Identification and characterization of Protic and Aprotic Polar Solvent Mixture Extract of Gynura procumbens Leaves

By

Munjiatunnesha

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in Chemistry

Khulna University of Engineering & Technology
Khulna 9203, Bangladesh
January, 2019
Declaration

This is to certify that the thesis work entitled "Identification and characterization of Protic and Aprotic Polar Solvent Mixture Extract of Gynura procumbens Leaves" has been carried out by Munjiatunnesha in the Department of Chemistry, Khulna University of Engineering & Technology, Khulna, Bangladesh. The above thesis work has not been submitted anywhere for the award of any degree or diploma.

07.01.19
Signature of Supervisor

Munjiatunnesha
Signature of Candidate
Approval

This is to certify that the thesis work submitted by Munjiatunnesha entitled “Identification and Characterization of Protil and Aprotic Polar Solvent Mixture Extract of Gynura Procumbens Leaves” has been approved by the board of examiners for the partial fulfillment of the requirements for the degree of Master of Science in the Department of Chemistry, Khulna University of Engineering & Technology, Khulna, Bangladesh in January 2019.

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Abstract

The leaves of *Gynura procumbens* were extracted with three solvents such as ethyl acetate-ethanol at the ratio of (25:75), (50:50) and (75:25) mixtures by using standard chromatographic techniques and isolated compounds characterized by IR and NMR (\(^1\)H-NMR and \(^{13}\)C-NMR) spectroscopy. One known compound β-sitosterol-3-O-β-D-glucopyranoside was isolated from ethyl acetate-ethanol (75:25) mixture extract.

The investigation of antimicrobial activity on the *Gynura procumbens* was carried out by extraction of leaf using ethyl acetate (100%), ethanol (100%), and at the ratio of ethyl acetate-ethanol (25:75), (50:50) and (75:25) mixture extracts. Antibacterial activity of the extracts at a concentration 250 µg/disc and 500 µg/disc were performed against two gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two gram negative (*Escherichia coli* and *Salmonella enterica*) bacteria by Kirby-Bauer disc diffusion method using Kanamycin as standard. The ethyl acetate-ethanol (25:75) extracts exhibited promising antimicrobial activities against *Staphylococcus aureus* and ethyl acetate-ethanol (75:25) extracts exhibited moderate antimicrobial activities against *Bacillus subtilis* and *Staphylococcus aureus*, whereas, the other crude extracts did not show any sort of sensitivity.
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Introduction

1.1 Background

Over 3,000 million years ago, the first living-organism which approached a plant appeared. It was blue-green algae which lived in the sea and can still be found in the water today. When the plants made their first appearance on planet earth the atmosphere was unlivable for all oxygen breathing creatures. The air was made out of carbon dioxide, a gas which to us is deadly. Then photosynthetic plants came along and slowly over several million years, cleaned the atmosphere and filled it with oxygen.

There are various ways to categorize plants. Two of the most common are plant structure and life cycle.

Plant structure: Plants can be either herbaceous or woody. Herbaceous plants generally have stems that are soft, green, and contain little woody tissue. These plants are ones that usually die to the ground each year. Most annual and perennial flowers fall into this category along with vegetables and houseplants.

Life cycle: A designation which usually describes how long a plant lives or how long it takes to complete its life cycle (grow, flower, set seed). Plants are classified as either an annual, perennial or biennial.

Plants are important to human in many other ways. The role of forests and other types of natural vegetation in controlling foods and climate regulation are well known. Plant products have played an important role in the need of human being from the ancient time. The primary compounds carbon dioxide and water in the presence of sunlight and chlorophyll produce oxygen by the process of photosynthesis. But this is not possible for animals. So, they depend on plants. Plants are universally avowed to be helpful for the mankind as well as to ensure the existence of all forms of life. Plants also play a vital role in consuming CO$_2$ from the atmosphere and save it from the negative impact of greenhouse effect [1].
Bangladesh is a country that is envisaged rich in medicinal plants genetic resources by virtue of its favorable agro climatic condition as well as seasonal diversity. Bangladesh holds about 6500 plants species including bryophytes, pteridophytes, gymnosperms and angiosperms; among them, 500 plant species have medicinal values [2].

The use of traditional medicine and medicinal plants in most developing countries, as a basis for the maintenance of good health has been widely accomplished by UNESCO, 1996 [3]. Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally applied rural herbal remedies [4]. Hence in the importance of plant and its derivative chemistry utilizing medicinal plants started to develop. Scientists are now performing together to find out new drugs from plants for incurable diseases like diabetes, cancer and AIDS [5].

1.2 Sources of Natural products

Throughout the ages humans have relied on nature to cater for their basic needs, not the least of which are medicines for the treatment of a wide spectrum of diseases. Plants in particular have formed the basis of sophisticated traditional medicine systems, with the earliest records, dating from around 2600 BCE, documenting the uses of approximately 1000 plant-derived substances in Mesopotamia. These include oils of Cedrus species (cedar) and Cupressus sempervirens (cypress), Glycyrrhiza glabra (licorice), Commiphora species (myrrh), and Papaver somniferum (poppy juice), all of which are still used today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation. Egyptian medicine dates from about 2900 BCE, but the best known record is the "Ebers Papyrus" dating from 1500 BCE, documenting over 700 drugs, mostly of plant origin [6]. Some medicines are developed from the natural product drive originally obtained from the natural source. This means the lead may be:

- Revealed by total synthesis or
- A starting point (precursor) for a semi-synthetic compound or
- A outward show that serves as the basis for a structurally different compound arrived at by total/semi-synthesis.
1.3 Medicinal importance of plant materials

Medicinal plants, also called medicinal herbs have been discovered and used in traditional medicine practices since prehistoric times. Plants synthesise hundreds of chemical compounds for functions including defence against insects, fungi, diseases and herbivorous mammals. Numerous phytochemicals with potential or established biological activity have been identified. However, since a single plant contains widely diverse phytochemicals, the effects of using a whole plant as medicine are uncertain. Further, the phytochemical content and pharmacological actions, if any of many plants having medicinal potential remain unassessed by rigorous scientific research to define efficacy and safety. In the United States over the period 1999 to 2012, despite several hundred applications for new drug status, only two botanical drug candidates had sufficient evidence of medicinal value to be approved by the Food and Drug Administration. In modern medicine, around a quarter of the drugs prescribed to patients are derived from medicinal plants and they are rigorously tested [7, 8]. However, development of plants or extracts having potential medicinal uses is blunted by weak scientific evidence, poor practices in the process of drug development and insufficient financing.

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>
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<td>Betulinic acid</td>
<td>Anticancerous</td>
<td>Betula alba</td>
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<td>Menthol</td>
<td>Rubefacient</td>
<td>Mentha species</td>
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<tr>
<td>Papain</td>
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<td>Carica papaya</td>
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</tr>
<tr>
<td>Taxol</td>
<td>Antitumor agent</td>
<td>Taxus brevifolia</td>
</tr>
<tr>
<td>Vasicine</td>
<td>Cerebral stimulant</td>
<td>Vinca minor</td>
</tr>
</tbody>
</table>
1.4 Medicinal Plants in Folk Tradition

Since the beginning of the nineteenth century when modern chemistry and pharmacy began to develop the original impetus was on the study of natural products chemistry utilizing medicinal plants. Scientists today also engaged themselves searching for compounds from plants, which can be used in important medicinal purposes. Folkloric reputations in many cases are found true and modern drugs like penicillin, morphine, quinine etc. are exceedingly useful for treatment of various diseases in animals and humans alike. Advances in biotechnology, particularly methods for culturing plant cells and tissues should provide new means for the commercial processing of even rare plants and the chemicals they produce.

For the foreseeable future, there is no doubt that plants will continue to provide the mankind with valuable agents of potential use in the investigation, prevention and treatment of diseases such as cancer, AIDS, asthma and other vital infections such as malaria, disorders of the cardiovascular and central nervous systems and many others [9]. Of the 2,50,000 higher plant species on earth, more than 80,000 species are reported to have at least some medicinal value [10, 11]. There are about 400 families in the world of flowering plants of which at least 315 are represented in India. According to WHO, approximately 21,000 plant species have the potential for being used as medicinal plants [12]. Although some of the therapeutic properties attributed to plants have been proven to be erroneous [13], the use of traditional medicinal plants for the treatment of various diseases is well known and documented since ancient times [14]. According to Jiaxiang [15], 4877 plant species belongs to different plant groups having potential therapeutic value.

**Table: 1.2:-** Plant species with therapeutic value under different plant groups.

<table>
<thead>
<tr>
<th>Number</th>
<th>Name of the plant</th>
<th>Number of species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thalophytes</td>
<td>230</td>
</tr>
<tr>
<td>2</td>
<td>Bryophytes</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>Pteridophytes</td>
<td>382</td>
</tr>
<tr>
<td>4</td>
<td>Gymnospermae</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>Angiospermae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(a) Monocotyledones</td>
<td>676</td>
</tr>
<tr>
<td></td>
<td>(b) Dicotyledones</td>
<td>3495</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>4877</td>
</tr>
</tbody>
</table>
Ayurvedic medicine is used in India. Ayurveda stresses the use of plant-based medicines and treatments. Hundreds of plant-based medicines are employed, including two commonly used spices: cardamom and cinnamon. The plant *Alhagi maurorum* Medik (Camels thorn) secretes a sweet, gummy material from the stems and leaves during hot days [16]. Native Americans also have a long history of use of traditional medicines. They use sage which was believed to heal multiple problems of the digestive system. Remedies for common colds include American ginseng; herbs for aches and pains include Pennyroyal and hops and dogwood and willow bark are remedies for fever. Salvia was used by Indian tribes of southern California as an aid in childbirth [17].

1.5 Nature as Source of New Drug Compounds

That Nature in one guise or another has continued to influence design of small molecules is shown by inspection of the information given below where with the advantage of now 30 years of data, the system has been able to be refined. In particular, as behooves authors from the National Cancer Institute (NCI), in the specific case of cancer treatments, we have continued to consult the records of the FDA and added comments from investigators who have informed us of compounds that may have been approved in other countries and that were not captured in our earlier searches. As was done previously, the cancer data will be presented as a stand-alone section from the beginning of formal chemotherapy in the very late 1930s or early 1940s to the present, but information from the last 30 years will be included in the datasets used in the overall discussion.

Examples for medical treatments from natural sources include the discovery of the beneficial effects of *cardiotonic digitalis* extracts from foxglove for treating some manifestations of heart disease in the 18th century, the use of the bark of the willow and cinchona trees in treating fever and the effectiveness of poppy extracts in the treatment of dysenteries [18]. *Digitalis purpurea* L. (foxglove) had been traced back to Europe in the 10th century but it was not until the 1700s that the active constituent digitoxin (1), a cardiotonic glycoside was found to enhance cardiac conduction, thereby improving the strength of cardiac contractibility. Morphine (2), largely reproducing the analgesic and sedative effect of opium, was isolated from *Papaver somniferum* (opium) obtained from the seed pots of the poppy plant in 1804 and was the first Nature-derived compound to be commercialized [18].
Figure 1.1: Some of the earliest isolated and characterized drugs obtain from Nature; (a) quinine from *Cinchona officinalis* bark (b) morphine from *Papaver somniferum* (c) penicillin from the bacteria *Penicillium notatum*

Quinine (3) was an antimalarial agent, isolated from *Cinchona officinalis* bark found by the French scientists Caventou and Pelletier in 1820 [19]. By 1829, scientists discovered that the compound salicin (4), in *Salix alba* (willow trees) was responsible for pain relief and in 1838 salicylic acid was isolated [20] in its acetylated form (acetylsalicylic acid), more commonly known as aspirin (5). Aspirin was first isolated and synthesized by Felix Hoffmann, a chemist with the German company Bayer and marketed in 1897. Thus, the most widely used drug in the world continues to be aspirin. Penicillin (6), discovered in 1928 by Fleming was isolated from the culture broth of *Penicillium notatum* as a bioactive principle inhibiting the growth of Gram-positive bacteria [21]. Soon thereafter when research of natural products had become popular, the isolation of new compounds included strychnine and brucine from *Strychnos nux-vomica*, colchicine from *Colchicum autumnale*, caffeine from *Coffea arabica*, nicotine from *Nicotiana tabacum*, atropine from *Atropa belladonna* and cocaine from *Erythroxylum coca* [22]. Vinca alkaloids, vincristine sulphate and vinblastine sulphate are also the first plant derived anticancer agents to be approved for use in cancer [23].
Chapter I

Introduction

Digitoxin (1)

Morphine (2)

Quinine (3)

Salicin (4)

Acetylsalicylic acid (5)

Penicillin (6)
1.6 Key factors for success in natural products research

It is generally accepted that the number of antibiotics in nature is vast \cite{24}. Data generated in our lab show that the percentage of actinomycete and fungal strains producing antimicrobial activities in standard agar diffusion assays ranges between 30% and 80% depending on the ecological or taxonomic groups \cite{25, 26}. The only field in which some similarities could be found is in the search for natural antitumor agents, since cytotoxic metabolites are equally abundant in nature. One of the perceived liabilities of this field is that it requires a substantial amount of manual work. Automation has been regarded as necessary to improve the efficiency of the early steps in the drug discovery process, but it cannot be used with equal success and efficiency throughout the process of natural products lead discovery, the screening step being clearly more amenable to automation than the generation of natural products libraries \cite{27}.

![Diagram of antibiotic discovery process]

Figure 1.2: The process of antibiotic discovery from microbial natural products.
1.7 Natural product sources

Natural products isolated from various sources especially derived from plants, have long been used in treatment of human ailments. For long time, the approach to new drugs through natural products was proved to be the single most successful strategy for the discovery of new drugs. Despite the initial success, chemical diversity and specific action on target, drug discovery from natural products, has been deemphasized by many pharmaceutical companies in favour of approaches based on combinatorial chemistry and genomics. Besides covering the historical notes and the drugs already isolated from different natural sources like plants, fungi, marine organisms and animals, this review article is also presenting the reasons for the leg back in the field. Natural products have a large unexplored range of compounds which is almost impossible to imitate; they will always remain a potential source of future drug discovery.

In recent years, other less conventional sources like alcoholic and non-alcoholic beverages, spices, animal and human excreta and many more have generated interest for natural product researchers [28].

1.7.1 Natural products from microorganisms

Microorganisms are capable of producing natural products with widely divergent chemical structures. Greatest attention in the past has been paid to natural products that have antibiotic properties. Natural products accumulate in fermentation broths during secondary metabolism, a characteristic of the incomplete metabolic control operative in growth-inhibited microorganisms. With this general mechanism of biosynthesis, the natural products synthesized by microorganisms would be expected to have a broad range of pharmacological activities. The directed screening for non-antibiotic natural products has been of limited scope. The expectation that new compounds of interest would be found has been validated. The pharmacologically active natural products provide previously unrecognized structures as tools for fundamental
research programs as well as offering the possibility of direct use in medicine or in industrial processes. Microorganisms have a wide variety of potentially active substances and have led to the discovery of antibacterial agents like cephalosporins, antidiabetic agents like acarbose and anticancer agents like epirubicin [29].

Epirubicin

Acarbose

Cefprozil (Cephalosporin)

1.7.2 Natural products from marine organisms

Marine organisms are an excellent source of novel chemicals and enzymes, which include a variety of primary and secondary metabolites with significant biological activities. Variations within species can produce differences in compounds and in their concentrations in different marine environments. Therefore, it is essential to study organisms from various locations. The
first active compounds to be isolated from marine species were spongouridine and spongothymididine from the Caribbean sponge Cryptotheca crypta in the 1950s. These compounds are nucleotides and show great potential as anticancer and antiviral agents. Their discovery led to an extensive research to identify novel drug candidates from marine sources.

These chemicals can serve as possible remedies for various ailments, especially cancer. One such example is discodermolide, isolated from the marine sponge, Discodermia dissoluta which has a similar mode of action to that of paclitaxol and possesses a strong antitumor activity. It also exhibits better water solubility as compared to paclitaxol. A combination therapy of the two drugs has led to reduced tumor growth in certain cancers [30].

![Spongouridine](image1.png) ![Discodermolide](image2.png)

**Spongouridine** **(+-) Discodermolide**

### 1.7.3 Natural products from animal sources

Comparative analysis of the timetables depicting breakthrough events in the development of the steroid hormone and the prostaglandin fields when viewed in relation to certain developments in the field of science as a whole reveals some of the factors which most influenced their genesis. The most prominent factor in regulating the pace of progress was the emergence of new supporting technology in other fields of science.

Animals have also been a source of some interesting compounds that can be used as drugs. Epibatidine obtained from the skin of an Ecuadorian poison frog is ten times more potent than morphine [31]. Venoms and toxins from animals have played a significant role in designing a
multitude of cures for several diseases. Teprotide (pry-trp-pro-arg-glu-ile-propro), for example, extracted from a Brazilian viper has led to the development of cilazapril and captopril which are effective against hypertension [32].

1.7.4 Natural products from plant sources:

Plants produce a huge array of natural products (secondary metabolites). These compounds have important ecological functions, providing protection against attack by herbivores and microbes and serving as attractants for pollinators and seed-dispersing agents. They may also contribute to competition and invasiveness by suppressing the growth of neighbouring plant species (a phenomenon known as allelopathy). Humans exploit natural products as sources of drugs, flavouring agents, fragrances and for a wide range of other applications.

A significant number of drugs have been derived from plants that were traditionally employed in ethnomedicine or ethnobotany (the use of plants by humans as medicine as in Ayurvedic or Traditional Chinese Medicine) while others were discovered initially (through random screening of plant extracts in animals) or later, by determining their in vitro activity against HIV or cancer cell lines [33].

Their ethnopharmacological properties have been used as a primary source of medicines for early drug discovery. According to the World Health Organization (WHO) 80% of people still rely on plant-based traditional medicines for primary health care and 80% of 122 plant derived drugs were related to their original ethnopharmacological purpose [34]. The knowledge associated with traditional medicine (complementary or alternative herbal products) has
promoted further investigations of medicinal plants as potential medicines and has led to the isolation of many natural products that have become well known pharmaceuticals. Several important drugs such as Taxol, camptothecin, morphine and quinine have been isolated from plant sources. The first two are widely used as anticancer drugs while the remaining are analgesic and antimalarial agents, respectively.

1.8 Economic importance of medicinal plants (An over-view with special reference to Bangladesh)

Global statistics has revealed the continued growing economic importance of medicinal plants and plant based pharmaceuticals which developing countries can harness to improve their pharmaceutical supplies that can impact positively on their healthcare delivery system. This is premised on the fact that most of these medicinal plants are abundantly indigenous in these developing countries that export them at very cheap rates to developed countries only to import pharmaceuticals made from them at very exorbitant prices that most often affect their foreign exchange. It is for these reasons and perhaps among others not mentioned that this review suggests the need to intensify research into ethnomedicine as this can turn to address the current precarious supply and reduce the burden of import of essential medicines by the developing nations.

There are many countries in the world which earn a substantial amount of foreign currency by exporting medicinal plants and crude plant drugs. India and Thailand are two glaring examples of such countries in this subcontinent which earn crores of rupees by exporting medicinal plants and their semi-processed products to other countries including Bangladesh [1].

Every year Bangladesh transports a large quantity of pharmaceuticals raw materials including medicinal plants and semi-processed plant products to feed its various drug manufacturing industries. These expenditures seriously aggravate the already bad situation with regard to our dwindling foreign exchange position and something has to be done to reduce this spending [1].

Bangladesh possessing a rich flora of medicinal plants should make serious efforts to derive maximum economic benefit from these plants by using them as raw materials for its indigenous drug manufacturing industries [1]
1.9 General description about *Gynura procumbens*

1.9.1 Description of the genus *Gynura*

*Gynura* is a genus of flowering plants in the daisy family Asteraceae endemic to Asia. Their leaves grow alternately in dentate or pinnate divided shape. They have typical discoid capitula, solitary or few to numerous corymbose in yellow or purple color. Involucres are campanulate or cylindric with 9-13 uniseriate phyllaries and scarious margins. Receptacle is flat areolate or shortly fimbriate. Anthers are entire or subauriculate at base. Style branches are slender and puberulent. Achenes are cylindric with ribs. Species of *Gynura* grow either in open place in primary vegetation, such as tree fall gaps or along river margins, or in disturbed and secondary vegetation such as roadsides. They can be found from sea level to up to 3,000 m altitude. The genus *Gynura* Cass. (Asteraceae-Senecioneae) comprises 44 species and is distributed from tropical Africa to South and East Asia and Australasia with one species in tropical Australia (Vanijajiva & Kadereit, submitted). The highest specific diversity is found in Southeast Asia(Davies, 1981) but the genus is least well understood particular in Thailand (Davies, 1978; Koyama, 1988).

They are systematic classified as below:

1. *Gynura abbreviata* F.G.Davies
2. *Gynura bicolor* (Roxb. ex Willd.) DC. (Okinawa spinach; nutritious cooked vegetable. Known as KinJiSo in Japan. Known as Hong FengCai in Taiwan.)
3. *Gynura calciiphila* Kerr
4. *Gynura divaricata* (L.) DC.
5. *Gynura elberti* J.Kost.
7. *Gynura hispida* Thwaites
8. *Gynura japonica* (Thunb.) Juel
9. *Gynura malaccensis* Belcher
10. *Gynura nepalensis* DC. (cholesterol spinach; supposedly cholesterol-lowering)
11. *Gynura procumbens* (Lour.) Merr.
12. *Gynura sarmentosa* "Aureo-variegata", "Pink Ice"
1.9.2 Naming of *Gynura procumbens*

*Gynura procumbens* belongs to Asteracea family and is an annual evergreen shrub with fleshy stems [35]. *Gynura procumbens* was firstly described and the name validly published by Joao de Loureiro in 1790. It was Elmer Drew Merrill, however, who reclassified it into today’s valid botanical systematics in 1923 [36]. It was also called *Gynura sarmentosa* DC. and *Calacia procumbens* Lour.[37].

**Common names:** In our country it known as *Gynura procumbens* and diabetic plant [38].

**Vernacular names:** *Gynura procumbens* has various local names in various countries, like-

<table>
<thead>
<tr>
<th>Country</th>
<th>Vernacular names</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaysia</td>
<td>Mollucan spinach, “Akar Sebiak,” “Kecam Akar,” and “Sambung Nyawa”</td>
<td>35</td>
</tr>
<tr>
<td>Indonesia</td>
<td>Daun Dewa, Sambung Nyawa (which means Extending Live)</td>
<td>37</td>
</tr>
<tr>
<td>China</td>
<td>“pointed phoenix tail”, Akar Sebiak, Kelemai Mearh, Nan fei Ye, Bai Bing Ca</td>
<td>39</td>
</tr>
<tr>
<td>Myanmar</td>
<td>‘Pyar Mee Swae’</td>
<td>37</td>
</tr>
<tr>
<td>Thailand</td>
<td>longevity spinach, Paetumpung, Pra kum dee kwai</td>
<td>37</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>Longevity spinach</td>
<td>39</td>
</tr>
<tr>
<td>England</td>
<td>Mollucan spinach, Leaves of Gods, Cholesterol spinach</td>
<td>39</td>
</tr>
<tr>
<td>Cambodia</td>
<td>Chi angkam</td>
<td>39</td>
</tr>
<tr>
<td>Vietnam</td>
<td>Kim That Tai. Cay Tieu Duong, Tiem Vinh</td>
<td>39</td>
</tr>
<tr>
<td>Philippine</td>
<td>Kamangi</td>
<td>39</td>
</tr>
</tbody>
</table>
1.9.3 Taxonomy of *Gynura procumbens*

**Binomial name:** *Gynura procumbens* (Lour.) Merr. (1923)

**Scientific classification:** [39]

- **Kingdom:** Plantae
- **Division:** Magnoliophyta
- **Class:** Magnoliopsida
- **Order:** Asterales
- **Family:** Asteraceae
- **Tribe:** Senecioneae
- **Genus:** *Gynura*
- **Species:** *Gynura procumbens* (Lour.) Merr.

1.9.4 Description of Plant

*Gynura procumbens* (Also known as Sabuṅgai or Sambung Nyawa) [40, 41] sometimes called "longevity spinach," is an edible vine found in China, Southeast Asia and Africa. Leaves are ovate-elliptic or lanceolate, 3.5 to 8 centimeters long, and 0.8 to 3.5 centimeters wide. Flowering heads are panicked, narrow, yellow and 1 to 1.5 centimeters long [42, 43]. *Gynura procumbens* is an evergreen herbaceous plant (Figure 1.3). It occurs in a wide range of habitats but it is best grown under partly-shaded area and moist soil. *Gynura procumbens* is a perennial, climbing vine, growing wild on edges of tropical forests and bushes, meadows, near streams or other wet but well drained soils and semi-shady places. It like rich soils and can quickly spread 6m in length. It also can grow on poor, bit dry soil and cope with full sun but its growth will be slow. This plant is not hardy at all and temperatures just below 5°C can damage it. [37, 44].
Figure 1.3: *Gynura procumbens* (a) shrub plant; (b) fleshy green leaves and shoot system.

**Height:** *Plants* 2-5 m high or more, stems scrambling to climbing, sparsely pubescent to glabrescent.

**Stems:** fleshy, procumbent, brownish or purple striate, glabrous or pubescent when young, branched. [37, 44]

**Leaves:** bright to darker green in colour, rather smooth to touch, stalked (uppermost ones stalkless), ovate-elliptic or lanceolate, 3.5-8 cm long and 0.8-3.5 cm wide, with somewhat entire or toothed margins, leaf petiole 5-15 mm, glabrous, blade abaxially purplish, adaxially green, ovate, ovate-oblong or elliptic, 3-8 × 1.5-3.5 cm, both surfaces glabrous, rarely sparsely pubescent, lateral veins 5-7-paired, curved, veinlets inconspicuous, base rounded-obtuse or cuneately attenuate into petiole, margin entire or repand-dentate, apex acute or acuminate; upper stem leaves and leaves on synflorescence branches reduced, lanceolate or linear-lanceolate, sessile or subsessile. [37, 44]

**Capitula:** 3-5 in each corymb, in terminal or axillary corymbs; peduncles long, slender, often with 1-3 linear bracts, sparsely shortly pubescent or glabrous; bracts involucral, involucres campanulate or funnelform, smooth, 15-17 × 5-10 mm, bracteoles at base 5 or 6, linear; phyllaries (9 or) 11-13, becoming purplish, oblong-lanceolate, 15-17 × 1.5 mm, glabrous, 1-3-veined, margin narrowly scarious, apically acuminate; flowering heads panicked, narrow, yellow and 1-1.5 cm long. [37]
**Florets:** 20-30; corolla orange, 12-15 mm, with slender 8-10 mm tube and dilated limb, lobes ovate-lanceolate, apically acute; anthers obtuse at base, appendages triangular; style branch tips conical; papillose. [37, 44]

**Achenes:** brown, very small, smooth, with very close and slender ribs, cylindric, 4-6 mm, glabrous, 10-ribbed; pappus white, silky. [37, 44]

**Flower:** *Gynura procumbens* produces flowers in long hanging inflorescence. The flowers head are panicked, orange to yellow in colour, bisexual with narrow bracts and purple involucres. The style is slender and hairy. The achenes are narrow and puberulous and the pappus (modified calyx) is white, fine and silky. [37, 44].

1.9.5 Habitat and distribution of *Gynura procumbens*

*Gynura procumbens* (Lour.) Merr. is a common medicinal plant distributed in western and central Africa, and from southern China throughout continental South-East Asia and Papua New Guinea [37]. *Gynura* was first described in 1838 and it can be found abundantly in Borneo, Java, the Philippines and Peninsula Malaysia [35] and widely distributed in Indonesia, Malaysia and Thailand [45]. It comprises about 44 species native to the humid tropics of Africa to Southeast Asia.

However, this plant is not native to Bangladesh. But at present it is cultivating in Bangladesh especially in Khulna region [38].

1.10 Use of *Gynura procumbens*

1.10.1 Culinary Uses

*Gynura procumbens* is well known in South East Asia and the leaves of the plant are eaten as a vegetable. In fact, in Malaysia *Gynura procumbens* young shoots are eaten raw as ‘ulam’. Leaves have a mild flavor and are used raw in salads, added to soups, stir-fries, casseroles, condiments and sauces, rice dishes and other savory meals. It is also used for sauces, as flavoring and in sandwich for making taste. Dried or fresh, cutted leaves and stems, are used for making herbal tea [46].
➢ Leaf vegetable - taste like green beans
➢ Young leaves/ shoots can be eaten fresh by itself/ included in rice dishes/ mixed in with salads (like lettuce or spinach)
➢ Cook with butter /add to a casserole /soup/ stir-fried (fry garlic in a little oil and then sauté the leaves and young shoots in oyster sauce)/ sautéed lightly like one would spinach
➢ Tea.

Figure 1.4: *Gynura procumbens*  anti-age cream and tea

1.10.2 Traditional Uses

Traditional medicinal plants has been widely used as major sources of drugs in pharmaceutical industry. The research into medicinal plants with alleged folkloric use as pain relievers, anti-inflammatory agents [47] . Medicinal plant is any plant from which valuable drugs can be synthesized as it contains substances that can be used for medicinal purpose [48]. The cultivation of medicinal plants on large scale is increasing in order to provide raw material for the manufacturing of herbal products. In developing countries, farmers are practicing co-cultivation of medicinal (herbal) plants to get additional income. Each parts of medicinal plants have it owns used for treatment of certain disease. Numerous examples had impressively demonstrated the innovative potential of natural compounds and their impacts on the progress of drugs discovery and development [49, 50, and 51]. About 30% of drugs used worldwide are based on natural
products [52]. Plants have been the basis of traditional medicines throughout the world for thousands of years and continue to provide new remedies to humankind. Several authors have reviewed the beneficial uses of these plant species [53, 54, and 55]. Nowadays, the usage of medicinal plants in forms of traditional medicine, herbal medicine and botanical dietary supplement and so on increase considerably [56].

1.10.3 Medicinal Uses

**Anti-hypertensive remedy:** *Gynura procumbens* has been used as a therapy for lowering high blood pressure for decades. Scientific research has proven its efficacy as an effective anti-hypertensive remedy [57].

**Anti-herpetic actions:** Research has proven anti-herpetic actions of *Gynura procumbens*. It does so by reducing replication ability of virus. It has potent anti-microbial properties, which make it effective against the little trouble makers invading our body [58].

**Lowers blood sugar levels:** Research shows that Gynura lowers blood glucose levels and can be used as an effective remedy against diabetes. Its leaf extract has bioactive constituents that ensure anti-diabetic action [59].

**Anti-oxidant effects:** Research shows that *Gynura procumbens* has strong anti-oxidant potential. This miraculous plant has phenol compounds trapped in its leaves. These phenolic compounds scavenge free radicals in our body [60].

**Anti-cancer nature:** *Gynura procumbens* is an emerging herbal anti-cancer remedy. Proteins extracted from its leaves include miraculin, peroxidase, thaumatin-like proteins. These proteins control abnormal cellular growth, thus have therapeutic effects on tumors, especially tumors of the colon and bones. *Gynura* is also effective in alleviating breast cancer [61].

**Anti-hyperlipidemic effect:** Along with glucose lowering effects, ethanolic extract of *Gynura procumben’s* leaves has anti-hyperlipidemic effects. It can lower cholesterol and triglycerides levels in our body [62].
Anti-ulcerogenic activity: *Gynura procumbens* has been used for gastrointestinal ailments. Research shows that Gynura has anti-ulcerogenic activity. It protects mucosa (the inner layer of gut) from damage, thus ensures a healthy gut [63].

Enhances wound healing: Wound healing is a physiological process that starts right after the injury occurs. *Gynura procumbens* show its efficacy in speeding up wound injury at a considerably fast pace [64].

Anti-inflammatory effect: *Gynura procumbens* has been famous for anti-inflammatory effects. It contains many bioactive constituents, alkaloids and steroids. These alkaloids play vital role in warding off inflammation. Steroids present in this plant are also responsible of anti-inflammatory actions [65].

Effective remedy for rheumatism: Alternative practitioners have used *Gynura procumbens* for rheumatism for ages.

Immune modulating effects: *Gynura procumbens* is one of those medicinal plants that have immune modulating effects. They stimulate growth of human lymphocytes- cells of immune systems. Consuming these plants also impacts growth of natural killer (NK) cells- another variety of immune cells. Both these cell- lymphocytes and NK- make vital part of immune system and ensure that bodily defenses are high enough [66].

Impacts on kidney: *Gynura procumbens* effective remedy for the management of kidney diseases. Researchers have found that Gynura has healthy impacts on progressive kidney disease. Bioactive compounds present in its leaf extract inhibit proliferation of mesangial cells (cells present in kidney). They also decrease expression of some harmful proteins, thus act as protective agents for kidneys [67].

Fertility activity: *Gynura procumbens* decrease toxins- oxidative stress- from body and increase both the motility and quantity of sperm [68].

Anti-photoaging activity: *Gynura procumbens* show anti-photoaging activity. It prevents production of free radicals by UV rays and makes sure safety from UV induced damage and premature aging [69].
**Taste making agent:** *Gynura procumbens* has a protein miraculin which makes up more than 0.1% of its protein extract. Miraculin protein is used to make taste of bitter medicine, better and useable [70].

**Anti-fungal effects:** *Gynura procumbens* have high anti-fungal activity. So, it can be claimed that besides having anti-viral effects, it also wards off fungal infections [71].

**Medicinal tooth paste:** *Gynura procumbens* is used to make a medicinal tooth paste with 1-20% extract of it. It also contains compounds like glycol, diglycol, abradant, carboxymethyl cellulose and others in various concentrations. This mixture is used as a dental cream and acts as an effective anti-bacterial, anti-viral and anti-phlogosis (anti-inflammatory for external parts of body) [72].

**Liver protection:** Flavonoids present in *Gynura procumbens* extract scavenge free radicals and protects liver. They act like a detox therapy, freeing body from poisons and giving liver a break [72].

![Figure-1.5: Biological activities of Gynura procumbens and its main bioactive constituents that contributed to the biological activities.](image)
1.10.4 Commercial uses

Among the existing patents related to *Gynura procumbens*, the majority of them are for preparations of traditional Chinese medicine intended for the treatment of various ailments including uterine cancer, cervical spondylosis and chronic skin ulcer. Besides, it has also been used as an ingredient in special diets for patients with medical conditions such as heart and liver disease. In the food industry, it has been incorporated into products such as tea, kimchi, coffee powder, chocolate, candy and chewing gum. The applications of *Gynura procumbens* in personal care and cosmetic products have also been reported which including hand-washing solution, hand sanitizer, oral spray, facial masks and skin care creams [73]. These patents have demonstrated the high commercial value of *Gynura procumbens* and its variety of uses in a number of industries.

1.11 Objectives of the Research Work

The present investigation using an ethnomedical drug discovery program, evaluated the antidiabetic activity of *Gynura procumbens* used in the traditional health system of the Southeast Asia, as an effective remedy and management for diabetes mellitus and other ailments. On literature review showed that investigations on these species have been carried out by different researchers but more investigations are needed to explore its therapeutic value. The aim of the present study will carry out preliminary phytochemical screening and physicochemical of the plant to assure the quality, safety and efficacy of this formulation. So the specific aims of this study are:

1. Isolation and identification of the chemical constituents from *Gynura procumbens* leaves
2. Deducing their structures by spectroscopic technique
3. Determination of moisture, ash and iron content
CHAPTER II
LITERATURE REVIEW
2.1 Literature survey of *Gynura procumbens*

There is a growing focus on the importance of medicinal plants and traditional health systems in solving the health care problems of the world. Use of plants as a source of medicine has come to new developing world from ancient practices. Most of studies addressed antioxidant effects of medicinal plants followed by antimicrobial, anti-diabetic anti-inflammatory, hepatoprotective, antifungal and anticancer properties. Fabaceae was found to be the most abundantly studied family with a total of eleven studied plants, followed by Lamiaceae, Combretaceae, Euphorbiaceae, Leguminosae, Malvaceae, Asteraceae, Apocynaceae, Cucurbitaceae, Rubiaceae, Zingiberaceae, Apiaceae, Compositae, Anarcadiaceae, Acanthaceae, Asciepiadaceae and Rutaceae. In spite of the fact that tremendous efforts were done by the researchers by providing an alternate with minimum side effects, easy accessibility and excellent compatibility, future clinical trials and standardization are still desired as an important steps in drug discovery.

From literature survey it was found that mainly the leaves of *Gynura procumbens* are used in the treatment of various diseases. *Gynura procumbens* shoot, root and leaf callus are also used. Due to the wide application of this plant in traditional medicine, a number of studies have been conducted to investigate its pharmacological activities. Pharmacological studies have indicated that *Gynura procumbens* exhibited different extents of antioxidant activity. Especially the ethyl acetate fractions, which contain the highest amount of phenolic compounds, exhibited the greatest antioxidant activity. The high scavenging property of *Gynura procumbens* may be due to hydroxyl groups existing in the chemical structure of the phenolic compound that can provide the necessary component as a radical scavenger and antioxidant [58].

Li *et al.* (2017) investigated the antioxidant activities of four new polysaccharides (GPP-20, GPP-40, GPP-60 and GPP-80) which were fractionated from *Gynura procumbens* leaves by 20%, 40%, 60% and 80% (v/v) ethanol, via the reducing power assay and scavenging capacities
of 2, 2 diphenyl-1-picrylhydrazyl (DPPH) free radicals and hydroxyl free radicals, respectively. [74].

Teoh et al. (2016) evaluated antioxidant property of *Gynura procumbens* extracts using DPPH radical scavenging, metal chelating and β-carotene bleaching assays. Methanol extract showed the highest DPPH radical scavenging activity (IC$_{50}$ value of 1.13 mg/mL), followed by water (IC$_{50}$ 1.51 mg/mL), hexane (IC$_{50}$ 2.44 mg/mL) and ethyl acetate (IC$_{50}$ 4.16 mg/mL) extracts. Metal chelating activity of hexane extract of *Gynura procumbens* was significantly higher than the other extracts tested (IC$_{50}$ value of 1.99 mg/mL), followed by methanol (IC$_{50}$ 4.59 mg/mL), ethyl acetate (IC$_{50}$ 11.37 mg/mL) and water (IC$_{50}$ 16.50 mg/mL). This indicated that non-polar constituents of *Gynura procumbens* appeared to be the main contributor in chelating metal ions and preventing oxidative stress. [75].

Rahman et al. (2013) evaluated the antioxidant potential of *Gynura procumbens*, where tert-butyl-1-hydroxytoluene (TBHT) and ascorbic acid (ASA) was used as reference standard. Methanol (ME) extract showed the highest antioxidant activity with IC$_{50}$ value of 20.35μg/mL, which is comparable to that of the reference standard tert-butyl-1-hydroxytoluene (TBHT) (27.5μg/mL) and it is clear that ME extract might play an important role in preventing free radical induced-diseases. At the same time, n-hexane extract also showed potent antioxidant activity (48.0μg/mL). On the other hand, DCM and Ethyl Acetate extracts showed only mild antioxidant activity, whose IC$_{50}$ values were 355.0μg/mL and 221.0μg/mL, respectively [76].

Vijendren et al. (2015) utilized several nonenzymatic antioxidants methods to study the antioxidant capacity, which include ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, total flavonoid content, total phenolic content, and ascorbic acid content determination. DPPH assay reveals *Gynura procumbens* shoot as the lowest (66.885%) and *Gynura procumbens* root as the highest (93.499%) DPPH radical inhibitor. In all antioxidant assays, *Gynura procumbens* callus culture exhibits very low antioxidant activity. However, *Gynura procumbens* root exhibited highest phenolic content, flavonoid content, and ascorbic acid content with 4.957 TEAC mg/g FW, 543.529 QE μg/g FW, and 54.723 μg/g FW, respectively [77].
Puangprontipitag et al. (2010) evaluated the anti-oxidative properties in *Gynura procumbens* leaves extract and they found highest anti-oxidative activity in ethanolic extract of *Gynura procumbens* (EC$_{50}$ = 1.63 in hydroxyl scavenging activity, EC$_{50}$ = 2.17 in iron chelating activity compared to EDTA control and EC$_{50}$ = 2.75 in inhibition of lipid peroxidation compared to malondialdehyde). They suggest that *Gynura* Extract may have positive effects on free radical scavenging and iron chelating [78].

The potential of antioxidant activities of the plant extract *Gynura procumbens*, *Achyranthes aspera* and *Polygenum tomentosum* were studied by Maw et al. (2011) using DPPH. Antioxidant activity was qualitatively and quantitatively determined. In this analysis, Ascorbic acid (Vitamin C) was used as the standard. The antioxidant activities were observed all three plant extracts and the EC$_{50}$ values of *Gynura procumbens*, *Achyranthes aspera* and *Polygenum tomentosum* were 13.7 µg/mL, 14.37 µg/mL and 14.35 µg/mL compared to vitamin C (EC$_{50}$ 8.31 ± 0.327 mg/mL). Among these plants, *Gynura procumbens* ethanol (95% v/v) extract is more potent antioxidant activity then others. [79].

Mustafizur et al. (2013) showed the crude n-hexane (HX), dichloromethane (DCM), methanol (ME) and ethyl acetate (EA) extracts of the leaves of *Gynura procumbens* were subjected to antioxidant, antibacterial and antifungal activities and cytotoxicity against brine shrimp nauplii. The DCM and EA extracts exhibited mild antimicrobial activities, whereas, HX and ME extracts did not show any sort of sensitivity. On the other hand, the brine shrimp lethality with LC$_{50}$ values was 70.71, 0.78, 4.42 and 59.46 µg/mL for HX, DCM, ME and EA extracts, respectively indicates the presence of potent bioactive compounds. At the same time, among the four crude extracts, only ME and HX extracts showed potential antioxidant activity with IC$_{50}$ values of 20.35 and 48.0 µg/mL. A pure compound was isolated from HX extract and the structure of the compound was elucidated as stigmasterol by means of $^1$H NMR spectroscopy [80].

Pusparanee et al. (2008) identified diabetes mellitus believed to bring negative effects on the male reproductive system through an increase in oxidative stress. Diabetic rats (n=5) force-fed with *Gynura procumbens* aqueous extract with 100 mg/kg dosage (n=5) showed increased sperm count and motility by 25.12% and 23.97±1.09 % respectively while sperm mortality decreased by 38.43% as compared to the controls. Testicular LDH specific activities in *Gynura procumbens* aqueous extract treated rats were increased by 72.53%. On the other hand, diabetic rats force-
fed with glibenclamide with 5 mg/kg dosage (n=5) showed decreased sperm count and slightly increased sperm motility by 4.65% and 19.94±1.26 %, respectively, while sperm mortality increased significantly (p<0.05) by 38.43% as compared to the controls. Testicular LDH specific activities in glibenclamide treated rats were increased by 26.58%. [81].

Zurina et al. (2010) evaluated the in vivo hypoglycemic properties of the water extract of Gynura procumbens following 14 days of treatment and in vitro in RIN-5F cells. The antidiabetic effect of water extract of Gynura procumbens leaves was investigated in streptozotocin-induced diabetic rats. The intraperitoneal glucose tolerance test (IPGTT) was performed in diabetic rats treated with Gynura procumbens water extract for 14 days. In the IPGTT, blood was collected for insulin and blood glucose measurement. Gynura procumbens also showed minimal effects on β-cells of the islets of Langerhans in the pancreas [82].

Gansau et al. (2012) showed that Gynura procumbens extract (GPE) promotes hyperglycemia by enhancing insulin sensitivity in the skeletal muscle and suppressing glucose production in the liver of type 2 diabetic db/db mice. Gynura procumbens extract increased the expression of PM-GLUT4 in the skeletal muscle through pAMPK and pAS160 activation and reduced hepatic gluconeogenesis [83].

Chan et al. (2009) identified that repeated oral administration of hexane, ethyl acetate and n-butanol fractions from the ethanol (95 % v/v) extract of Gynura procumbens into diabetic rats for 14 days caused significant (p < 0.05) reduction in blood glucose levels of 29.7 %, 60.1 % and 33.5 %, respectively. At the dose tested (250 mg/kg bw/day), the ethyl acetate Gynura procumbens fraction exhibited glucose-reducing activity that is doubled the activities detected in the other two fractions and was as pronounced as the activity observed with the standard oral hypoglycemic drug, Glibenclamide which reduced blood glucose by 61.7 % (p < 0.0001) [84].

Wu et al. (2011) the water extract of this herb significantly inhibited (p < 0.05) ACE activity (IC$_{50}$ = 0.37 mg/mL) and showed a moderate potential hypoglycemic effect via in vitro α-amylase (IC$_{50}$ = 1.36 mg/mL) and α-glycosidase (IC$_{50}$ = 2.17 mg/mL) inhibition in dose-dependent manner. Further partitioning of the water extract (extracts 2–4) resulted in higher α-amylase inhibitory activities in extract 2 and 3. For α-glycosidase inhibition, extract 3 gave the highest inhibition. ACE inhibitory activities of the extracts were not improved by partitioning.
Sugar, protein, flavonoid and alkaloid were found in water extract but only a small portion was partitioned in the n-butanol extract. However, a large portion of the flavonoids and alkaloids were found in ethyl acetate extract [85].

Algariri et al. (2014) showed biochemical and haematological data obtained from both acute and sub-chronic procedures suggest that the extract is safe at the limit test dose (2000 mg/kg); thus, the oral lethal dose (LD$_{50}$) exceeds 2000 mg/kg. The acceptable daily intake (ADI) was determined to be 700 mg/kg/day. In the acute antihyperglycaemic study, the n-butanol fraction (n-BF) was found to consistently lower glucose levels the most effectively, which was also demonstrated in the 14-day study. The estimated amounts of flavonoids in n-BF were 62.90% and 79.68% higher than the ethyl acetate fraction (EAF) and aqueous fraction (AF) with a corresponding value of 61.22% [86].

Dalimartha et al. (2001) reported that Gynura procumbens leaves were beneficial to cure inflammation of eyes, toothache, rheumatic, cysts and tumors [87].

Mahmood et al. (2010) investigated Gynura procumbens ethanolic extracts gastroprotective affect in adult sprague dawley rats which were divided into six groups. The rats were orally pre-treated with carboxymethyl cellulose (CMC) solution (ulcer control groups), omeprazole 20 mg/kg (reference group), 50, 100, 200 and 400 mg/kg of GPELE in CMC solution (experimental groups). The ulcer control group exhibited severe mucosal injury, whereas groups pre-treated with GPELE exhibited significant protection of gastric mucosal injury [63].

Kim et al. (2006) showed that oral administration of 500 mg/kg of Gynura procumbens extract (GPE) resulted in significantly lower blood pressure in spontaneously hypertensive (SHR) rats compared with SHR rats not given GPE (p < 0.05). Furthermore, GPE-administered rats had significantly lower serum lactate dehydrogenase, creating phosphate kinase and increased nitric oxide (NO), a known vasodilator, compared with the non–GPE administered SHR group (p < 0.05). These results suggest that oral administration of aqueous GPE may be useful for prevention and treatment of hypertension through increasing NO production in blood vessels [88].
Hoe et al. (2007) explained that a partially purified fraction (FA-1) of aqueous extract from *Gynura procumbens* appears to be able to bring about hypotensive effect in hypertensive rats (SHR) and normotensive rats (WKY) by acting through inhibition of the angiotensin-converting enzyme (ACE). The FA-I produced a marked dose-dependent reduction in mean arterial pressure (MAP) in SHR and WKY rats, with an ED$_{50}$ of 1.09 and 1.05 mg/kg, respectively (p < 0.01). Furthermore, FA-I at 10 mg/kg strongly inhibited the angiotensin I-induced rise in MAP (p < 0.01). This response was comparable to that of captopril (synthetic ACE inhibitors) at 20 μg/kg. In the in vitro assay, ACE activity was inhibited with an IC$_{50}$ of 0.8 mg/mL [89].

Hew et al. (2013) showed that protein fraction SN-F11/12 from *Gynura procumbens* leaves inhibited the growth of MDA-MB-231 breast cancer cells (at an EC$_{50}$ value of 3.8 μg/mL) using lactate dehydrogenase cytotoxicity assay. The protein fraction was also decreased the expression of proliferation markers Ki67 (p < 0.05) and proliferating cell nuclear antigen (PCNA) (p < 0.05) after 36 hours of incubation of the cancer cells with the protein fraction compared to untreated cancer cells. The invasion marker chemokine (C-C motif) ligand 2 (CCL2) (p < 0.05) was decreased after 48 hours incubation compared to untreated cancer cells [90].

Zhara et al. (2010) showed the effects of topical application of ethanol extract of *Gynura procumbens* leaf extract on the rate of wound healing closure and histology of wound area. An area of uniform wound 2.00 cm in diameter using circular stamp was excised from the nape of the dorsal neck of all rats with the aid of round seal. The animal groups were topically treated with 0.2 ml of each vehicle (gum acacia), Intrasite gel, 100 and 200 mg/ml of ethanol extract, respectively. Macroscopically, wound dressed with *Gynura procumbens* leaf extract and Intrasite gel significantly healed earlier than those treated with vehicle [64].

Kaewseejan et al. (2012) revealed the contents of chlorophyll-a, b and carotenoids in ethanolic *Gynura procumbens* extract were 365.20±0.049, 132.40±0.029 and 53.20±0.034 μg g$^{-1}$ dry weight, respectively. Moreover, the ethanolic *Gynura procumbens* extract showed the presence of alkaloids and volatile oils, whereas saponins and anthraquinone glycosides were absent [91].

Afandi et al. (2014) identified total phenolic content (TPC) of *Gynura procumbens* by applying Folin-Ciocalteu method. The methanol extract of *Gynura procumbens* exhibits the highest total phenolic content (75.70 ± 2.69 mg GAE/g extract) followed by ethanol extract (70.70 ± 2.58 mg
GAE/g extract). On the other hand, the total phenolic content in an aqueous extract was observed to be lower than the latter (40.00 ± 0.59 mg GAE/g extract) [92].

Teoh et al. (2016) determined total phenolic content of Gynura procumbens extracts and they determined that ethyl acetate extract of Gynura procumbens showed the highest phenolic content (172.68 mg of GAE/g of extract). Similar phenolic content ranging from 67.18 to 67.59 mg of GAE/g of extract was observed in methanol and water extracts. The non-polar hexane extract which lacked polar and semi-polar phenolic constituents showed significantly the lowest phenolic content (22.03 mg of GAE/g of extract) [75].
CHAPTER III
EXPERIMENTAL
CHAPTER III

Experimental

3.1.1 General Methodology

3.1.1.1 Solvents and chemicals

All chemicals and reagents used in the study were of analytical grade. n-Hexane (95.0 % purity), ethanol (99.9 % purity), chloroform (99.0 % purity), acetone (99.6 % purity), DMSO (99.98 % purity) were purchased from Fisher Scientific Co. (Leicestershire, UK). Methanol (99.9 % purity) was purchased from Sigma-Adrich (Steinheim, Germany). Dichloromethane (99.5 % purity) and ethyl acetate (99.5 % purity) were purchased from Daejung, Korea and Loba Chemie, India respectively. All other chemicals used were analytical grade and all stock solutions were prepared using purified distilled water.

3.1.1.2 Evaporation

All evaporations were carried out under reduced pressure using a rotary evaporator at a bath temperature of 45 °C. The residual solvent in the extract and compounds were removed under high vacuum.

Figure-3.1: Rotary vacuum evaporator
3.1.2 Preparation of the reagents

3.1.2.1 Vanillin-sulphuric acid reagent

1% vanillin in concentrated sulfuric acid is used as a general spray reagent followed by heating the plates to 100 for 10 minutes.

![Image of Vanillin-sulphuric acid spray]

Figure-3.2: Vanillin-sulphuric acid spray

3.1.3 Preparation of extracts

The leaf of the plant was collected in fresh condition. It was sun-dried and then, dried in an oven at reduced temperature (not more than 40 °C) to make it suitable for grinding purpose. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for further use.

3.1.3.1 Initial extraction

Extraction can be done in two ways such as-

i) Cold extraction    ii) hot extraction
Chapter III

Experimental

i) Cold extraction

In cold extraction the powdered plant materials is submerged in a suitable solvent or solvent systems in an air-tight flat bottomed container for several days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant material will be dissolved in the solvent during this time and hence extracted as solution.

ii) Hot extraction

In hot extraction the powdered plant material is successively extracted to exhaustion in an Extraction apparatus at elevated temperature and reflux with several solvents of increasing polarity.

3.1.3.2 Final extraction procedures

The plant material extracted exhaustively in Extraction apparatus first with n-hexane, then with ethyl acetate - ethanol mixture (75:25), then with ethyl acetate - ethanol mixture (50:50) and last with ethyl acetate - ethanol mixture (25:75). All the extracts were filtered (with Whatman No 1 filter paper) individually and then concentrated with a rotary evaporator at low temperature (40-50 °C) under reduced pressure.

Figure-3.3: Extraction apparatus
3.1.4 Chromatographic techniques

Chromatography has been defined as primarily a separation process which is used for the separation of essentially molecular mixtures. It depends upon the redistribution of molecules of the mixture between two or more phases. It is the best and modern separation technique.

For separation of extracted compounds into individual pure ones, various types of chromatographic techniques are used such as column chromatography, thin layer chromatography (TLC) and preparative thin layer chromatography (PTLC).

3.1.4.1 Column chromatography (CC)

Column chromatography is the most common separation technique based on the principle of distribution (partition/adsorption) of compounds between a stationary and mobile phase.

A normal chromatographic column is packed with silica gel (kiesel gel 60, mesh 70-230). Slurry of silica gel in a suitable solvent is added into a glass column of appropriate height and diameter. When the desired height of adsorbent bed is obtained, a few hundred milliliter of solvent is run through the column for proper packing of the column. After packing, the sample to be separated is applied as a concentrated solution in a suitable solvent or the sample is adsorbed onto silica gel allowed to dry and subsequently applied on top of the adsorbent layer.

Then the column is developed with suitable solvent mixtures of increasing polarity. The elutes are collected either in test tubes or in conical flasks.
3.1.4.2 Thin layer chromatography (TLC)

Ascending one-dimensional thin layer chromatographic technique is used for the initial screening of the extracts and column fractions and checking the purity of isolated compounds. For the latter purpose commercially available precoated silica gel plates are usually used. For initial screening, TLC plates are made on glass plates with silica gel.

Two types of TLC plates were used throughout the experiments.

- a) Pre-coated TLC plates: 0.2 mm thin coatings of silica gel on the glass plates or aluminium sheets were used.

- b) Manually prepared silica gel coated plates were used.
3.1.4.3.1 Solvent treatment

Solvent treatment is a process by which a compound consisting of the major portion of a mixture of compounds can be purified utilizing selective solvent washing. Initially, a solvent or a solvent mixture in which the desired compound is practically insoluble and other components are soluble is chosen.

The undesired components are separated with repeated washing with this solvent or solvent mixture. If required other solvent or solvent mixture can be used until a pure compound is obtained.

3.1.4.3.2 Sample application (Spotting the plates)

The TLC and PTLC plates were spotted with a small amount of the crude extract by using a narrow glass capillary. The capillary must be washed in a solvent (either acetone or ethanol) before each sample was applied.
3.1.4.3.3 Solvent Systems

The solvents of different polarity used for TLC are given below:

(i) n-Hexane
(ii) Dichloromethane (DCM)
(iii) Chloroform
(iv) Acetone
(v) Ethyl acetate (EA)
(vi) Ethanol (EtOH)
(vii) Methanol (MeOH)
(viii) EA: EtOH (in different ratio)

3.1.4.3.4 Developing TLC plate with solvent

A number of glass plates measuring 20cm x 5cm are thoroughly washed and dried in an oven. The dried plates are then swabbed with acetone-soaked cotton in order to remove any fatty residue. To make the slurry required amount of silica gel and appropriate volume of distilled water (2ml/gm of silica gel) are mixed in a conical flask and the flask is gently shaken. The slurry is then evenly distributed over the plates using TLC spreader. After air drying the coated plates are subjected to activation by heating in an oven at 110 °C for 70 minutes.
3.1.4.3.5 Visualization/detection of compounds

Detection of compounds in TLC plates is a very important topic in analyzing extractives to isolate pure compounds. The following techniques are used for detecting the compounds in TLC/PTLC plates.

1. The developed chromatogram is viewed visually to detect the presence of colored compounds.
2. The developed and dried plates are observed under UV light of both long and short wavelength (254 nm and 366 nm) to detect the spot/band of any compound. Some of the compounds appear as fluorescent spots while the others as dark spots under UV light.
3. The developed chromatogram is placed in a closed chamber containing crystals of iodine and kept for few minutes. The compounds that appeared as brown spots are marked. Unsaturated compounds absorb iodine. Bound iodine is removed from the plate by air blowing.

Figure-3.6: Development of TLC plate
3.1.4.3.6 Determination of $R_f$ (retardation factor) values

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

$$R_f = \frac{\text{Distance (cm) traveled by solute}}{\text{Distance (cm) traveled by solvent}}$$

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

![Diagram of TLC plate for calculating $R_f$ value](image)

Figure 3.7: A TLC plate for the calculation of $R_f$ value

3.1.4.3.7 Crystallization

Crystallization was employed as a final purification process. The solvent in which the compound was dissolved in a minimum volume of solvent in hot condition and was left undisturbed for crystallization. Sometimes mixture of solvents was used. The compound was dissolved in a suitable solvent and then a solvent in which the compound was insoluble, was gradually until cloudiness developed in the solution.
3.1.4.4 Spectroscopic techniques

3.1.4.4.1 Infra-red (IR) spectroscopy

A Shimadzu IR-470 spectrometer was used to record the infra-red spectrum. Before making the pellets, the samples were dried in the desiccator to remove moisture completely and to avoid unexpected O-H peak for accumulated water molecules in the sample. The IR was taken from Khulna University of Engineering & Technology, Khulna.

3.1.4.4.2 Nuclear Magnetic Resonance (NMR) spectroscopy

$^{13}$C NMR and $^1$H NMR spectra were recorded using 400MHz AVANCE Bruker NMR spectrometer. The spectra were recorded using MeOH with tetramethylsilane (TMS) as the standard reference. The NMR was taken from Wazed Miah Science Research Centre (WMSRC), Jahangirnagar University, Dhaka.

3.1.4.4.3 Determination of melting points

All melting points were taken in a Stuart’s electrothermal melting point apparatus (Model no. SMP 30). The heating was done carefully so as to maintain a steady temperature.

Figure-3.8: Melting point apparatus
3.2 Experimental

3.2.1 Chemical investigation of *Gynura procumbens*
In this study, leaves of *Gynura procumbens* belonging to the family Asteraceae were chemically investigated.

3.2.2 Collection and plant material preparation
The leaves of *Gynura procumbens* was collected from local market of Rupsha nursery, Khulna district of Bangladesh. The collected fresh leaves were cleaned thoroughly with distilled water. Then the leaves were dried under mild sunlight. Afterwards the plants were powdered. The powder was used throughout the investigation.

3.2.3 Extraction, isolation and characterization of compounds from the leaves of *Gynura procumbens*
The air-dried and powdered plant leaves (~200gm) was extracted to exhaustion in an extraction apparatus by n-hexane in a round bottom flux and heated by heating metal about one hour. Then it was kept for 24 hours and after that total content was filtered by filter paper (Whatman no.1). The residue obtained after extraction with n-hexane was further extracted with the ratio of EA- EtOH (25:75), (50:50) and (75:25) mixtures in the similar way. The volume of the filtrate was concentrated with a rotary evaporator at low temperature (40-50 °C) and reduced pressure. The extraction procedure was shown in the following scheme:
Figure-3.9: Extraction scheme of *Gynura procumbens*
Thus, four types of extract were found.

1) n-Hexane extract (~17.5g).
2) EA:EtOH (75:25) extract (~13.55g)
3) EA:EtOH (50:50) extract (~25.12g)
4) EA:EtOH (25:75) extracts (~19.85g).

### 3.2.3.1 Isolation and characterization of compounds from EA- EtOH (75:25) extract of *Gynura procumbens*

#### 3.2.3.1.1 Thin Layer chromatography (TLC) of EA- EtOH (75:25) extract

By TLC screening the EA- EtOH (75:25) crude extract was exposed to see the type of compounds present in the extract. The whole portion of the EA- EtOH (75:25) extract was subjected to Liquid Chromatography (LC) for rapid fractionation. The LC fractions were screened by TLC to find out interesting fractions and it showed several spots in iodine chamber on TLC plate, indicating presence of several compounds.

#### 3.2.3.1.2 Fractionation of the extract by column chromatography (LC)

The EA- EtOH (75:25) extract was concentrated by rotary evaporator. The concentrated EA-EtOH (75:25) extract of *Gynura procumbens* was mixed with column grade silica gel (Kieselgel 60). Then the sample mixture was placed on the top of the bed of column. The column was first eluted with 100% n-hexane and then eluted with mixtures n-hexane and increasing amount of EA, then eluted with mixtures EA and increasing amount of EtOH and finally with MeOH and EtOH mixtures. The eluents were collected in an amount of about 20 mL in a series of test tubes and conical flask. Solvent systems used as mobile phases in the analysis of ethyl acetate and ethanol extract were listed in Table 3.1.
Table-3.1: Number of fraction collected in test tubes from column chromatography of EA-EtOH (75:25) extract using different solvent systems is as follows:

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Total Amount of solvent (mL)</th>
<th>No. of test tubes/Conical flask</th>
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<tr>
<td>n-hexane (mL)</td>
<td>Ethyl acetate (mL)</td>
<td>Ethanol (mL)</td>
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<td>100</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
3.2.3.1.3 Screening of the fractions

Each of the fractions was monitored by TLC. Fractions of the similar TLC behavior were combined together and were designated as F₁-F₉ (Table-3.2).

Table-3.2: TLC behaviors of fractions are listed in table-

<table>
<thead>
<tr>
<th>No. of test tubes or conical flask</th>
<th>Fraction no.</th>
<th>Solvent system for TLC</th>
<th>No. of spots (After keeping iodine chamber)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.flask-01-04</td>
<td>F₁</td>
<td>100% DCM</td>
<td>Spot merged into tailing</td>
</tr>
<tr>
<td>C.flask-05-06</td>
<td>F₂</td>
<td>70% n-Hexane:30% EA</td>
<td>Spot merged into tailing</td>
</tr>
<tr>
<td>C.flask-07-08</td>
<td>F₃</td>
<td>30% n-Hexane:70% EA</td>
<td>No spot</td>
</tr>
<tr>
<td>01-26</td>
<td>F₄</td>
<td>40% n-Hexane:60% EA</td>
<td>No spot</td>
</tr>
<tr>
<td>27-35</td>
<td>F₅</td>
<td>90% EA : 10% Acetone</td>
<td>No spot</td>
</tr>
<tr>
<td>36-50</td>
<td>F₆</td>
<td>90% EA : 10% EtOH</td>
<td>Single Spot</td>
</tr>
<tr>
<td>51-65</td>
<td>F₇</td>
<td>90% Acetone:10% EtOH</td>
<td>No spot</td>
</tr>
<tr>
<td>66-75</td>
<td>F₈</td>
<td>70% Acetone:30% EtOH</td>
<td>Spot merged into tailing</td>
</tr>
<tr>
<td>76-98</td>
<td>F₉</td>
<td>80%EtOH:20%MeOH</td>
<td>No spot</td>
</tr>
</tbody>
</table>

3.2.3.1.4 Analysis of the fraction by TLC

TLC analysis of the fractions was further carried, fractions F₆ were found to contain single compound. The rest of the fractions were found to contain mixture of compounds. So attempt was taken to characterize the fractions F₆.

3.2.3.1.5 Analysis of fraction F₆

Fraction F₆ was left undisturbed at room temperature for several days. A white shaped crystal was obtained. The crystal were separated from mother liquor and washed with different solvents. Its TLC study also showed a single spot (Rₜ value=0.533) in EA and EtOH (90:10) mixture. It was designated as EE-1.
3.2.3.1.6 Properties and Characterization of the compound EE-1 by spectroscopic methods

i) Physical properties:

<table>
<thead>
<tr>
<th>Physical state</th>
<th>White crystal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>Soluble in MeOH</td>
</tr>
<tr>
<td>R_f value</td>
<td>0.533 in EA:EtOH (90:10)</td>
</tr>
<tr>
<td>Amount</td>
<td>27mg</td>
</tr>
</tbody>
</table>

ii) Infrared (IR) spectroscopy:

The IR spectrum of the compound EE-1 (KBr pellet) was recorded.

iii) ^1H NMR spectroscopy:

The ^1H NMR spectrum of the compound EE-1 was taken in MeOH at 400 MHz and TMS was used as reference.

iv) ^13C NMR spectroscopy:

The ^13C NMR spectrum of EE-1 was recorded using MeOH as solvent at 400 MHz.
3.2.3.2 Isolation and characterization of compounds from EA and EtOH (25:75) extract of *Gynura procumbens*

3.2.3.2.1 Thin Layer chromatography (TLC) of EA and EtOH (25:75) extract

By TLC screening the EA- EtOH (25:75) crude extract was exposed to see the type of compounds present in the extract. The whole portion of the EA- EtOH (25:75) extract was subjected to Liquid Chromatography (LC) for rapid fractionation. The LC fractions were screened by TLC to find out interesting fractions and it showed several spots in iodine chamber on TLC plate, indicating presence of several compounds.

3.2.3.2.2 Fractionation of the extract by column chromatography (CC)

The EA- EtOH (25:75) extract was concentrated by rotary evaporator. The concentrated EA- EtOH (25:75) extract of *Gynura procumbens* was mixed with column grade silica gel. Then the sample mixture was placed on the top of the bed of column. The column was first eluted with 100% n-hexane and then eluted with mixtures n-hexane and increasing amount of EA, then eluted with mixtures EA and increasing amount of EtOH and finally with MeOH and EtOH mixtures. The eluents were collected in an amount of about 20 ml in a series of test tubes and conical flask. Solvent systems used as mobile phases in the analysis of ethyl acetate extract were listed in Table-3.3
Table-3.3: Different solvent systems used for column chromatography (CC) analysis of EA-EtOH (25:75) extract as follows:

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Total Amount of solvent (mL)</th>
<th>No. of test tubes/Conical flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexane (mL)</td>
<td>Ethyl acetate (mL)</td>
<td>Ethanol (mL)</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>70</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>50</td>
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<tr>
<td>0</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>0</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>0</td>
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<td>100</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>80</td>
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<tr>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
3.2.3.2.3 Screening of the fractions

All the column fractions were screened by TLC and a number of similar fractions were mixed together and identified them by new fractions F₁-F₇ (Table-3.4).

Table-3.4: Screening of the fractions by similar TLC pattern

<table>
<thead>
<tr>
<th>No. of test tubes</th>
<th>Fraction no.</th>
<th>Solvent system for TLC</th>
<th>No. of spots (After keeping iodine chamber)</th>
</tr>
</thead>
<tbody>
<tr>
<td>07-10</td>
<td>F₁</td>
<td>n-Hexane (100%)</td>
<td>No spot</td>
</tr>
<tr>
<td>19-24</td>
<td>F₂</td>
<td>80% n-Hexane:20 % EA</td>
<td>Spot merged into tailing</td>
</tr>
<tr>
<td>27-31</td>
<td>F₃</td>
<td>90% EA:10% EtOH</td>
<td>Single spot</td>
</tr>
<tr>
<td>36-40</td>
<td>F₄</td>
<td>60% EA: 40% EtOH</td>
<td>No spot</td>
</tr>
<tr>
<td>47-52</td>
<td>F₅</td>
<td>60% EtOH: 40% EA</td>
<td>Spot merged into tailing</td>
</tr>
<tr>
<td>56-82</td>
<td>F₆</td>
<td>20% EA:80% EtOH</td>
<td>Spot merged into tailing</td>
</tr>
<tr>
<td>85-125</td>
<td>F₇</td>
<td>100% EA</td>
<td>Spot merged into tailing</td>
</tr>
</tbody>
</table>

3.2.3.1.4 Analysis of the fraction by TLC

TLC analysis of the fractions was further carried, fractions F₃ were found to contain single compound. The rest of the fractions were found to contain mixture of compounds. So attempt was taken to characterize the fractions F₃.

3.2.3.1.5 Analysis of fraction F₃

Fraction F₃ was left undisturbed at room temperature for several days. A white shaped crystal was obtained. The crystal were separated from mother liquor and washed with different solvents. Its TLC study also showed a single spot (Rᵢ value=0.68) in EA and EtOH (90:10) mixture. It was designated as EE-3.
3.2.3.1.6 Properties and Characterization of the compound EE-3 by spectroscopic methods

i) Physical properties:

<table>
<thead>
<tr>
<th>Physical state</th>
<th>White crystal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>Soluble in MeOH</td>
</tr>
<tr>
<td>R_f value</td>
<td>0.68 in EA:EtOH (90:10)</td>
</tr>
<tr>
<td>Amount</td>
<td>(~20.6 mg)</td>
</tr>
</tbody>
</table>

ii) Infrared (IR) spectroscopy:

The IR spectrum of the compound EE-3 (KBr pellet) was recorded.

iii) ^1H NMR spectroscopy:

The ^1H NMR spectrum of the compound EE-3 was taken in MeOH at 400 MHz and TMS was used as reference.

iv) ^13C NMR spectroscopy:

The ^13C NMR spectrum of EE-3 was recorded using MeOH as solvent at 400 MHz.

3.3 Antimicrobial Screening

3.3.1 Introduction

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity, and due to new bacterial strains, which are multi-resistant. Consequently, new infections can occur in hospitals resulting in high mortality.
From 1980 to 1990, Montelli and Levy documented a high incidence of resistant microorganisms in clinical microbiology in Brazil. This fact has also been verified in other clinics around all over the world. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, and develop research to better understand the genetic mechanisms of resistance and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient.

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in Brazil. According to World Health Organization medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency.

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils, as well as in tannin.

The antimicrobial properties of plants have been investigated by a number of researchers worldwide, especially in Latin America. In Argentina, a research tested 122 known plant species used for therapeutic treatments. It was documented that among the compounds extracted from these plants, twelve inhibited the growth of *Staphylococcus aureus*, ten inhibited *Escherichia coli*, and four inhibited *Aspergillus niger* and also reported that the most potent compound was one extracted from *Tabebuia impetiginosa*. [93].
3.3.2 Antimicrobial susceptibility testing methods

The performance of antimicrobial susceptibility testing by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antimicrobial agents, or to detect resistance in individual bacterial isolates. Empirical therapy continues to be effective for some bacterial pathogens because resistance mechanisms have not been observed e.g., continued penicillin susceptibility of *Streptococcus pyogenes*. Susceptibility testing of individual isolates is important with species that may possess acquired resistance mechanisms. The following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly:

i) Disk diffusion,

ii) Broth dilution,

iii) Agar dilution.

Among the above mentioned techniques the disk diffusion is a widely accepted in vitro investigation for preliminary screening of test agents because of its low cost, ease in modifying test antimicrobial disks when required and can be used as a screening test against large numbers of isolates. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method.

3.3.3 Disc Diffusion Method

The agar diffusion assay is one method for quantifying the ability of antibiotics to inhibit bacterial growth. Interpretation of results from this assay relies on model-dependent analysis, which is based on the assumption that antibiotics diffuse freely in the solid nutrient medium. In many cases, this assumption may be incorrect, which leads to significant deviations of the predicted behavior from the experiment and to inaccurate assessment of bacterial susceptibility to antibiotics. We sought a theoretical description of the agar diffusion assay that takes into consideration loss of antibiotic during diffusion and provides higher accuracy of the MIC determined from the assay. We propose a new theoretical framework for analysis of agar diffusion assays. MIC was determined by this technique for a number of antibiotics and analysis
was carried out using both the existing free diffusion and the new dissipative diffusion models. A theory for analysis of antibiotic diffusion in solid media is described, in which we consider possible interactions of the test antibiotic with the solid medium or partial antibiotic inactivation during diffusion. This is particularly relevant to the analysis of diffusion of hydrophobic or amphipathic compounds. The model is based on a generalized diffusion equation, which includes the existing theory as a special case and contains an additional, dissipative term. Analysis of agar diffusion experiments using the new model allows significantly more accurate interpretation of experimental results and determination of MICs. The model has more general validity and is applicable to analysis of other dissipative processes, for example to antigen diffusion and to calculations of substrate load in affinity purification [94].

3.3.4 Principle of Disc Diffusion Method

Solutions of known concentration (µg/mL) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6mm diameter) are then impregnated with known amounts of the test substances using micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature (4°C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel. As a result there is a gradual change of test materials concentration in the media surrounding the discs [95].

The plates are then incubated at 37 °C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter.
The experiment is carried out more than once and the mean of the readings is required. In the present study all the crude extracts were tested for antimicrobial activity by disc diffusion method. Some pure compounds could not be tested due to scarcity of samples.

### 3.3.5 Experimental

#### 3.3.5.1 Antibacterial screening

The disk diffusion method was used as a screening test for antibacterial activity. Filter-paper disks (6 mm in diameter) impregnated with extract solutions were placed on Müller-Hinton agar plates which were inoculated with test organisms according to the standard protocol described by the National Committee of Clinical Laboratory Standard Performance Standards of Antimicrobial Disk Susceptibility Testing. The filter-paper disks were impregnated with 20 µL of the extract solutions in order to obtain final concentrations of 1000, 200, 100, 50, and 25 µg of extract in the disks.

The plates were incubated at 35 °C (±1 °C), and after 18 hours the diameters of the inhibition zones were measured. Filter-paper disks containing sterile water without any test compound were negative control and no inhibition was observed. Standard antibiotic disks were selected according to the sensitivity of the bacteria tested.

#### 3.3.5.2 Antifungal screening

Screening for antifungal effect can be carried out by using the disc diffusion method. The plate containing 25 mL of PDA medium will be seeded with 1 mL of fungal conidial spore suspension containing $10^5$ spores per mL from a 120-h-old culture. Three Whatman filter paper No. 1 discs of 6-mm diameter can be used to screen the antifungal activity. Each sterile disk will be impregnated with 20 mL of the extract corresponding with 100 mg/mL of crude extract, myconazole 30 µg/mL, as positive control, or vehicle as negative control. The plates will be refrigerated for 2 hours to allow the compounds presents in the extract diffused and then will be incubated at 28 °C for 5 days. Diameter of the inhibition zone will be measured, and the mean of
the three replicates are taken (Bauer et al., 1966). The disc diffusion method is a qualitative test which could provide the information whether the crude extract possessed antifungal properties.

3.3.5.3 Determination of antimicrobial activity by the zone of inhibition

The disk-diffusion agar method tests the effectiveness of antibiotics on a specific microorganism. An agar plate is first spread with bacteria and then paper disks of antibiotics are added. The bacteria is allowed to grow on the agar media and then observed. The amount of space around every antibiotic plate indicates the lethality of that antibiotic on the bacteria in question. Highly effective antibiotics (disk C) will produce a wide ring of no bacterial growth while an ineffective antibiotic (disk A) will show no change in the surrounding bacterial concentration at all. The effectiveness of intermediate antibiotics (disk B) can be measured using their zone of inhibition. This method is used to determine the best antibiotic to use against a new or drug-resistant pathogen.

![Inhibition Zone measurement](image)

Figure 3.10: Inhibition Zone measurement

3.3.6 Kirby-Bauer Antimicrobial Susceptibility Test

The Kirby-Bauer and Stokes' methods are usually used for antimicrobial susceptibility testing, with the Kirby-Bauer method being recommended by the National Council of Clinical
Laboratory Service (NCCLS). The accuracy and reproducibility of this test are dependent on maintaining a standard set of procedures as described here.

### 3.3.6.1 Materials used

1. Test materials: Five crude extract [EA, EtOH and three ratio of EA: EtOH (75:25), (50:50) and (25:75)] of *Gynura procumbens*.

2. Test Organisms: The bacterial strain used for the experiment was collected as pure cultures from the Khulna University of pharmacy department both gram-positive and gram-negative organisms were taken for the test and they are listed in the Table-3.5

#### Table 3.5: List of Test bacteria and fungi

<table>
<thead>
<tr>
<th>Gram positive Bacteria</th>
<th>Gram negative Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td><em>Salmonella enterica</em></td>
</tr>
</tbody>
</table>

3. Growth Media: The activity was conducted on the Nutrient Agar Media produced from TSA (Tryptone Soya Agar).

4. Apparatus used:
   - **Petri plate**: Plastic plate which was previously sterilized
   - **Pipette**: Micropipette was used for adding the required concentration of sample to the plates.
   - **Blank discs**: Susceptible blank discs were used which was stored in -20 °C to 8 °C.
   - **Glasswares**: 500 mL conical flask and test tubes were used.
   - **Compounds Screened**: All the synthesized compounds.
   - **Solvent used**: MeOH
   - **Standard used**: Kanamycin acid.
3.3.7 Antimicrobial Assay of 5 Samples

3.3.7.1 Principle

Measured amount of the test samples were dissolved in definite volumes of solvent to give solutions of known concentration (µg/ml). Then sterile Matricel (BBL, Cocksville, USA) filter paper discs were impregnated with known amount of test substances using micropipette and dried. Standard antibiotic discs and discs on which the solvent used to dissolve the samples was adsorbed and dried were used as positive and negative control, respectively. These discs were then placed in petridishes (120 mm in diameter) containing a suitable agar medium seeded with the test organisms using sterile transfer loop for antibacterial screening. The plates were then kept at 40 °C facilitating maximum diffusion. A number of events take place simultaneously including

- The dried discs absorb water from the agar medium and the material under test is dissolved.
- The test material diffuses from the discs to the surrounding medium. The diffusion takes place according to the physical law that controls the diffusion of molecules through agar gel.
- There is a gradual change of test material concentration in the agar surrounding each disc. The plates are then kept in an incubator (37 °C) for 12-18 hour to allow the growth of the microorganisms. If the test material has any antimicrobial activity, it will inhibit the growth of microorganism giving a clear, distinct zone called “zone of inhibition”. The antibacterial activity of the test agent is determined by measuring the diameter of the zone of inhibition in term of millimeter. The experiments were carried out more than twice and the mean of the reading are recorded.
3.3.7.2 Preparation of Media

Nutrient agar media were prepared by adding water to a dehydrated product that contains all the ingredients. Practically all media are available commercially in powdered form.

The following steps were followed in the preparation of bacteriological media:

- Definite amounts of nutrient agar were accurately weighed.
- It was taken in a volumetric flask containing distilled water (half of the required volume).
- A clear medium was obtained by thorough dissolving agar over a water bath with occasional shaking.
- Then the final volume was adjusted.
- The medium was then transferred in 16 mL and 5 mL volume respectively, to prepare plates and slants, in a number of required test tubes.
- The test tubes were then plugged with cotton and sterilized in an autoclave at a temperature of 121 °C and pressure of 15-lbs/sq inch for 20 minutes.
3.3.7.3 Sterilization of Different Equipments and Media

- Media, petridishes and other glasswares were sterilized by autoclaving at a temperature of 121 °C and a pressure of 15-lbs/sq inch for 20 minutes.
- Blank discs kept in a covered petridish, loop and forceps were subjected to dry heat sterilization at 160 °C for 1 hour. Later they were kept in a laminar hood under UV light for 30 minutes.
- UV light was switched on before one hour working in a laminar hood to avoid accidental contamination.

3.3.7.4 Preparation of Sub-culture

- The agar slants were used for making fresh culture of microorganisms that in turn would be used for sensitivity tests.
- With the help of an inoculating loop, the test organisms from the pure cultures were transferred to the agar slants in an aseptic condition using laminar air hood.
- The inoculated slants were then incubated at 37 °C for 18-24 hours (for bacteria) to assure the growth of test organisms. This culture was used within two days.

3.3.7.5 Preparation of the Seeded Test Plates

- Each of the test organisms were transferred from the subculture to the test tube containing 16 mL autoclaved media with the help of the sterilized inoculating loop at 45 °C under laminar air follow.
- The test tubes were shaken by rotation to get a uniform suspension of organism. The bacterial suspensions were immediately transferred to the sterile petridishes aseptically.
- The petridishes were rotated several times, first clockwise and then anticlockwise, to assure homogeneous distribution of the test organisms.
- The medium was poured into petridishes in such a way as to give a uniform layer of depth of approximately 4 mm. After the medium became cooled to room temperature, it was stored in a refrigerator (4 °C).
3.3.7.6 Preparation of Disks

Three types of discs were used for antibacterial screening:

a) Sample discs
b) Standard discs and
c) Blank discs

Their preparations described below:

a) **Sample Discs**

Sterile filter paper discs (5 mm in diameter) were taken in a blank petridish. Sample solution of the desired concentration was applied on the discs with the help of a micropipette in an aseptic condition. These discs were left for few minutes in aseptic condition for complete removal of solvent.

b) **Standard Discs**

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antibacterial agent.
with that produced by test samples. In this investigation Kanamycin (30 µg / disc) standard disc was used as the reference.

c) **Blank Discs**

These were used as negative control. They ensure that the residual solvents (left over the discs even after air drying) and the filter paper were not active themselves.

![Figure- 3.13: Application of discs](image)

**3.3.7.7 Determination of Antibacterial Activity**

After proper incubation, the antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition in term of millimeter with a digital slide calipers.

Sample impregnated discs, standard antibiotic discs and negative control discs were placed gently on the solidified agar plates, freshly seeded with the test organisms with the help of a sterile forceps to assure complete contact with medium surface. The spatial arrangement of the discs was such that the discs were not closer than 15mm to the edge of the plate and far enough apart to prevent overlapping the zones of inhibition. The plates were then inverted and kept in refrigeration for about 24 hours at 4 °C . This was sufficient time for the material to diffuse into a considerable area of the medium. Finally the plates were incubated upside down at 37 °C for 12-18 hour.
3.3.7.8 Preparation of sample discs with test samples of *Gynura procumbens* extract

The ratio of EA- EtOH (25:75), (50:50) and (75:25), EA and EtOH of *Gynura procumbens* was used for antimicrobial activity test against a number of both gram positive & gram negative bacteria. The Antimicrobial activity of the extracts where tested using sample concentration 250µg and 500µg per disc.

3.3.7.9 Application of samples on the discs

a) Compounds were dissolved in MeOH and diluted to get concentration of 12 µg/µL.

b) Five blank discs were placed in the petri plates. Reference standard kanamycin was impregnated on one of the discs and only solvent as a blank was impregnated on one of the discs and others experimental solutions were impregnated on others discs. Each disc’s was marked by a marker as a small symbol so that each of the discs could be easily identified 25µL of solution was injected on each disc.

![Fig 3.14: Application of Samples on the discs](Image)
CHAPTER IV
RESULTS AND DISCUSSION
CHAPTER IV

Results and Discussion

4.1 Preliminary investigation of the plant material

4.1.1 Plant material

A species of the Asteraceae family, *Gynura procumbens* has been investigated in this work. The leaves of the plant have been used in this experiment.

4.1.2 Extraction of the plant material

The leaves of *Gynura procumbens* was collected from local market of Rupsha nursery, Khulna district of Bangladesh. The collected fresh leaves were cleaned thoroughly with distilled water. Then the leaves were dried under mild sunlight. Afterwards the plants were powdered. The powder was used throughout the investigation. Then the powdered plant material (~200 g) was extracted with n-Hexane (100%), EA and EtOH (75:25) %, EA and EtOH (50:50) % and EA and EtOH (25:75) % mixture. Then the filtrates was concentrated in a vacuum rotary evaporator to yield the crude extract of n-Hexane(100%), EA and EtOH (75:25) %, EA and EtOH (50:50)% and EA and EtOH (25:75)% mixture. Then the crude extract was dried in room temperature to get dry mass.

4.1.3 Isolation and characterization of compounds from EA- EtOH (75:25) extract

From the extractives pure compounds were isolated applying various chromatographic techniques. After application of the sample, solvents of increasing polarities using n-Hexane, EA and EtOH were passed through the column. The fractions were collected in an amount of about 20 mL in a series of test tubes and conical flask (50 mL). The eluted samples, collected in
98 test tubes and 10 conical flasks. They were combined on the basis of their Rf values in TLC and finally nine fractions (F₁, F₉) were obtained. Among the fractions, one showed single spot on TLC and collected as pure compounds and was labeled as EE-01.

4.1.3.1 Characterization of compound EE-01 by spectroscopic methods

¹H-NMR and ¹³C-NMR spectra were recorded in MeOH. The chemical shifts are reported in ppm on scale downfield from TMS as internal standard and signal patterns are indicated as follows: s = singlet, d = doublet, dd = doublet of doublet, dt = doublet of triplet, t = triplet, q = quartet, qd = quartet of doublet, m = multiplet, br = broad. IR spectra were recorded on KBr pellets and reported in wave number (cm⁻¹).

i. Physical properties

It was whitish crystalline solid, soluble in MeOH, Rf value of the compound was 0.533 in EA: EtOH (4:1), the amount of the compound was ~27 mg and melting point of the compound was 280-282 °C.

ii. Infrared (IR) Spectra

Table:-4.1: IR data of the compound EE-01

<table>
<thead>
<tr>
<th>No</th>
<th>Groups</th>
<th>Types of Vibration</th>
<th>Frequency (cm⁻¹)</th>
<th>Actual Frequency (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkane</td>
<td>Stretching</td>
<td>2960.73</td>
<td>2850-3000</td>
</tr>
<tr>
<td>2</td>
<td>C=C</td>
<td>Stretching</td>
<td>1647.21</td>
<td>1670</td>
</tr>
<tr>
<td>3</td>
<td>-CH₂-</td>
<td>Bending</td>
<td>1458.18</td>
<td>1465</td>
</tr>
<tr>
<td>4</td>
<td>-OH</td>
<td>Broad absorption</td>
<td>3365.78</td>
<td>3200-3500</td>
</tr>
<tr>
<td>5</td>
<td>C-O</td>
<td>Stretching</td>
<td>1074.35</td>
<td>1100 ( 2° saturated alcohol )</td>
</tr>
</tbody>
</table>
Figure 4.1: IR spectrum of EE-01 compound
iii. $^1$H-NMR Spectroscopy (MeOH, 400 MHz)

The Table-4.2 of $^1$H-NMR spectrum of EE-01 indicated the aglycone and sugar moieties. The $^1$H-NMR spectrum (Figure-4.2) showed the chemical shift at $\delta_H$ 0.744-0.762 and 0.965 ppm indicated the presence of two angular methyl signals for the position at H-18 and H-19. The proton NMR spectrum also exhibited one olefinic double bond proton as a doublet at $\delta_H$ 5.387 ppm for the position at H-6, along with the two up field signals at $\delta_H$ 0.909 ppm and 0.889 ppm respectively, due to the presence of two secondary methyl groups at position H-26 and H-27 of the skeleton, i.e., the presence of an isopropyl group of the molecular structure.

The very up field chemical shift at $\delta_H$ 0.834 ppm as a triplet with the intensity of 3H was assigned for the terminal methyl group of H-29. Similarly, the other up field chemical shift at $\delta_H$ 1.110 ppm was assigned the secondary methyl group at position H-21 of the molecular structure.

The proton NMR spectrum also exhibited five tetrahydropyran proton in high field region chemical shift at $\delta_H$ 4.570 ppm for the position $H-1'$, at $\delta_H$ 3.691-3.580 ppm for the position $H-2'$, $\delta_H$ 3.883-3.855 ppm for the position $H-3'$ and $H-4'$ and $\delta_H$ 3.181-3.140 ppm for the position $H-5'$. Another high field signal at $\delta_H$ 4.410-4.391 ppm for the position $H-6'$ indicated a methylene group. Ten aglyconic methylene proton give signal at $\delta_H$ 2.463 ppm for the position $H-4$, $\delta_H$ 2.083-1.976 ppm for the position $H-2$, $\delta_H$ 1.923 ppm for the position $H-1$, $\delta_H$ 1.245 ppm for the position $H-28$, $\delta_H$ 1.054 ppm for the position $H-22$ and $H-23$ and $\delta_H$ 1.054-1.402 ppm for the position $H-7$, $H-11$, $H-12$, $H-15$ and $H-16$. Again seven aglyconic methine protons give signal at $\delta_H$ 1.883 ppm for the position $H-25$, $\delta_H$ 1.402 ppm for the position $H-20$, $\delta_H$ 1.701-1.479 ppm for the position $H-8$, $H-9$, $H-14$ and $H-17$, $\delta_H$ 0.981 ppm for the position $H-24$. 
Figure 4.2: $^1$H-NMR spectrum of EE-01 compound
Figure 4.3: $^1$H-NMR spectrum of EE-01 compound (Extended form -01)
Figure 4.4: $^1$H-NMR spectrum of EE-01 compound (Extended form-02)
Table 4.2: $^{13}$C-NMR and $^1$H-NMR spectral data of the compound EE-01

<table>
<thead>
<tr>
<th>Position of Carbon</th>
<th>$^{13}$C-NMR Peak $\delta_C$ in ppm</th>
<th>Types of carbon</th>
<th>Position of proton</th>
<th>$^1$H-NMR Peak $\delta_H$ in ppm</th>
<th>Types of proton</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>36.023</td>
<td>CH$_2$</td>
<td>H-1</td>
<td>1.923 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-2</td>
<td>29.018</td>
<td>CH$_2$</td>
<td>H-2</td>
<td>1.883-2.083 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-3</td>
<td>76.725</td>
<td>CH</td>
<td>H-3</td>
<td>3.140-3.818 (m)</td>
<td>1H</td>
</tr>
<tr>
<td>C-4</td>
<td>36.486</td>
<td>CH$_2$</td>
<td>H-4</td>
<td>2.463 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-5</td>
<td>140.776</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-6</td>
<td>121.355</td>
<td>CH</td>
<td>H-6</td>
<td>5.387 (d)</td>
<td>1H</td>
</tr>
<tr>
<td>C-7</td>
<td>31.662</td>
<td>CH$_2$</td>
<td>H-7</td>
<td>1.054-1.402 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-8</td>
<td>31.869</td>
<td>CH</td>
<td>H-8</td>
<td>1.479-1.701 (m)</td>
<td>1H</td>
</tr>
<tr>
<td>C-9</td>
<td>50.355</td>
<td>CH</td>
<td>H-9</td>
<td>1.479-1.701 (m)</td>
<td>1H</td>
</tr>
<tr>
<td>C-10</td>
<td>36.486</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-11</td>
<td>22.766</td>
<td>CH$_2$</td>
<td>H-11</td>
<td>1.054-1.402 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-12</td>
<td>40.624</td>
<td>CH$_2$</td>
<td>H-12</td>
<td>1.054-1.402 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-13</td>
<td>42.337</td>
<td>C</td>
<td>H-13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-14</td>
<td>56.791</td>
<td>CH</td>
<td>H-14</td>
<td>1.479-1.701 (m)</td>
<td>1H</td>
</tr>
<tr>
<td>C-15</td>
<td>23.907</td>
<td>CH$_2$</td>
<td>H-15</td>
<td>1.054-1.402 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-16</td>
<td>27.940</td>
<td>CH$_2$</td>
<td>H-16</td>
<td>1.054-1.402 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-17</td>
<td>56.791</td>
<td>CH</td>
<td>H-17</td>
<td>1.479-1.701 (m)</td>
<td>1H</td>
</tr>
<tr>
<td>C-18</td>
<td>10.927</td>
<td>CH$_3$</td>
<td>H-18</td>
<td>0.744-0.762 (d)</td>
<td>3H</td>
</tr>
<tr>
<td>C-19</td>
<td>18.427</td>
<td>CH$_3$</td>
<td>H-19</td>
<td>0.965 (s)</td>
<td>3H</td>
</tr>
<tr>
<td>C-20</td>
<td>36.023</td>
<td>CH</td>
<td>H-20</td>
<td>1.402 (m)</td>
<td>1H</td>
</tr>
<tr>
<td>C-21</td>
<td>18.765</td>
<td>CH$_3$</td>
<td>H-21</td>
<td>1.110 (d)</td>
<td>3H</td>
</tr>
<tr>
<td>C-22</td>
<td>33.720</td>
<td>CH$_2$</td>
<td>H-22</td>
<td>1.054 (m)</td>
<td>2H</td>
</tr>
<tr>
<td>C-23</td>
<td>29.018</td>
<td>CH$_2$</td>
<td>H-23</td>
<td>1.054 (m)</td>
<td>2H</td>
</tr>
</tbody>
</table>
### 13C-NMR Spectroscopy (MeOH, 400 MHz)

Figure 4.5 shows spectrum $^{13}$C NMR (MeOH, 400 MHz) of the compound EE-01 at $\delta_C$ 20.779 ppm and 18.765 ppm, which were for the two separate terminal methyl groups at the position C-26 and C-27. The three up field chemical shifts at $\delta_C$ 10.927, 18.427 and 18.765 ppm respectively, were assigned for the angular methyl carbons linked at C-18, C-19 and C-21 position.

The up field signals at $\delta_C$ 31.869 ppm, 36.486 ppm and 56.791 ppm were assignable to the carbon at positions C-8, C-10 and C-14 that was fused in the proposed $\beta$-sitosteryl-D-glycoside. Similarly, the relative down field chemical shifts at $\delta_C$ 50.355 ppm, and $\delta_C$ 45.906 ppm, respectively, were assigned for the carbon that was fused at positions C-9 and C-13, respectively, in the proposed skeleton. The up field chemical shift at $\delta_C$ 36.023, 29.018, 76.725, 36.486, 31.662, 22.766, 42.104, 23.907 and 27.940 ppm were appropriate for the cyclohexyl and
cyclopentyl carbons at positions C-1, C-2, C-3, C-4, C-7, C-11, C-12, C-15 and C-16, respectively.

The other shifts at $\delta_C$ 36.023, 33.720, 29.018, 46.954, 29.018, 23.907 and 10.896 ppm were assigned for the carbon numbers C-20, C-22, C-23, C-24, C-25, C-28 and C-29, respectively, which constitute the side chain of six carbons which were linked at position C-17 of the cyclopentyl ring. The chemical shift at $\delta_C$ 56.791 ppm was assigned for the carbon number C-17 which was the point of link of a side chain to the cyclopentyl ring. The very down field chemical shift at $\delta_C$ 73.758, 78.487, 70.314, 76.489 and 61.619 ppm were assigned for the carbon numbers of the sugar moiety at position C-2´, C-3´, C-4´, C-5´ and C-6´.

The very down field chemical shift at $\delta_C$ 140.776 ppm and 121.355 ppm was for the $>\text{C=C}<$ carbons between C-5 and C-6 and down field chemical shift at $\delta_C$ 101.077 ppm was for tetrahydropyran carbon at C-1´.
Figure 4.5: $^{13}$C-NMR spectrum of EE-01 compound
Figure-4.6: $^{13}$C-NMR spectrum of EE-01 compound (Extended form)
4.1.3.2 Structure of the compound EE-01

On the basis of IR, \(^1\)H-NMR and \(^{13}\)C-NMR data the name of the compound EE-01 was \(\beta\)-sitosterol-3-O-\(\beta\)-D-glucopyranoside. The IR, \(^1\)H-NMR and \(^{13}\)C-NMR data almost same with the published data for \(\beta\)-sitosterol-3-O-\(\beta\)-D-glucopyranoside and the relative configuration was confirmed on the basis of comparison with the literature values (N. P. Rai, et al., 2006) [102]. The structure of \(\beta\)-sitosterol-3-O-\(\beta\)-D-glucopyranoside is given below:

![Structure of the compound EE-01](image)

**Name of the compound:** \(\beta\)-sitosterol-3-O-\(\beta\)-D-glucopyranoside

4.1.3.4 Characterization of compound EE-03 by spectroscopic methods

By characterization of spectroscopic method of compound EE-03, it identified that compound EE-03 is same as characterized of compound EE-01.
4.2 Antimicrobial screening

4.2.1 Reading of plates

The culture plates were incubated at 37 °C for 24 hours. The zones of inhibition produced by compounds and Kanamycin were recorded in mm and compared. Inhibition zone of some selected active compounds are given in-

Figure-4.7: Antibacterial activity of compounds (a), D(6.5), E(7), F(0) against; *Staphylococcus aureus* (b), D(0), E(0), F(0) against *Salmonella enterica*

Figure-4.8: Antibacterial activity of compounds (b) D(7), E(0), E(0) against *Bacillus subtilis*; (b) D(0), E(0), F(0) against *Escherichia coli*.
Table-4.3: Antimicrobial activities of the crude extract EA, EtOH and EA-EtOH (75:25), (25:75) and (50:50) mixtures of *Gynura procumbens* (Inhibition zone in mm) in 250 µg/disc.

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Ethanol extract (B) (250 µg/disc)</th>
<th>Ethyl acetate extract (C) (250 µg/disc)</th>
<th>Ethyl acetate ethanol(75:25) extract (D) (250 µg/disc)</th>
<th>Ethyl acetate ethanol(25:75) extract (E) (250 µg/disc)</th>
<th>Ethyl acetate ethanol(50:50) extract (F) (250 µg/disc)</th>
<th>Standard (Kanamycin) (30µg/disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0</td>
<td>0</td>
<td>5.5</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0</td>
<td>0</td>
<td>5.5</td>
<td>6</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td><strong>Gram negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>
Table 4.4: Antimicrobial activities of the crude extract EA, EtOH and EA-EtOH (75:25), (25:75) and (50:50) mixtures of *Gynura procumbens* (Inhibition zone in mm) in 500 µg/disc.

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Ethanol extract (B) (500 µg/disc)</th>
<th>Ethyl acetate extract (C) (500 µg/disc)</th>
<th>Ethyl acetate-ethanol (75:25) extract (D) (500 µg/disc)</th>
<th>Ethyl acetate-ethanol (25:75) extract (E) (500 µg/disc)</th>
<th>Ethyl acetate-ethanol (50:50) extract (F) (500 µg/disc)</th>
<th>Standard (Kanamycin) (30 µg/disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0</td>
<td>0</td>
<td>6.5</td>
<td>7</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Gram negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>
4.2.2 Antimicrobial activity of EA-EtOH (75:25) extract (D) of *Gynura procumbens* leaves

250 μg/disc EA-EtOH (75:25) extract of *Gynura procumbens* leaves was shown poor antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* (Gram positive bacteria) and no activity against *Escherichia coli* and *Salmonella enterica* (Gram negative bacteria) –

Figure 4.9: Zone of inhibition vs bacterial strain of 250 μg/disc EA-EtOH (75:25) extract

And 500 μg/disc EA-EtOH (75:25) extract of *Gynura procumbens* leaves was shown moderate antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* (Gram positive bacteria) and no activity against *Escherichia coli* and *Salmonella enterica* (Gram negative bacteria) –
Chapter IV

Result and Discussion

4.2.3 Antimicrobial activity of EA-EtOH (25:75) extract (E) of *Gynura procumbens* leaves

250 µg/disc EA-EtOH (25:75) extract of *Gynura procumbens* leaves was shown mild antibacterial activity against only *Staphylococcus aureus* (Gram positive bacteria) and no activity against *Escherichia coli* and *Salmonella enterica* (Gram negative bacteria) –

Figure-4.10: Zone of inhibition vs bacterial strain of 500 µg/disc EA-EtOH (75:25) extract

Figure-4.11: Zone of inhibition vs bacterial strain of 250 µg/disc EA-EtOH (25:75) extract
And 500 µg/disc EA-EtOH (25:75) extract of *Gynura procumbens* leaves was shown moderate antibacterial activity against only *Staphylococcus aureus* (Gram positive bacteria) and no activity against *Escherichia coli* and *Salmonella enterica* (Gram negative bacteria) –

![Impact of EA- EtOH (25:75) extract on bacterial strain](image)

**Figure-4.12** Zone of inhibition vs bacterial strain of 500 µg/disc EA-EtOH (25:75) extract

### 4.2.4 Results and discussion

The antimicrobial screening of *Gynura procumbens* leaves was carried out by using EA, EtOH and the ratio of EA-EtOH (75:25), (25:75) and (50:50) mixtures. Antibacterial activity of the extracts at a concentration 250 µg/disc and 500 µg/disc were performed against four bacterial strains (*Escherichia coli, Staphylococcus aureus, Salmonella enterica* and *Bacillus subtilis*) by Kirby-Bauer disc diffusion method using Kanamycin as standard. EA-EtOH (75:25) crude extracts exhibited poor to moderate antimicrobial activity against two of the test organisms (Table-4.4), whereas, the crude EA-EtOH (25:75) extract showed mild to moderate antimicrobial activity and other crude such as EA, EtOH and EA-EtOH (50:50) extract showed no antimicrobial activity in the most cases (Table-4.3). EA-EtOH (75:25) crude extracts exhibited poor antibacterial activity in 250 µg/disc having inhibition zone 5.5 mm and moderate antibacterial activity in 500 µg/disc having inhibition zone 7 mm against *Bacillus subtilis*. In 250 µg/disc EA-EtOH (75:25) crude extracts exhibited poor and EA-EtOH (25:75) crude extract
showed mild antibacterial activity against *Staphylococcus aureus* having inhibition zone 5.5 and 6 mm. And in 500 µg/disc EA-EtOH (75:25) crude extracts exhibited mild and EA-EtOH (25:75) crude extract showed moderate antibacterial activity against *Staphylococcus aureus* having inhibition zone 6.5 and 7 mm. No crude extract show any activity against *Escherichia coli*. And other such as EA, EtOH and EA-EtOH (50:50) crude showed no activity against four bacteria *Escherichia coli, Staphylococcus aureus, Salmonella enterica* and *Bacillus subtilis*.

From the above result it can be reported that the EA-EtOH (75:25) and (25:75) crude extract inhibit the growth of *Bacillus subtilis* and *Staphylococcus aureus* which are responsible for a minority of foodborne illnesses, skin infections, fungicide, animal infections and life-threatening diseases.

### 4.3 Moisture analysis

#### 4.3.1 Result

The amount of moisture content of the leaves powder of *Gynura procumbens* is 19.17 %.

### 4.4 Ash content analysis

#### 4.4.1 Result

The amount of ash content of *Gynura procumbens* leaves powder is 18.11 %.

### 4.5 Iron content analysis

#### 4.5.1 Result

*Gynura procumbens* leaves powder contains 0.084 g iron/100 g dry powders.
CHAPTER V
CONCLUSIONS
&
RECOMMENDATIONS
5.1 Conclusions

This M.Sc thesis was focused on the identification and characterization of protic and aprotic polar solvent mixture extract of *Gynura procumbens* leaves and also its anti-microbial, ash content, moisture percentage and iron content was determined.

5.1.1 Identification and characterization of chemical constituents from *Gynura procumbens* leaves

On the basis of IR, $^1$H-NMR and $^{13}$C-NMR spectral data the structure of compound EE-01 was identified as $\beta$-sitosterol-3-O-$\beta$-D-glucopyranoside. The structure of the compound is given below:

**Name of the compound:** $\beta$-sitosterol-3-O-$\beta$-D-glucopyranoside
5.1.2 Antimicrobial activities of *Gynura procumbens* leaves

i) The EA and EtOH (75:25) extracts of *Gynura procumbens* exhibited small antimicrobial activity.

ii) EA and EtOH (75:25) crude extracts exhibited mild antimicrobial activity, whereas, the crude EA and EtOH (25:75) extract showed moderate antimicrobial activity against all gram positive bacteria and showed no activity against all gram negative bacterial strain.

iii) Among the crude extract, EA and EtOH (75:25) extracts of *Gynura procumbens* leaves exhibited mild antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and EA and EtOH (25:75) moderate activity against *Staphylococcus aureus* which implies that they could be used to treat such microbial and fungicide diseases.

5.1.3 Ash, moisture, iron content analysis of *Gynura procumbens* leaves

**Moisture:**

The amount of moisture content in *Gynura procumbens* leaves is 19.17%.

**Ash:**

The amount of ash content in *Gynura procumbens* leaves is 18.11%.

**Iron content analysis:**

*Gynura procumbens* leaves powder contains 0.084 g iron/100 g dry powders.
5.2 Recommendations

Based on the findings from this study, it is recommended that future research should focus on:

i ) The compounds isolated from *Gynura procumbens* leaves which have medicinal values. *Gynura procumbens* shows good activity against bacteria. Farther research to be carried out on the leaves extracts of *Gynura procumbens* to isolate and identify the compounds responsible for their antimicrobial, cytotoxic and antioxidant properties.

ii ) More spectroscopic technique should be utilized (like DEPT, COSY, HMBC and HSQC) for analyzing and establishing a complete structure.

iii ) The disc diffusion method was not a final method to determine the antimicrobial content of the leaves extracts. Other test was therefore carried out using the micro dilution method to determine the bactericidal concentrations of the leaves extracts.
References


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