td>&gt;C&lt;</td>
<td>48.1</td>
<td>48.4</td>
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<tr>
<td>C-15</td>
<td>—CH$_2$—</td>
<td>27.6</td>
<td>26.2</td>
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</tr>
<tr>
<td>C-16</td>
<td>—CH$_2$—</td>
<td>27.9</td>
<td>27.0</td>
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</tr>
<tr>
<td>C-17</td>
<td>&gt;C&lt;</td>
<td>46.4</td>
<td>47.2</td>
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<tr>
<td>C-18</td>
<td>&gt;CH—</td>
<td>47.3</td>
<td>47.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-19</td>
<td>—CH$_2$—</td>
<td>45.8</td>
<td>46.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-20</td>
<td>&gt;C&lt;</td>
<td>32.4</td>
<td>31.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-21</td>
<td>—CH$_2$—</td>
<td>36.7</td>
<td>34.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-22</td>
<td>—CH$_2$—</td>
<td>30.6</td>
<td>31.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-23</td>
<td>—CH$_3$</td>
<td>28.5</td>
<td>28.1</td>
<td>0.73</td>
<td>(3H, s)</td>
</tr>
<tr>
<td>C-24</td>
<td>—CH$_3$</td>
<td>17.1</td>
<td>17.1</td>
<td>0.84</td>
<td>(3H, s)</td>
</tr>
<tr>
<td>C-25</td>
<td>—CH$_3$</td>
<td>16.3</td>
<td>16.6</td>
<td>1.01</td>
<td>(3H, s)</td>
</tr>
<tr>
<td>C-26</td>
<td>—CH$_3$</td>
<td>16.5</td>
<td>16.8</td>
<td>0.89</td>
<td>(3H, s)</td>
</tr>
<tr>
<td>C-27</td>
<td>—COOH</td>
<td>178.8</td>
<td>178.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-28</td>
<td>—COOH</td>
<td>182.8</td>
<td>180.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-29</td>
<td>—CH$_3$</td>
<td>33.0</td>
<td>33.8</td>
<td>0.92</td>
<td>(3H, s)</td>
</tr>
<tr>
<td>C-30</td>
<td>—CH$_3$</td>
<td>23.5</td>
<td>23.7</td>
<td>0.94</td>
<td>(3H, s)</td>
</tr>
</tbody>
</table>

The molecular formula of compound-1 was assigned as C$_{30}$H$_{46}$O$_6$ from the $^1$H-NMR, $^{13}$C-NMR and DEPT-135 data. By the studies of IR, $^1$H-NMR and $^{13}$C-NMR, the compound-1 was assigned as 2α, 3β-dihydroxy olean-12-en-27, 28-dioic acid. Literature survey showed that the compound-1 was collated for the first time from the plant *Vitex peduncularis*. 


Fig-3.1.1 FT-IR Spectra of Compound-1
Fig-3.1.2 $^1$H-NMR Spectra of Compound-1
Fig-3.1.2a Expanded $^1$H-NMR Spectra of Compound-1
Fig-3.1.2b Expanded $^1$H-NMR Spectra of Compound-1
Chapter Three

Result & Discussion

Fig-3.1.3 $^{13}$C-NMR Spectra of Compound-1

$2\alpha, 3\beta$-dihydroxy olean-12-en-27, 28-dioic acid
Fig-3.1.3a Expanded $^{13}$C-NMR Spectra of Compound-1
Fig-3.1.4 DEPT-135 NMR Spectra of Compound-1
Fig-5.1.4a Expanded DEPT-135 NMR Spectra of Compound-1
3.2.2.1 Characterization of Compound-2 as triterpenoid

The compound-2 was isolated as white amorphous powder (22 mg) from the CH₂Cl₂ soluble part of Vitex peduncularis by repeated column chromatography. It gave a pink colour with ceric sulphate spraying reagent on TLC plate indicating that the compound might be a triterpenoids. It is soluble in few drops MeOH in CH₂Cl₂ solvent. The R₇ value of the compound is 0.514 (DCM: MeOH=4.7:0.2).

![3.2 Structure of compound-2](image)

2α, 3β, 23-trihydroxy olean-12-en-27,28-dioic acid

Fig: 3.2 Structure of compound-2

3.2.2.2 Characterization of Compound-2 by spectroscopic methods

The structure of the compound-1 Fig: (3.2) has been established by IR, ¹H-NMR, ¹³C-NMR and DEPT-135 spectral evidences.

3.2.2.3 Infrared (IR) spectroscopy of Compound-2

The IR spectrum (Fig-3.2.1) of compound-2 showed absorption of hydroxy groups at 3419.6 cm⁻¹. The absorption at 2929.7 cm⁻¹ was the indication of C—H asymmetric and symmetric stretching vibrations. The absorption band at 1652.9 cm⁻¹ indicated the non-conjugated double bond (C=C) stretching vibrations. The absorption band at 1693.4 cm⁻¹ indicated the C=O stretching vibrations in —COOH group. Absorption at 1458.1 cm⁻¹ was due to —CH₂— bending of methylene group. The absorption frequencies at 1379 cm⁻¹ indicated the presence of methyl groups. Two absorption bands 1271 cm⁻¹ at 1234.4 cm⁻¹ with about the equal intensities were the characteristics peak for geminal dimethyl groups. The absorption frequency at 1039.6 cm⁻¹ was due to C—O stretching vibration.
3.2.2.4 $^1$H-NMR spectroscopy of Compound-2

The $^1$H-NMR spectrum of compound-2 (Fig-3.2.2, 2a and 2b) showed the signals for five methyl groups. The broad singlet at 3.84 ppm is the indication of an oxygenated methane proton in cyclohexane ring adjacent to a methane proton ($>\text{CH}–$) and a quaternary carbon. The broad singlet at 3.56 is the indication of another oxygenated methane proton. A proton signal (1H, d, J= 12.4 Hz, H-12) at 5.20 ppm with coupling constant 12.4 Hz clearly showed the presence of a double bond in the ring system in between a methane proton and a quaternary carbon. Five signals at 0.67, 0.91, 0.71, 0.80, and 0.83 ppm each for three protons indicated the presence of five methyl group. A double of doublet with the signals at 3.45 ppm (1H, d, J=11.2 Hz) and at 3.37 ppm (1H, d, J = 10.8 Hz) proton which linked with same carbon and indicated the presence of an exocyclic —CH$_2$— group.

Table-3.3: $^1$H-NMR data of compound-2 compared with published data$^5$ of triterpenoid

<table>
<thead>
<tr>
<th>Position of carbon</th>
<th>Type of proton</th>
<th>Compound-2 (δ$_H$ ppm)</th>
<th>Triterpenoid (δ$_H$ ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>—CH$_2$—</td>
<td>1.6, 1.2 (2H, m)</td>
<td>1.6, 1.1</td>
</tr>
<tr>
<td>C-2</td>
<td>&gt;CH—</td>
<td>3.56 (1H, s)</td>
<td>3.48</td>
</tr>
<tr>
<td>C-3</td>
<td>&gt;CH—</td>
<td>3.84 (1H, s, br)</td>
<td>3.9</td>
</tr>
<tr>
<td>C-5</td>
<td>&gt;CH—</td>
<td>1.04 (1H, s)</td>
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<tr>
<td>C-6</td>
<td>—CH$_2$—</td>
<td>1.5, 1.1 (2H, m,)</td>
<td>1.5, 1.2</td>
</tr>
<tr>
<td>C-7</td>
<td>—CH$_2$—</td>
<td>1.5, 1.1 (2H, m,)</td>
<td>1.5, 1.2</td>
</tr>
<tr>
<td>C-9</td>
<td>&gt;CH—</td>
<td>1.50 (1H, s)</td>
<td>1.50</td>
</tr>
<tr>
<td>C-11</td>
<td>—CH$_2$—</td>
<td>2.1, 1.8 (2H, m)</td>
<td>2.0, 1.7</td>
</tr>
<tr>
<td>C-12</td>
<td>&gt;CH—</td>
<td>5.21 (1H, d, J=12.4 Hz)</td>
<td>5.12</td>
</tr>
<tr>
<td>C-15</td>
<td>—CH$_2$—</td>
<td>1.6, 1.2 (2H, m)</td>
<td>1.6, 1.3</td>
</tr>
<tr>
<td>C-16</td>
<td>—CH$_2$—</td>
<td>1.6, 1.2 (2H, m)</td>
<td>1.5, 1.2</td>
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<tr>
<td>C-18</td>
<td>&gt;CH—</td>
<td>2.2 (1H, s)</td>
<td>2.12</td>
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<tr>
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<td>—CH$_2$—</td>
<td>1.5, 1.1 (2H, m)</td>
<td>1.5, 1.2</td>
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<tr>
<td>C-21</td>
<td>—CH$_2$—</td>
<td>1.5, 1.1 (2H, m)</td>
<td>1.5, 1.2</td>
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<tr>
<td>C-22</td>
<td>—CH$_2$—</td>
<td>1.9, 1.5 (2H, m)</td>
<td>1.8, 1.5</td>
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<td>C-23</td>
<td>—CH$_2$—</td>
<td>3.4, 3.3 (2H, dd, J=11 Hz)</td>
<td>3.5, 3.3</td>
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<tr>
<td>C-24</td>
<td>—CH$_3$</td>
<td>0.67 (3H, s)</td>
<td>0.78</td>
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<tr>
<td>C-25</td>
<td>—CH$_3$</td>
<td>0.91 (3H, s)</td>
<td>1.0</td>
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<tr>
<td>C-26</td>
<td>—CH$_3$</td>
<td>0.71 (3H, s)</td>
<td>0.89</td>
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<tr>
<td>C-29</td>
<td>—CH$_3$</td>
<td>0.80 (3H, s)</td>
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<td>C-30</td>
<td>—CH$_3$</td>
<td>0.83 (3H, s)</td>
<td>0.91</td>
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</table>
3.2.2.5 $^{13}$C-NMR spectroscopy of Compound-2

The presence of 30 carbons in the $^{13}$C-NMR spectrum revealed that the compound-2 also contained 30 carbons (Fig.3.2.3, 3a, 3b, and 3c). The basic skeleton of compound-2 was thus assigned as a pentacyclic triterpenoids by comparing the $^{13}$C-NMR and DEPT-135 data of the compound with the literature values.

Table 3.4: $^{13}$C-NMR data of compound-2 compared with published data\(^{95,96}\) of triterpenoid

<table>
<thead>
<tr>
<th>Position of carbon</th>
<th>Type of carbon</th>
<th>Compound-2 ($\delta_c$ ppm)</th>
<th>Triterpenoid ($\delta_c$ ppm)</th>
<th>Chemical shift ($\delta_H$ ppm)</th>
<th>Coupling constant (J)</th>
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</thead>
<tbody>
<tr>
<td>C-1</td>
<td>$\text{CH}_2$</td>
<td>41.2</td>
<td>38.7</td>
<td></td>
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</tr>
<tr>
<td>C-2</td>
<td>$\text{CH}$</td>
<td>66.2</td>
<td>68.1</td>
<td>3.56 (1H, s)</td>
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</tr>
<tr>
<td>C-3</td>
<td>$\text{CH}$</td>
<td>78.1</td>
<td>78.0</td>
<td>3.84 (1H, s)</td>
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</tr>
<tr>
<td>C-4</td>
<td>$\text{C}&lt;\text{C}$</td>
<td>39.4</td>
<td>39.8</td>
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<tr>
<td>C-5</td>
<td>$\text{CH}$</td>
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<td>55.3</td>
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<tr>
<td>C-6</td>
<td>$\text{CH}_2$</td>
<td>17.7</td>
<td>18.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-7</td>
<td>$\text{CH}_2$</td>
<td>33.7</td>
<td>33.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-8</td>
<td>$\text{C}&lt;\text{C}$</td>
<td>39.2</td>
<td>39.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-9</td>
<td>$\text{CH}$</td>
<td>47.2</td>
<td>47.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-10</td>
<td>$\text{C}&lt;\text{C}$</td>
<td>37.7</td>
<td>37.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-11</td>
<td>$\text{CH}_2$</td>
<td>23.3</td>
<td>23.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-12</td>
<td>$\text{CH}$</td>
<td>121.9</td>
<td>121.7</td>
<td>5.20 (1H, d), 12.4 Hz</td>
<td></td>
</tr>
<tr>
<td>C-13</td>
<td>$\text{C}&lt;\text{C}$</td>
<td>143.9</td>
<td>145.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-14</td>
<td>$\text{C}&lt;\text{C}$</td>
<td>47.8</td>
<td>48.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-15</td>
<td>$\text{CH}_2$</td>
<td>27.8</td>
<td>26.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-16</td>
<td>$\text{CH}_2$</td>
<td>27.5</td>
<td>27.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-17</td>
<td>$\text{C}&lt;\text{C}$</td>
<td>47.7</td>
<td>47.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-18</td>
<td>$\text{CH}$</td>
<td>47.1</td>
<td>47.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-19</td>
<td>$\text{CH}_2$</td>
<td>45.8</td>
<td>46.8</td>
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<td></td>
</tr>
<tr>
<td>C-20</td>
<td>$\text{C}&lt;\text{C}$</td>
<td>29.5</td>
<td>31.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-21</td>
<td>$\text{CH}_2$</td>
<td>36.7</td>
<td>34.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-22</td>
<td>$\text{CH}_2$</td>
<td>32.4</td>
<td>31.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-23</td>
<td>$\text{CH}_3\text{OH}$</td>
<td>70.6</td>
<td>65.7</td>
<td>3.4, 3.3 (2H, d), 11 Hz</td>
<td></td>
</tr>
<tr>
<td>C-24</td>
<td>$\text{CH}_3$</td>
<td>16.0</td>
<td>16.8</td>
<td>0.67 (3H, s)</td>
<td></td>
</tr>
<tr>
<td>C-25</td>
<td>$\text{CH}_3$</td>
<td>16.8</td>
<td>16.6</td>
<td>0.91 (3H, s)</td>
<td></td>
</tr>
<tr>
<td>C-26</td>
<td>$\text{CH}_3$</td>
<td>17.2</td>
<td>16.8</td>
<td>0.71 (3H, s)</td>
<td></td>
</tr>
<tr>
<td>C-27</td>
<td>$\text{COOH}$</td>
<td>180.8</td>
<td>178.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-28</td>
<td>$\text{COOH}$</td>
<td>181.0</td>
<td>180.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-29</td>
<td>$\text{CH}_3$</td>
<td>32.9</td>
<td>33.8</td>
<td>0.80 (3H, s)</td>
<td></td>
</tr>
<tr>
<td>C-30</td>
<td>$\text{CH}_3$</td>
<td>23.5</td>
<td>23.7</td>
<td>0.83 (3H, s)</td>
<td></td>
</tr>
</tbody>
</table>
Two signals at 121.95 and 143.92 ppm were due to the two olefinic carbons of C-12 and C-13 respectively which are characteristic carbon signals for olean-12-type triterpenoid. The most downfield chemical shift at 143.92 ppm must be due to the presence of carboxyl group (—COOH) attached at C-14 quaternary olefinic carbon. The signals at 80.88 and 181.07 ppm were due to the presence of two (—COOH) carboxylic groups. A comparative study with the literature survey it was suggested that the position of these two —COOH groups should be at C-27 and C-28 which were attached to the C-14 and C-17 carbons respectively.

The \(^{13}\)C-NMR (Fig-3.2.3) and DEPT-135° (Fig-3.2.4) expressed the presence of methyl, methylene, methine and quaternary carbons. Five signals for methyl group at 16.00, 16.89, 17.22, 32.96, 23.53 ppm assigned to C-24, C-25, C-26, C-29 and C-30 positions respectively. Ten signals for methylene carbons at 41.21, 17.71, 33.75, 23.30, 27.84, 27.50, 45.84, 36.70, 32.42 and 70.69 ppm were assigned to C-1, C-6, C-7, C-11, C-15, C-16, C-19, C-21, C-22 and C-23 respectively. Six signals for methine at 66.21, 78.12, 52.66, 47.27, 121.95 and 38.95 ppm were assigned for C-2, C-3, C-5, C-9, C-12 and C-18 respectively. Rest of the signals at 39.46, 39.24, 37.78, 47.83, 47.70 and 29.59 ppm was assigned as quaternary carbons, for C-4, C-8, C-10, C-14, C-17 and C-20 positions respectively. The molecular formula of compound-2 was assigned as C\(_{30}\)H\(_{46}\)O\(_7\) from the \(^1\)H-NMR, \(^{13}\)C-NMR (Fig-3.2.3, 3a, 3b, and 3c) and DEPT-135° (Fig-3.2.4 and 4a) data. By analyzing the IR, \(^1\)H-NMR and \(^{13}\)C-NMR data of compound-2 with the reported triterpenoids in the literature\(^96\), the structure of compound-2 was determined as 2α, 3β, 23-tri-hydroxy olean-12-en-27, 28-dioic acid. As per literature review, the compound-2 was isolated for the first time from \textit{Vitex peduncularis}. 
Fig-3.2.1 FT-IR Spectra of Compound-2
Fig-3.2.2 $^1$H-NMR Spectra of Compound-2
Fig-3.2.2a Expanded $^1$H-NMR Spectra of Compound-2
Fig-3.2.2b Expanded $^1$H-NMR Spectra of Compound-2
Fig-3.2.3 $^{13}$C-NMR Spectra of Compound-2

2α, 3β, 23-trihydroxy olean-12-ен-27,28-dioic acid
Fig-3.2.3a Expanded $^{13}$C-NMR Spectra of Compound-2
Fig-3.2.3b Expanded $^{13}$C-NMR Spectra of Compound-2
Fig 3.2.3c Expanded $^13$C-NMR Spectra of Compound-2
Fig-3.2.4 DEPT-135 NMR Spectra of Compound-2
Fig-3.2.4a Expanded DEPT-135 NMR Spectra of Compound-2
3.2.3.1 Characterization of Compound-3 as isocoumarin

The compound-3 is a pink color solid compound. The R_f value of the compound is 0.1 (n-hexane : Acetone ≡ 0.9:0.1). It is soluble in dichloromethane, methanol and acetonitrile.

![Structure of compound-3](image)

Fig: 3.3 Structure of compound-3

3.2.3.2 Characterization of Compound-3 by spectroscopic methods

The structure of the compound-3 (Fig: 3.3) has been established by UV, IR, ^1_H-NMR, ^13_C-NMR, DEPT-135 and COSY-45.

3.2.3.3 Ultraviolet (UV) spectroscopy of Compound-3

The UV spectrum of the compound-3 (Fig: 3.3.1) has absorption at λ_max 308 nm in methanol and calculated λ_max is 313 nm.

3.2.3.4 Infrared (IR) spectroscopy of Compound-3

The IR Spectroscopic analysis (in KBr) of the compound-3 (Fig: 3.3.2), the observed absorption bands are at 1726.99 cm⁻¹ that is characteristic of C=O stretching. Absorption at 2959.44 cm⁻¹ is due to >C—H stretching, 2930.31 cm⁻¹ and 2866 cm⁻¹ assigned to aliphatic C—H str. Absorption at 1599.78 cm⁻¹, 1580.19 cm⁻¹ and 1462.00 cm⁻¹ indicate the >C=C< group. This compound shows the C—O absorption at 1270.54 cm⁻¹. These absorption frequencies resemble the absorption frequencies observed for isocoumarin.

3.2.3.5 ^1_H-NMR spectroscopy of Compound-3

The ^1_H-NMR spectrum of the compound-3 (Fig: 3.3.3, 3a and 3b) revealed the double triplet at same place between δ 0.88-0.92 ppm due to two methyl groups of isocoumarin. A number of multiplate between δ 1.30 - 1.33 ppm was due to three methylene protons present in the compound. The peak at 1.24 ppm showed a singlet for other methylene proton. This is because three multiplate is the same position and one at immediate previous position. The multiplate at δ 1.38-1.48 ppm indicated the presence of two methyne protons. The quartet between δ 1.64-1.68 indicated the presence of one methylene protons. The triplet between δ 4.24-4.20 revealed the presence of one methylene protons near to oxygen atom. The two
triplets between δ 7.49-7.52 and δ 7.68-7.70 indicated the presence of unsaturated two CH protons.

Table 3.5: $^1$H-NMR data of compound-3 compared with published data$^{97}$ of isocoumarin:

<table>
<thead>
<tr>
<th>Position of carbon</th>
<th>Type of proton</th>
<th>Compound-3 (δ_H ppm)</th>
<th>Isocoumarin (δ_H ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-3</td>
<td>—CH₂—</td>
<td>4.22 (2H, t, J=5.2 Hz)</td>
<td>4.22</td>
</tr>
<tr>
<td>C-4</td>
<td>—CH₂—</td>
<td>1.67 (2H, q, J=6 Hz)</td>
<td>1.67</td>
</tr>
<tr>
<td>C-5</td>
<td>&gt;CH—</td>
<td>1.41 (1H, m, J=6.8 Hz)</td>
<td>1.52</td>
</tr>
<tr>
<td>C-7</td>
<td>&gt;CH—</td>
<td>7.51 (1H, t, J=3.2 Hz)</td>
<td>6.90</td>
</tr>
<tr>
<td>C-8</td>
<td>&gt;CH—</td>
<td>7.69 (2H, t, J=3.6 Hz)</td>
<td>7.23</td>
</tr>
<tr>
<td>C-10</td>
<td>&gt;CH—</td>
<td>1.41 (1H, m, J=6.8 Hz)</td>
<td>1.53</td>
</tr>
<tr>
<td>C-11</td>
<td>—CH₂—</td>
<td>1.24 (2H, s)</td>
<td>1.32</td>
</tr>
<tr>
<td>C-12</td>
<td>—CH₃</td>
<td>0.92 (3H, m)</td>
<td>1.00</td>
</tr>
<tr>
<td>C-13</td>
<td>—CH₂—</td>
<td>1.30 (2H, m)</td>
<td>1.31</td>
</tr>
<tr>
<td>C-14</td>
<td>—CH₂—</td>
<td>1.30 (2H, m)</td>
<td>1.31</td>
</tr>
<tr>
<td>C-15</td>
<td>—CH₃</td>
<td>1.33 (2H, m)</td>
<td>1.34</td>
</tr>
<tr>
<td>C-16</td>
<td>—CH₃</td>
<td>0.88 (3H, m)</td>
<td>0.90</td>
</tr>
</tbody>
</table>

3.2.3.6 $^{13}$C-NMR spectroscopy of Compound-3

In $^{13}$C-NMR spectrum in CDCl$_3$ of the compound-3 (Fig: 3.3.4, 4a and 4b) the peak at δ 167.73 ppm (C=O) ppm confirmed the presence of carbonyl group in the compound. The peaks at δ 128.80 and 130.85 ppm (C-7 & C-8) ppm indicated the presence of two >CH— carbon. The two quaternary carbon (C-6 & C-9) showed at 132.49 and 129.03 ppm. The peaks at δ 68.16 ppm (C-3) indicated the presence of methylene carbon near to oxygen atom. The peaks at δ 38.76 ppm (C-5, C-10) indicated the presence of methyne carbon. The peaks at δ 22.98, 23.74, 28.94, 29.70, and 30.39 ppm (C-15, C-11, C-14, C-13 and C-4) indicated the presence of five methylene carbons. The peak at δ 10.96 ppm (C-12) and δ 14.03 ppm (C-16) indicated the presence of methyl carbon. The comparison of all these values with the literature survey$^{97}$ has been showed in the following table 3.6:
Table 3.6: $^{13}$C-NMR data of compound-3 compared with published data\textsuperscript{97} of isocoumarin:

<table>
<thead>
<tr>
<th>Position of carbon</th>
<th>Type of carbon</th>
<th>Compound-3 ($\delta_c$ ppm)</th>
<th>Isocoumarin ($\delta_c$ ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>—CO—</td>
<td>167.7</td>
<td>170.8</td>
</tr>
<tr>
<td>C-3</td>
<td>—CH$_2$—</td>
<td>68.1</td>
<td>69.7</td>
</tr>
<tr>
<td>C-4</td>
<td>—CH$_2$—</td>
<td>30.3</td>
<td>30.4</td>
</tr>
<tr>
<td>C-5</td>
<td>&gt;CH—</td>
<td>38.7</td>
<td>38.9</td>
</tr>
<tr>
<td>C-6</td>
<td>&gt;C&lt;</td>
<td>132.4</td>
<td>135.2</td>
</tr>
<tr>
<td>C-7</td>
<td>&gt;CH—</td>
<td>128.7</td>
<td>123.7</td>
</tr>
<tr>
<td>C-8</td>
<td>&gt;CH—</td>
<td>130.8</td>
<td>132.5</td>
</tr>
<tr>
<td>C-9</td>
<td>&gt;C&lt;</td>
<td>129.0</td>
<td>131.5</td>
</tr>
<tr>
<td>C-10</td>
<td>&gt;CH—</td>
<td>38.7</td>
<td>39.2</td>
</tr>
<tr>
<td>C-11</td>
<td>—CH$_2$—</td>
<td>23.7</td>
<td>22.3</td>
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<tr>
<td>C-12</td>
<td>—CH$_3$</td>
<td>10.9</td>
<td>10.5</td>
</tr>
<tr>
<td>C-13</td>
<td>—CH$_2$—</td>
<td>29.6</td>
<td>30.5</td>
</tr>
<tr>
<td>C-14</td>
<td>—CH$_2$—</td>
<td>28.9</td>
<td>29.4</td>
</tr>
<tr>
<td>C-15</td>
<td>—CH$_2$—</td>
<td>22.9</td>
<td>23.2</td>
</tr>
<tr>
<td>C-16</td>
<td>—CH$_3$</td>
<td>14.1</td>
<td>14.2</td>
</tr>
</tbody>
</table>

The molecular formula of compound-3 was assigned as C$_{15}$H$_{22}$O$_2$ from the $^1$H-NMR (Fig: 5.3.2, 2a and 2b), $^{13}$C-NMR (Fig: 5.3.3, 3a and 3b), DEPT-135 (Fig: 3.3.5 and 5a) and COSY-45 (Fig: 3.3.6) data. By analyzing the IR, $^1$H-NMR, $^{13}$C-NMR, DEPT-135 and COSY-45 NMR data of compound-3 with the reported isocoumarin in the literature\textsuperscript{97}, the structure of compound-3 was determined as 5-butyl-6-ethyl-3,4,5,10-tetrahydroisochromen-1-one. As per literature review, the compound-3 was isolated for the first time from *Vitex peduncularis*. 

\textsuperscript{97}This reference number is not visible in the image, it is assumed to be a citation or a note from the source.
3.2.3.7 COSY-45 NMR spectroscopy of Compound-3

The COSY-45 spectrum (400 MHz) in CDCl$_3$ of the compound-3 (Fig: 5.3.5) indicates that the correlation between $\delta_H$ (ppm) 7.5 (C-7) & 7.6 (C-8) and 4.2 (C-3), 1.6 (C-4), 1.4 (C-10), 1.3 (C-15) & 0.9 (C-16).
Fig-3.3.1 UV Spectra of Compound-3
Fig-3.3.2 IR Spectra of Compound-3
Fig-3.3.3 $^1$H-NMR Spectra of Compound-3

INARS, BCSIR, 1H spectrum, Compound-3 in CDCl$_3$, Ismet Ara
Fig-3.3.3a Expanded $^1$H-NMR Spectra of Compound-3
Fig-3.3.3b Expanded $^1$H-NMR Spectra of Compound-3
Fig-3.3.4 $^{13}$C-NMR Spectra of Compound-3
Fig-3.3.4a Expanded $^{13}$C-NMR Spectra of Compound-3
Fig-3.3.4b Expanded $^{13}$C-NMR Spectra of Compound-3
Fig-3.3.5 DEPT-135 Spectra of Compound-3
Fig-3.3.5a Expanded DEPT-135 Spectra of Compound-3
Fig-3.3.6 COSY Spectra of Compound-3
CHAPTER THREE
RESULT & DISCUSSION

INARS, BCSIR, Cosy 45, Compound-3, CDCl3, Ismet Ara

Fig-3.3.6a Expanded COSY Spectra of Compound-3
Fig-3.3.6b Expanded COSY Spectra of Compound-3
Fig. 3.3.6c Expanded COSY Spectra of Compound-3
Fig-3.3.6d Expanded COSY Spectra of Compound-3
3.2.4.1 Characterization of Compound-4 as phenolic compound
The compound-4 is a white amorphous solid. The $R_f$ value of the compound is 0.34 in dichloromethane : methanol $\equiv$ 90:10. It is soluble in methanol and acetone. Melting point of this compound is 136$^\circ$C. The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green color indicated the presence of phenolic compound.

![Methyl 3,4-dihydroxybenzoate](image)

Fig: 3.4 Structure of phenolic compound

3.2.4.2 Characterization of Compound-4 by spectroscopic methods
The structure of the Compound-4 (Fig: 3.4) has been established by IR, $^1$H-NMR, $^{13}$C-NMR, DEPT-135, COSY and HMBC.

3.2.4.3 Infrared (IR) spectroscopy of Compound-4
The IR Spectroscopic analysis (in KBr) of the compound-4 (Fig: 3.4.1), the absorption bands are at 3484.19 cm$^{-1}$ that is characteristic of O-H stretching. The observed absorption bands are at 1728.19 cm$^{-1}$ indicate the C=O stretching. Due to hydrogen bond C=O stretching absorption bands appear at 1680.6 cm$^{-1}$. Absorption at 2921.34 cm$^{-1}$ is due to aromatic >C-H stretching, 2852.20 cm$^{-1}$ assigned to aliphatic >C—H stretching. Absorption at 1597.06 cm$^{-1}$, 1504.36 cm$^{-1}$ and 1453.98 cm$^{-1}$ indicate the aromatic >C=C< group. The IR frequency at 1279.66 cm$^{-1}$ is due to C-O stretching. The finger print region at 805.88 cm$^{-1}$ & 762.65 cm$^{-1}$ indicate meta & para substituted benzene ring. These absorption frequencies revealed this compound is phenolic compound.

3.2.4.4 $^1$H-NMR spectroscopy of Compound-4
The $^1$H-NMR spectrum of compound-2 (Fig: 3.4.2 and 2a) indicated the presence of methyl group with proton signal at $\delta$ 3.88 ppm showed a singlet near to oxygen atom. A doublet at $\delta$ 6.8 (1H) represented the aromatic proton. A doublet and a singlet resembled in a same position and finally exhibited a singlet at the value of $\delta$ 7.5 (2H).
Table 3.7: $^1$H-NMR data of compound-4 compared with published data$^{98}$ of phenolic compound:

<table>
<thead>
<tr>
<th>Position of carbon</th>
<th>Type of proton</th>
<th>Compound-4 ($\delta_{H}$ ppm)</th>
<th>Phenolic Compound ($\delta_{H}$ ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>&gt;CH—</td>
<td>7.5 (1H, s)</td>
<td>7.4</td>
</tr>
<tr>
<td>C-5</td>
<td>&gt;CH—</td>
<td>6.8 (1H, d, $J=7.6$ Hz)</td>
<td>7.1</td>
</tr>
<tr>
<td>C-6</td>
<td>&gt;CH—</td>
<td>7.5 (1H, s)</td>
<td>7.3</td>
</tr>
<tr>
<td>O-Me</td>
<td>—CH$_3$</td>
<td>3.88 (3H, s)</td>
<td>3.88</td>
</tr>
</tbody>
</table>

3.2.4.5 $^{13}$C-NMR spectroscopy of Compound-4

In $^{13}$C-NMR spectrum in CD$_3$OD of the compound-4 (Fig: 3.4.3 and 3a) the peak at $\delta$ 168.67 ppm confirmed the presence of carbonyl group in the compound. The peaks at $\delta$ 151.27, 147.28, 123.88, 121.80, 114.45, 112.55 ppm in the spectrum indicated the presence of aromatic carbon. Methoxy carbon exhibited at $\delta$ 55.06 ppm in the compound. These values give us the conformation of the compound-4 is phenolic compound. The comparison of all these values of with the literature survey$^{99}$ has been showed in the following table 3.8:

Table 3.8: $^{13}$C-NMR data of compound-4 compared with published data$^{99}$ of phenolic compound:

<table>
<thead>
<tr>
<th>Position of carbon</th>
<th>Type of carbon</th>
<th>Compound-4 ($\delta_{C}$ ppm)</th>
<th>Phenolic Compound ($\delta_{C}$ ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>&gt;C&lt;</td>
<td>121.8</td>
<td>124.2</td>
</tr>
<tr>
<td>C-2</td>
<td>&gt;CH—</td>
<td>112.5</td>
<td>116.0</td>
</tr>
<tr>
<td>C-3</td>
<td>&gt;C&lt;</td>
<td>147.2</td>
<td>147.0</td>
</tr>
<tr>
<td>C-4</td>
<td>&gt;C&lt;</td>
<td>151.2</td>
<td>151.6</td>
</tr>
<tr>
<td>C-5</td>
<td>&gt;CH—</td>
<td>114.4</td>
<td>116.4</td>
</tr>
<tr>
<td>C-6</td>
<td>&gt;CH—</td>
<td>123.8</td>
<td>123.9</td>
</tr>
<tr>
<td>—CO</td>
<td></td>
<td>168.6</td>
<td>166.0</td>
</tr>
<tr>
<td>—CH$_3$-O</td>
<td></td>
<td>55.0</td>
<td>51.5</td>
</tr>
</tbody>
</table>

The molecular formula of compound-4 was assigned as C$_8$H$_8$O$_4$ from the $^1$H-NMR (Fig: 5.4.2 and 2a), $^{13}$C-NMR (Fig: 3.4.3 and 3a), DEPT-135 (Fig-3.4.4), COSY-45 (Fig: 3.4.5) and HMBC (Fig: 3.4.6) data. By analyzing the IR, $^1$H-NMR, $^{13}$C-NMR, DEPT-135, COSY-45 and HMBC data of compound-4 with the reported phenolic compound in the literature$^{99}$, the structure of compound-4 was determined as methyl-3,4-dihydroxy benzoate. As per literature review, the compound-4 was isolated for the first time from *Vitex peduncularis*. 
3.2.4.6 COSY-45 NMR spectroscopy of Compound-4
The COSY-45 spectrum (400 MHz) in CD$_3$OD of the compound-4 (Fig: 3.4.5) indicated that the correlation between $\delta_H$ (ppm) 7.5 (C-5) & 6.8 (C-6).

3.2.4.7 HMBC spectroscopy of Compound-4
The HMBC spectrum (100 MHz) in CD$_3$OD of the compound-4 (Fig: 3.4.6) indicated the following correlation.
Fig-3.4.1 IR Spectra of Compound-4
Fig-3.4.2 $^1$H-NMR Spectra of Compound-4
Fig-3.4.2a Expanded $^1$H-NMR Spectra of Compound-4

Methyl 3,4-dihydroxybenzoate
Fig-3.4.3 $^{13}$C-NMR Spectra of Compound-4
Fig-3.4.3a Expanded $^{13}$C-NMR Spectra of Compound-4
Fig-3.4.4 DEPT-135 NMR Spectra of Compound-4
Fig-3.4.5 COSY Spectra of Compound-4
Fig-3.4.5a Expanded COSY Spectra of Compound-4
Fig-3.4.5b Expanded COSY Spectra of Compound-4
Fig-3.4.5c Expanded COSY Spectra of Compound-4
Fig-3.4.6 HMBC Spectra of Compound-4
Fig-3.4.6a Expanded HMBC Spectra of Compound-4
CHAPTER FOUR

BIOLOGICAL ACTIVITIES
4.0 Introduction

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid (vitamin C), or polyphenols. Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Oxidative stress is damage to cell structure and cell function by overly reactive oxygen-containing molecules and chronic excessive inflammation. Oxidative stress seems to play a significant role in many human diseases, including cancers. The use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. For these reasons, oxidative stress can be considered to be both the cause and the consequence of some diseases. They are very important for the defense of living systems against oxidative stress. Antioxidants are nutrients (vitamins and minerals) as well as enzymes. In biological systems, the antioxidants are defined as “any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate.” This covers all oxidizable cellular substrates, i.e., above-mentioned lipids, proteins, DNA, and carbohydrates.

Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis, cataracts and even altitude sickness.

Antioxidants also have many industrial uses, such as preservatives in food and cosmetics and to prevent the degradation of rubber and gasoline.

Free radical species & reactive oxygen species (ROS) may cause oxidative damage. Free radicals induced by ultraviolet light or oxidative stress contributes to skin & can adversely affect the skin health, which means antioxidants active in skin cells may support skin health.
Free radicals are an atom or group of atoms containing at least one unpaired electron. An electron is a particle that normally occurs in pairs and is negatively charged which is a stable arrangement. When an electron become unpaired, it becomes unstable, and to create balance again, it will bond with another molecule, which then sets off a whole chain reaction.

Free radicals are generated when oxygen is used to produce energy but are also produced by other external factors.

Free radicals can:
1. Affect the rate at which we age.
2. Start cancers by damaging the DNA in cells.
3. Increase heart disease by making LDL cholesterol more likely to stick to artery walls.
4. Produce cataracts and encourage degeneration of the lens of the eye that ultimately leads to blindness.
5. Contribute to inflammation of the joints, as in arthritis.
6. Damage brain cells, promoting neurological conditions such as Parkinson's or Alzheimer's disease.

Foods rich in vitamin E and flavanoids are recommended components of a healthy diet. These foods include vegetables, fruits, unsaturated and monounsaturated fats (especially extra virgin olive oils), nuts, soybeans, tea and grape products. Possible benefits of supplemental antioxidants are limited to vitamin E on current evidence, although even in this case the evidence is equivocal.

However, because of the complexity of the antioxidant system, it may be that optimal benefit from antioxidant supplementation will only be obtained from a mixture of these substances. Ideally, the best way to get them is from food – this will ensure the consumption of other unidentified but beneficial compounds.

![Free radical scavenging reaction between DPPH & active compound](image)

**Fig: 4.1** Free radical scavenging reaction between DPPH & active compound
4.1 Classification of Antioxidants

Antioxidants may be broadly grouped according to their mechanism of action:

a) Primary or chain breaking antioxidants and
b) Secondary or preventive antioxidants

Classification of antioxidants (primary and secondary antioxidants) is discussed below.

4.1.1 Primary Antioxidants

Primary antioxidants are also referred to as chain-breaking antioxidants. Because of the chemical nature of these molecules, they can act as free radical acceptors/scavengers and delay or inhibit the initiation step or interrupt the propagation step of autoxidation. Primary antioxidants may interfere along the lipid autoxidation pathway. Primary antioxidants cannot inhibit photosensitized oxidation or scavenge singlet oxygen. The compounds that exhibit primary antioxidant activity include polyhydroxy phenolics. There are several synthetic ring substituted phenolics as well as naturally occurring phenolic compounds that may perform via the primary antioxidant mechanism. The common feature of all of these antioxidants is that they are mono or polyhydroxy phenols with various ring substitutes (Fig-25). Substitution with an electron-donating group/s ortho and/or para to the hydroxyl group of phenol increases the antioxidant activity of the compound by an inductive effect (e.g. BHA). Thus, the presence of a second hydroxyl group in the 2- (ortho) or the 4-position (para) of a phenol increases the antioxidant activity (e.g. TBHQ). The formed antioxidant radical is stabilized by delocalization of the unpaired electron around the phenol ring to form a stable resonance hybrid (Fig-26) and as a result attained low-energy levels.
4.1.2 Secondary Antioxidants

Secondary antioxidants are also classified as preventive or class II antioxidants. They offer their antioxidant activity through various mechanisms to slow the rate of oxidation reactions. The main difference with primary antioxidants is that the secondary antioxidants do not convert free radicals into stable molecules. They act as chelators for prooxidant or catalyst metal ions, provide H to primary antioxidants, decompose hydroperoxide to nonradical species, deactivate singlet oxygen, absorb ultraviolet radiation, or act as oxygen scavengers. They often enhance the antioxidant activity of primary antioxidants. Ascorbic acid, Ascorbyl palmitate, Erythorbic acid, Sodium erythorbate, Carotenoids are Secondary Antioxidants.

4.2 Natural Antioxidants

Use of plant parts (bark, leaves, seeds, etc.) and their extracts to preserve food from developing a rancid taste is a practice that has continued since prehistoric time. There is evidence that even for the industrial materials, plant-based components were used as antidrying agents to prevent oxidation and polymerization of polyunsaturated fatty acid-rich plant oils.\textsuperscript{105,106,107} During the past two decades, intensive research has been carried out on naturally occurring antioxidative compounds from different sources. The main drive behind this search was to reduce the use of synthetic compounds as food additives because of their potential negative health effects and as a result of consumer demand. Plant-based components have increasingly been advocated as “safe and natural” antioxidants considering their existence in regular foods that are consumed. Much of the interest on naturally occurring antioxidants is developed because of the trend to minimize or avoid the use of synthetic food additives. Continuous effort in searching for naturally occurring antioxidative compounds during the past 20 years has helped to develop efficient models for activity screening, structure function relationship assessment, categorizing sources of antioxidant groups, developing methods of isolating purified antioxidative compounds from natural sources, and developing branded foods (e.g., claims for marketing purpose). There are many naturally occurring compounds that act as antioxidants in fats and lipid-containing foods. Among these, only a few are currently
approved and employed in foods. Groups of compounds that are found naturally and exhibit strong antioxidant activity are discussed here.

### 4.2.1 Ascorbic Acid and Ascorbic Acid Esters

Vitamin C or ascorbic acid is widespread in nature but sparingly associated with fats of oils because of its hydrophilic nature. Ascorbic acid in the free form, salts of sodium and calcium, and esters of stearic and palmitic are commonly used as antioxidants in foods. Erythorbic acid is the D-isomer of naturally present L-ascorbic acid (Struc-28) and is often used as an antioxidant in dried fruits. In foods, water-soluble ascorbic acid acts as a secondary antioxidant and participates in various antioxidative and related functions. *In vivo* the acid acts as a primary antioxidant and in tissues it is essential for the prevention of oxidative cellular damage by hydrogen peroxide.

As an antioxidant, ascorbic acid is very attractive as it carries GRAS (generally recognized as safe) status with no usage limits; it is a natural or nature-identical product and is highly recognized as an antioxidant among the nutrient category. Ascorbic acid and its salts (Na- and Ca-ascorbate) are water soluble and are not applicable as antioxidants in oils and fats. These salts are used extensively for stabilizing beverages that contain oxidizable substrates.

### 4.2.2 Carotenoids

Carotenoids are ubiquitously found lipid-soluble-colored compounds, mainly from green plants, fruits, and vegetables. Carotenes are polyene hydrocarbons and vary in their degree of unsaturation (e.g., β-carotene, lycopene; Struc-29).
Carotenoids can act as primary antioxidants by trapping free radicals or as secondary antioxidants by quenching singlet oxygen. In foods, carotenoids usually act as a secondary antioxidant; however, at low oxygen partial pressure (in the absence of singlet oxygen), carotenoids may trap free radicals and act as a chain-breaking antioxidant.\textsuperscript{109,110}

4.3 Disease prevention
Antioxidants can cancel out the cell-damaging effects of free radicals.\textsuperscript{111} Furthermore, people who eat fruits and vegetables, which happen to be good sources of antioxidants, have a lower risk of heart disease and some neurological diseases,\textsuperscript{112} and there is evidence that some types of vegetables, and fruits in general, protect against a number of cancers. These observations suggested the idea that antioxidants might help prevent these conditions. This suggests that other substances in fruit and vegetables (possibly flavonoids), or a complex mix of substances, may contribute to the better cardiovascular health of those who consume more fruit and vegetables. However, there is some evidence that antioxidants might help prevent other diseases such as macular degeneration suppressed immunity due to poor nutrition, and neurodegeneration. It is thought that oxidation of low density lipoprotein in the blood contributes to heart disease, and initial observational studies found that people taking Vitamin E supplements had a lower risk of developing heart disease.

Many nutraceutical and health food companies sell formulations of antioxidants as dietary supplements and these are widely used in industrialized countries. These supplements may include specific antioxidant chemicals, like resveratrol (from grape seeds or knotweed roots), combination of antioxidants, like the “ACES” products that contain Betacarotene (provitamin A), vitamin C, vitamin E and Selenium, or herbs that contain antioxidants – such as green tea and jiaogulan.

4.4 Technological Considerations in Using Antioxidants
The type of food to which antioxidants may be added is variable and ranges from baked goods, biscuits, chewing gum, dry snacks, fruit drinks, mayonnaise, meat products, nuts, and oils and fats, among others. For food applications, the antioxidants must be effective at low concentrations (below 0.02%, w/w) because at high concentrations, they may act as pro-oxidants. The antioxidants should also be nontoxic. Usually the antioxidant is directly added to the food as a concentrate in lipid/oil, dissolved in a food grade solvent, or in an emulsified form that may be sprayed onto the food product. Antioxidants must be thoroughly blended with the lipid to obtain their maximum potency. To be effective, antioxidant(s) should partition between oil–air interfaces in bulk oil systems or between oil–water interfaces in the emulsion systems. Antioxidants must be added, as soon as
possible, to the fresh product as they cannot reverse any oxidation reactions that have already occurred.\textsuperscript{113} Most synthetic antioxidants are formulated with polypropylene glycol, glyceryl monooleate, mono- and diacylglycerols, or vegetable oils as carriers to enhance their solubility or dispersibility in foods. Several synergistic mixtures are available commercially, especially citric acid with synthetic antioxidants. Commercial preparations of natural antioxidants are predominantly tocopherol- and ascorbic acid ester-based. Few formulations are available with rosemary, sage, and tea catechin-based antioxidative ingredients. In these formulations, vegetable oils and starches are used as carriers and citric acid is also included.

4.5 Plant extract

We first screened for the presence of phytochemical group test in methanol, n-hexane, DCM, ethylacetate and n-butanol extract of the samples. And significant present of flavonoids & phenolics were observed. We know that flavonoids & phenolics are antioxidant compounds. So we therefore tried to determine the antioxidant activity of the \textit{Vitex peduncularis} samples. To evaluate the antioxidant activity we followed the following methods.

1. Total Phenolic Content
2. Total Flavonoid Content
3. Total Antioxidant Capacity
4. DPPH scavenging activity
5. ABTS scavenging activity
6. Reducing power assay

4.5.1 Method for Determination of Total Phenolic Content

The total phenolic contents were determined by the modified folin-ciocalteu method.\textsuperscript{114} 1 ml of the aqueous and methanolic extracts was collected in two 10 ml volumetric flasks separately. To each flask, 5 ml of Folin-Ciocalteu reagent (1: 10 v/v distilled water) and 4ml (75 g/L) of sodium carbonate were added. The solutions were vortexed for 15 seconds and allowed to stand for 30 min at 40°C for color development. The absorbance was measured against the blank in a double beam UV/Visible spectrophotometer (Analykjen, Model 205, Jena, Germany) at absorption maxima 765 nm. Three readings were taken per solution to get reproducible results. The total phenolic content was determined and expressed as µg gallic acid equivalents per gram of dry extract using the equation obtained from a standard gallic acid calibration curve, \( y = 0.008x + 0.038, R^2 = 0.998 \).
4.5.2 Method for Determination of Total Flavonoid Content
Aluminium chloride colorimetric method was used for the determination of total flavonoid concentration of the extracts. \(^{115}\) 1 ml of the extracts was individually mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The solution was allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 415 nm with a double beam Analykjen UV spectrophotometer (Model 205, Jena, Germany). The total flavonoid content was determined as µg of Quercetin equivalent per gram using the equation obtained from a standard Quercetin calibration curve, \(y = 0.036x + 0.044; R^2 = 0.999\).

4.5.3 Determination of total antioxidant capacity:
The total antioxidant capacity of the extracts were evaluated by the phosphomolybdenum assay method\(^{116}\) which is based on the reduction of Mo (VI) to Mo (V) and the subsequent formation of a green phosphate-Mo (V) complex in acidic condition. The extracts were allowed to mix with 3.0 ml of the reagent solution (0.6 M H\(_2\)SO\(_4\), 28 mM Na\(_3\)PO\(_4\), 4 mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 min. After letting the solution cool back to room temperature, the absorbance was measured at 695 nm using a UV-Visible spectrophotometer against a blank solution. The total antioxidant capacity was determined as µg of ascorbic acid equivalent per gram using the equation obtained from a standard ascorbic acid calibration curve, \(y = 0.002x+0.001; R^2 = 0.999\).

4.5.4 DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity
DPPH radical serves as the oxidizing radical to be reduced by the antioxidant (AH) and as the indicator for the reaction

\[
\text{DPPH}^- + \text{AH} \rightarrow \text{DPPH-H} + \text{A}^- \quad ^{117}
\]

\[
\[\text{DPPH (free radical)} \quad \text{DPPH (non radical)}\]
\]

\[
\[\text{Inhibition of polymer chain, } R, \text{ by DPPH}\]
\]

Fig-4.5: DPPH radical, non radical & inhibition mechanism.
The stable DPPH radical-scavenging activity was measured using the modified method described by Gupta\textsuperscript{118} was used. In this assay, 2 ml of 0.2 m\textsubscript{u} methanolic DPPH solution was added to 2 ml of extract solution at different concentrations and the contents were stirred vigorously for 15 seconds. Then the solutions were allowed to stand at dark place at room temperature for 30 min for reaction to occur. After 30 min, absorbance was measured against a blank at 517 nm with a double beam Analykjena UV/Visible spectrophotometer (Model 205, Germany). The percentage of DPPH radical-scavenging activity of each plant extract was calculated as:

\[
\text{DPPH radical-scavenging activity} \ (%I) = \frac{A_0 - A}{A_0} \times 100
\]

Where, \(A_0\) is the absorbance of the control solution (containing all reagents except plant extracts); \(A\) is the absorbance of the DPPH solution containing plant extract.

The DPPH radical-scavenging activity (%) was plotted against the plant extract concentration to determine the concentration of extract necessary to decrease DPPH radical-scavenging by 50% (called IC\textsubscript{50}). The IC\textsubscript{50} value of each extract was estimated by sigmoid non-linear regression, using Sigma Plot 2000 Demo (SPSS Inc., Chicago, IL, USA). These values were changed to antiradical activity, defined as 1/EC\textsuperscript{50}, since this parameter increases with antioxidant activity. All determinations were performed in triplicate. Ascorbic acid was used as positive control standard.

4.5.5 ABTS {2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)} radical scavenging activity

ABTS radical serves as the oxidizing radical to be reduced by the antioxidant (AOH) and as the indicator for the reaction

\[\text{Oxidation of ABTS by potassium persulfate to generate radical cation ABTS' and its reaction with an antiradical compound (AOH)}\]
The antioxidant capacity of the extracts was determined by ABTS radical cation as described by Fan YJ and coworkers with some modifications. The ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 16 h. The ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. 1 ml of sample at different concentrations (100 to 500μg/ml) was added to 1 ml of the ABTS solution and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 6 min before the absorbance at 734 nm was recorded. The ABTS scavenging effect was calculated as per the equation: ABTS scavenging effect = (Ao – As / Ao) x 100, where, Ao = absorbance of control and As = absorbance of sample.

4.5.6 Reducing Power Assay
The reducing power of the *V. peduncularis* extracts was determined according to the method followed by Dehpour and co-workers. Different concentrations of the plant extracts (100500μg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A 10% solution of trichloroacetic acid (2.5 ml) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%) and the absorbance of the mixture was measured at 700 nm with spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. All the tests were carried out in triplicate and average of the absorptions was recorded. Ascorbic acid was used as the standard reference compounds.

4.6 Result and discussion of antioxidant scavenging activity of extract of *Vitex peduncularis* samples along with standard ascorbic acid.

Result and discussion of Total Phenolic Content, Total Flavonoid Content, Total Antioxidant Capacity and Antioxidant Scavenging Activity (DPPH, ABTS & Reducing Power) of VPM, VPH, VPD, VPE and VPB of *Vitex peduncularis* samples along with standard Ascorbic Acid are given below.
4.6.1 Amount of total phenolic content in different solvent extraction/fractions of *Vitex peduncularis* bark samples

Table-4.1 to 4.2 and Figure-4.1 to 4.2 are showing the amount of total phenolic content (µg gallic acid equivalent per gram of dry extract) in different solvent extracts of *Vitex peduncularis* bark samples and gallic acid standard compound.

Table-4.1: Absorbance of gallic acid (a standard phenolic compound) at 765nm

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Avg. absorbance at 765 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>12.5</td>
<td>0.1235</td>
</tr>
<tr>
<td>(Standard)</td>
<td>25</td>
<td>0.2651</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.4069</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0.6342</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.9276</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>2.2291</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD (n=3)

![Gallic Acid Standard Curve](image)

\[ y = 0.008x + 0.038 \]

\[ R^2 = 0.998 \]

Figure-4.1: Concentration Vs Absorbance (at 765nm) curve of Gallic acid

Figure-4.1 is representing the standard calibration curve for determination of total phenolic content in different solvent extracts of *Vitex peduncularis* samples.
Table 4.2: Comparison of total phenolic content of different extraction/fractions of *Vitex peduncularis* bark samples.

<table>
<thead>
<tr>
<th>Extractives Name</th>
<th>Average Absorbance at 765nm</th>
<th>Total Phenolic Content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol Extract (VPM)</td>
<td>0.5275</td>
<td>611.875</td>
</tr>
<tr>
<td>n-Hexane Fraction (VPH)</td>
<td>0.0952</td>
<td>71.500</td>
</tr>
<tr>
<td>Dichloromethane Fraction (VPD)</td>
<td>0.2769</td>
<td>298.625</td>
</tr>
<tr>
<td>Ethylacetate Fraction (VPE)</td>
<td>0.8632</td>
<td>1031.500</td>
</tr>
<tr>
<td>1-Butanol Fraction (VPB)</td>
<td>0.8531</td>
<td>1018.875</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD (n=3)

The crude methanol extract and all the solvent fractions were found to contain good amount of phenolic compounds and the ethyl acetate and 1-butanol solvent fractions were contained very significant amount of total phenolic content (1031.5 mg/g and 1018.875 mg/g respectively)
4.6.2 Amount of total flavonoid content in different extraction/fractions of *Vitex peduncularis* bark samples

Table-4.3 to 4.4 and figure-4.3 to 4.4 are representing the amount of total flavonoids content (µg Quercetin equivalent per gram of dry extract) in different solvent extractives of *Vitex peduncularis* bark samples.

**Table-4.3: Absorbance of quercetin (a standard flavonoid compound) at 430 nm**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Avg. absorbance at 430 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Standard)</td>
<td>12.5</td>
<td>0.5256</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.9681</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.8438</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>2.2134</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.6368</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>4.8651</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD (n=3)

**Figure-4.3: Concentration Vs absorbance (at 430) curve of Quercetin (Standard)**

Figure-4.3 is representing the standard calibration curve for determination of total flavonoids content in solvent extracts of *Vitex peduncularis* samples.
Table 4.4: Comparison of total flavonoid content of different extraction/fractions of *Vitex peduncularis* bark samples

<table>
<thead>
<tr>
<th>Extractives Name</th>
<th>Average Absorbance at 765 nm</th>
<th>Total Flavonoid Content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol Extract (VPM)</td>
<td>1.3951</td>
<td>56.295</td>
</tr>
<tr>
<td><em>n</em>-Hexane Fraction (VPH)</td>
<td>1.0835</td>
<td>43.312</td>
</tr>
<tr>
<td>DCM Fraction (VPD)</td>
<td>1.5912</td>
<td>64.467</td>
</tr>
<tr>
<td>Ethylacetate Fraction (VPE)</td>
<td>2.7443</td>
<td>112.512</td>
</tr>
<tr>
<td>1-Butanol Fraction (VPB)</td>
<td>1.5280</td>
<td>61.853</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD (n=3)

Figure 4.4: Total flavonoid content of different solvent extractives of *Vitex peduncularis* VPM=Vitex Peduncularis Methanol extract VPH=Vitex Peduncularis *n*-Hexane fraction, VPD=Vitex Peduncularis DCM fraction, VPE=Vitex Peduncularis Ethyl acetate fraction and VPB=Vitex Peduncularis 1-Butanol fraction.

Table 4.4 and figure 4.4 were showed the total flavonoid content of different extraction/fractions of *Vitex peduncularis* bark samples. Ethyl acetate solvent fraction (VPE) was found to contain maximum amount of total flavonoids (112.512 mg/g) whereas *n*-hexane solvent fraction (VPH) was found to contain less amount of total flavonoids (43.312 mg/g).
4.6.3 Amount of total antioxidant capacity in different extraction/fractions of *Vitex peduncularis* bark samples

Table-4.5 to 4.6 and Figure-4.5 to 4.6 are representing the amount of total antioxidant capacity (µg ascorbic acid equivalent per gram of dry extract) in different solvent extractives of *Vitex peduncularis* bark samples.

**Table-4.5: Absorbance of ascorbic acid (a standard compound) at 695 nm**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Avg. absorbance at 695 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>12.5</td>
<td>0.0278</td>
</tr>
<tr>
<td>(Standard)</td>
<td>25</td>
<td>0.0531</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.1001</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.2001</td>
</tr>
</tbody>
</table>

*Values are expressed as mean±SD (n=3)*

![Ascorbic acid standard curve](image)

*Figure-05: Concentration Vs absorbance (at 430) curve of Ascorbic acid (Standard)*

Figure-4.5 is representing the standard calibration curve for determination of total antioxidant capacity in solvent extracts of *Vitex peduncularis* bark samples.
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Table-4.6: Comparison of total antioxidant capacity of different extraction/fraction of *Vitex peduncularis* bark samples.

<table>
<thead>
<tr>
<th>Extractives Name</th>
<th>Average Absorbance at 695 nm</th>
<th>Total Antioxidant Capacity (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol Extract (VPM)</td>
<td>0.1574</td>
<td>86.020</td>
</tr>
<tr>
<td><em>n</em>-Hexane fraction (VPH)</td>
<td>0.0279</td>
<td>14.7950</td>
</tr>
<tr>
<td>DCM fraction (VPD)</td>
<td>0.1465</td>
<td>80.025</td>
</tr>
<tr>
<td>Ethylacetate fraction (VPE)</td>
<td>0.2108</td>
<td>115.390</td>
</tr>
<tr>
<td>1-Butanol fraction (VPB)</td>
<td>0.1735</td>
<td>94.875</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD (n=3)

Figure-4.6: Comparison of total antioxidant capacity of different extractives of *Vitex peduncularis*  
VPM=Vitex Peduncularis Methanol extract  VPH=Vitex Peduncularis *n*-Hexane fraction,  
VPD=Vitex Peduncularis DCM fraction, VPE=Vitex Peduncularis Ethyl acetate fraction and  
VPB=Vitex Peduncularis 1-Butanol fraction.

Table-4.6 & Fig-4.6 are showing the comparison of total antioxidant capacity of different extraction/fractions of *V. peduncularis* bark samples. Ethyl acetate fraction showed the highest amount of total antioxidant capacity (115.390 mg/g) whereas the *n*-hexane fraction showed the lowest amount of total antioxidant capacity (14.795 mg/g).
4.6.4 DPPH free radical scavenging activity of different extraction/fractions of *Vitex peduncularis* bark samples and standard ascorbic acid

Table-4.7 to 4.8 and Figure-4.7 to 4.8 are representing the DPPH radical scavenging activity & IC$_{50}$ values of different extractives of *Vitex peduncularis* bark samples and standard ascorbic acid.

**Table-4.7: Comparison of DPPH free radical scavenging activity of different extraction/fractions of *Vitex peduncularis* bark samples and standard ascorbic acid.**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VPM</td>
<td>VPH</td>
<td>VPE</td>
<td>VPB</td>
<td>AA</td>
</tr>
<tr>
<td>12.5</td>
<td>80.1059</td>
<td>38.1845</td>
<td>86.3345</td>
<td>89.4161</td>
<td>87.6632</td>
</tr>
<tr>
<td>25</td>
<td>78.4059</td>
<td>43.5475</td>
<td>91.2099</td>
<td>85.0365</td>
<td>92.6412</td>
</tr>
<tr>
<td>50</td>
<td>82.4155</td>
<td>51.1000</td>
<td>92.9833</td>
<td>85.0362</td>
<td>92.6703</td>
</tr>
<tr>
<td>80</td>
<td>84.0123</td>
<td>61.2400</td>
<td>90.5338</td>
<td>83.3112</td>
<td>94.6110</td>
</tr>
<tr>
<td>100</td>
<td>86.4560</td>
<td>73.5473</td>
<td>93.2064</td>
<td>90.123</td>
<td>95.1212</td>
</tr>
<tr>
<td>200</td>
<td>90.1245</td>
<td>82.4700</td>
<td>95.0107</td>
<td>94.0321</td>
<td>97.3220</td>
</tr>
</tbody>
</table>

| IC$_{50}$ (µg/ml)     | 7.9011       | 26.0002 | 6.3271 | 6.4891 | 4.2801 |

*Values are expressed as mean ±SD (n=3)*

![Graph showing comparison of DPPH Free Radical Scavenging activity of different Extractives](image)

*Figure-4.7 Comparison of DPPH Free Radical Scavenging activity of different Extractives, VPM=Vitex Peduncularis Methanol extract, VPH=Vitex Peduncularis n-Hexane fraction, VPE=Vitex Peduncularis Ethyl acetate fraction, VPB=Vitex Peduncularis 1-Butanol fraction and AA=Ascorbic Acid.*

Table-4.7 & fig-4.7 are showing the comparison of DPPH Free Radical Scavenging activity of *V. peduncularis* bark samples. Ethyl acetate fraction was determined maximum inhibition as 95.0107 was exhibited best scavenging activity in compare to the standard compound.
Table-4.8: Comparison of IC<sub>50</sub> values of DPPH free radical scavenging activity of *Vitex peduncularis* bark samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>VPM</th>
<th>VPH</th>
<th>VPE</th>
<th>VPB</th>
<th>AA (Standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
<td>7.9011</td>
<td>26.0002</td>
<td>6.3271</td>
<td>6.4891</td>
<td>4.2801</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD (n=3)

Fig-4.8 Comparison of IC<sub>50</sub> values of Vitex Peduncularis samples. VPM=Vitex Peduncularis Methanol extract, VPH=Vitex Peduncularis n-Hexane fraction, VPE=Vitex Peduncularis Ethyl acetate fraction, VPB=Vitex Peduncularis 1-Butanol fraction and AA=Ascorbic Acid.

Table-4.8 & fig-4.8 are showing the comparison of IC<sub>50</sub> values of DPPH free radical scavenging activity of *V. peduncularis* samples. Ethyl acetate fraction (VPE) showed the highest DPPH radical scavenging activity whereas the n-hexane fraction (VPH) showed the lowest DPPH free radical scavenging activity.
4.6.5 ABTS free radical scavenging activity of different extraction/fractions of *Vitex peduncularis* bark samples and standard ascorbic acid.

Table-4.9 to 4.10 and figure-4.9 to 4.10 are representing the ABTS radical scavenging activity & IC$_{50}$ values of different extractives of *Vitex peduncularis* bark samples and standard ascorbic acid.

Table-4.9: Comparison of ABTS free radical scavenging activity of different extraction/fractions of *Vitex peduncularis* bark samples and standard ascorbic acid.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VPM</td>
</tr>
<tr>
<td>12.5</td>
<td>91.762</td>
</tr>
<tr>
<td>25</td>
<td>93.020</td>
</tr>
<tr>
<td>50</td>
<td>96.690</td>
</tr>
<tr>
<td>80</td>
<td>97.432</td>
</tr>
<tr>
<td>100</td>
<td>97.639</td>
</tr>
<tr>
<td>200</td>
<td>98.432</td>
</tr>
</tbody>
</table>

IC$_{50}$ (µg/ml) | 7.111 | 7.325 | 7.259 | 6.946

*Values are expressed as mean ±SD (n=3)*

Figure-4.9 Comparison of ABTS Free Radical Scavenging activity of different extractives among VPM=Vitex Peduncularis Methanol extract, VPE=Vitex Peduncularis Ethyl acetate fraction, VPB=Vitex Peduncularis 1-Butanol fraction and AA=Ascorbic Acid.

Table-4.9 and fig-4.9 are showing the crude methanol extract and all of the solvent fractions were determined significant inhibition of ABTS free radical scavenging activity.
Table-4.10: Comparison of IC<sub>50</sub> values of ABTS radical scavenging activity of <i>Vitex peduncularis</i> samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>VPM</th>
<th>VPE</th>
<th>VPB</th>
<th>AA (Standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
<td>7.111</td>
<td>7.325</td>
<td>7.259</td>
<td>6.946</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD (n=3)

Fig-4.10 Comparison of IC<sub>50</sub> values of ABTS free radical scavenging activity among the extraction/fraction of <i>Vitex peduncularis</i> samples VPM=Vitex Peduncularis Methanol extract, VPE=Vitex Peduncularis Ethyl acetate fraction, VPB=Vitex Peduncularis 1-Butanol fraction and AA=Ascorbic Acid.

Table-4.10 & fig-4.10 are showing the comparison of IC<sub>50</sub> values of ABTS free radical scavenging activity of <i>V. peduncularis</i> samples. All the extraction/fractions showed significant IC<sub>50</sub> values of ABTS free radical scavenging activity.
4.6.6 Reducing power assay of different extraction/fractions of *Vitex peduncularis* bark samples and standard ascorbic acid

Table-4.11 and Figure-4.11 are representing the reducing power assay of different extractives of *Vitex peduncularis* bark samples and standard ascorbic acid.

**Table-4.11: Comparison of reducing power assay of different extraction/fractions of *Vitex peduncularis* bark samples and standard ascorbic acid.**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Avg. Absorbance at 700 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VPM</td>
</tr>
<tr>
<td>12.5</td>
<td>0.1726</td>
</tr>
<tr>
<td>25</td>
<td>0.1671</td>
</tr>
<tr>
<td>50</td>
<td>0.1849</td>
</tr>
<tr>
<td>80</td>
<td>0.2143</td>
</tr>
<tr>
<td>100</td>
<td>0.2621</td>
</tr>
<tr>
<td>200</td>
<td>0.2439</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD (n=3)

*Figure-4.11 Comparison of reducing power assay of different extractives among VPM=Vitex Peduncularis Methanol extract, VPH=Vitex Peduncularis n-Hexane fraction, VPD=Vitex Peduncularis DCM fraction, VPE=Vitex Peduncularis Ethyl acetate fraction, VPB=Vitex Peduncularis 1-Butanol fraction and AA=Ascorbic Acid.*

Table-4.11 & fig-4.11 are showing dichloromethane fraction (VPD) was found to have maximum absorbance at 0.5757 was exhibited best reducing power in compare to the standard compound and n-hexane and 1-butanol fraction showed moderate reducing power.
A detailed phytochemical analysis has been carried out on the bark of *Vitex peduncularis* plant. During this investigation five compounds were isolated from this plant extract. Among them structural elucidation of the four compounds were performed. All the compounds were identified preliminarily by chemical methods and then the structural elucidation of the compounds were performed by various spectroscopic methods (UV, IR, $^1$H-NMR, $^{13}$C-NMR, DEPT-135, COSY-45 and HMBC etc) and were confirmed by comparison with the results of available in the published literatures. The terpenoid and isocoumarin type compounds were isolated from DCM part and the phenolic type compound was isolated from ethyl acetate part. These compounds having the following chemical structure so far has been isolated for the first time from this plant.

The crude methanol extract and different solvent fractions of *Vitex peduncularis* bark showed moderate to significant anti-oxidant activities. Thus the investigation of the plant *Vitex peduncularis* might generate much synthetic and biological interest in the field of natural products for further study.
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