GCMS Analysis of Total Terpenoids from Baliospermum montanum and its Antimicrobial Activity

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Abstract -- Terpenoids and terpenes are aromatic compounds that are found in thousands of plant species. Many terpenoids have biological activities and are used for medical purposes. *Baliospermum montanum* is a medicinal as well as an endangered plant. In our present study we have done the specific extraction of total terpenoids from methanol extract of *Baliospermum montanum* leaves. Extraction was carried out by column chromatography. Identification of these terpenoids were performed by Gas Chromatography-Mass Spectroscopy. GCMS analysis yielded 5 terpenoids viz olean-12-ene, 3 methoxy, Alpha Amyrin, Lanosterol, Lap-20(29)-en-3-ol and Betulin. The extract was also screened for antimicrobial activity against different bacterial strains: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Streptococcus mutans* and *Bacillus cereus*, and fungal strains: *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. All the organism showed a significant amount of sensitivity. The study confirms that the *Baliospermum montanum* leaf extract contains phytotherapeutic chemicals that are very promising for the discovery and development of new pharmaceutical products.

Keyword -- **Terpenes, extraction, antibacterial, sensitivity, chromatography.**

I. INTRODUCTION

Terpenes are the largest group of natural bioactive compounds which are important for plant survival and also possess biological and pharmacological properties that are beneficial to humans [1]. Based on the number of building blocks, terpenoids are grouped into several classes, such as monoterpenes (carvone, geraniol, d-limonene and peril alcohol), diterpenes (e.g. retinol and retinoic acid), triterpenes (betulinic acid, lupeol, oleanolic acid and ursolic acid) and tetraterpenes (α-carotene, β-carotene, lutein, and lycopene) [2]- [4]. Plants produce various types of secondary metabolites, many of which have been subsequently exploited by humans for their beneficial roles in a diverse array of biological functions [5]. In particular, terpenoids present in many plants have been shown to be available for pharmaceutical applications, for example, artemisinin and taxol as malaria and cancer medicines respectively. Various terpenoids present in many plants are not only used as herbal medicine but also as dietary product [6], [7]. Terpenoid biosynthesis occur within specific tissues or at specific stages of development in plants [8], [9]. Terpenes, together with aromatic compounds, constitute the essential oils of plants, with the highest concentration usually found in the specialized storage cavities of leaves. Commercially, terpenes have industrial uses as agrichemicals, fragrances, nutraceuticals and pharmaceuticals [10].

Terpenes and isoprenoids in general have garnered much attention among the scientists because of their important physiological roles such as hormones, aliphatic membrane anchors, maintaining membrane structure, ecological roles (defense compounds, insect or animal attractants), and their wide uses in pharmaceutical and industrial applications ranging from flavors and fragrances to medicines [11]- [15]. Terpenoids have been found to be useful for the treatment of various types of diseases and disorders viz, antimicrobial, antifungal, antiparasitic, antiviral, antihyperglycemic, antihypoglycemic, anti-inflammatory and immunomodulatory properties [16]- [18].

Microbes play a major role in causing various infectious diseases. Some Gram positive pathogens like *Staphylococcus* spp. and *Streptococcus* spp. are involved in the respiratory and skin infections. *Pseudomonas aeruginosa* and members of the Enterobacteriaceae cause gastrointestinal, urogenital diseases and wound infection. Many clinical isolates are resistant to virtually all of the older antibiotics like penicillin, gentamicin, tobramycin, amikacin, ciprofloxacin, clindamycin, erythromycin, chloramphenicol, trimethoprim-sulfamethoxazole and vancomycin [19], [20]. One of the important persistent problems confronted by medical practitioners is the increasing prevalence of antimicrobial resistance [21].

A major challenge in global health care is the need for novel, effective and affordable medicines to treat microbial infections. Therefore, the development of an alternative drug line of treatment for infectious diseases is of utmost importance. Plants have an amazing ability to produce a wide variety of secondary metabolites, like terpenoids, steroids, alkaloids, glycosides, saponins, flavonoids, tannins, quinones and coumarins [22]. Some of these natural phytochemicals are highly resourceful in the treatment of bacterial and fungal infections.

Baliospermum montanum (Muell – Arg) is an important aromatic medicinal plant belonging to the family Euphorbiaceae includes 280 genera with 730 species with the largest genus Euphorbia [23]. Root, leaf and seeds of B. montanum are used medicinally and are documented from Asian countries, including Nepal, Burma, Malaya and India. The plant is commonly referred as Naga Danti, a threatened medicinal plant [24]. B. montanum is known for its ethnobotanical and traditional use [24]. The plant is well known for its antioxidant and anti-inflammatory activity [26], [27].

Keeping all the above things in mind, the present study was undertaken with an objective to extract the total terpenoids and identify the novel natural bioactive compounds present in the leaf extract of *Baliospermum montanum*. The extract was also screened for the antimicrobial property against pathogenic bacteria and fungi.

II. MATERIALS AND METHODS

A. Collection of Plant Material and Preperation of Extract

The plant material was collected from Gandhi Krishi Vignan Kendra (GKVK) Bangalore. Soon after collection, fresh and young leaves were removed, washed, shade dried and made into a fine powder (particle- 0.25 mm size). 100 grams of plant powder were taken in a soxhlet extractor and extracted with 500 ml of methanol (48 h). The solvent was recovered by distillation. The extract was concentrated in a rotary evaporator and the solid residue was stored for subsequent experiments.

B. Isolation of Terpenoids

The extract was evaporated completely and the solid residue was dissolved in saturated sodium bicarbonate solution. The sodium bicarbonate was mixed with 10 % HCl and extracted three times with ethyl acetate using separating funnel. The whole acidified ethyl acetate extract was chromatographed on a silica gel (Merck) column with hexane and ethyl acetate (1:1) mixture and the terpenoid fraction were collected [28], [29]. Thus collected fraction were again rechecked for terpenoids and employed for GCMS analysis and antimicrobial assay.

C. GCMS Analysis

GCMS was carried out by using an Agilant Technology-7890 system coupled with Aligant 7000 GCMS triplequaid and Agilant 7693 auto sampler equipped with a capillary column (30mmX 0,25mm). Helium was used as the carrier gas at a flow rate of 1ml/min. The oven temperature was initially held at 70^0C for 1 minute and increased to 180^0C at the rate of 30^0C /min. Finally the temperature was increased to 280^0 C for 30 minutes. Interpretation of mass spectrum GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the component stored in the NIST library and the name, molecular weight and structure of the components of the test materials were ascertained [30].

D. Antibacterial Assay

The terpenoid extract of leaves of B. montanum were evaluated for its antibacterial potential by resazurin microtitre plate method [31]. The concentrated terpenoids were re-dissolved in dimethyl sulfoxide (DMSO) to make 10 µg /ml solution. Different concentration of the extract were prepared (1000, 500, 125, 62.5, 31.2 15.6 and 7.8µg/ ml) and treated against selected bacteria *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus mutans*. To each well 30µL of 0.1% resazurin indicator solution was added and placed in an incubator at 37 °C for 18–24 h. The color change was then assessed visually. (Fig.1). Amoxicillin was taken as the standard. The presence of blue color indicates no growth of the organism, whereas the appearance of pink color indicates growth. The lowest concentration at which color change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the test material and bacterial strain.

E. Antifungal Assay

The extract was screened for anti-fungal activity by the plate diffusion technique on Potato Dextrose Agar (PDA) growth medium against three fungal pathogens - *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* [32]. A 10mg of the extract was dissolved in 1mL of DMSO (Dimethyl sulfoxide) in a sterilized eppendrof tube. From this stock various concentration of extract [100µg, 200µg, 300µg, 400µg] were prepared by adding DMSO (Dimethyl sulfoxide). A 100µL inoculum of *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* was pipetted into the PDA plates respectively and spread thoroughly using a plate spreader. On agar plates, five wells measuring 5.5 mm were prepared and were filled with 50 µL of different concentration of extract and 50 µL Control (DMSO) in the middle well. The culture plates were incubated at 25° C for 72 hours and the zone of inhibition was recorded in millimeters around the wells. Fluconazole was taken as the standard.

III. RESULTS

A. Extraction of trepenoids

Terpenoids were extracted from the ethanolic leaf extract of *Baliospermum montanum* through column chromatography, which generated a mixture of terpenoids.

B. GCMS analysis

The evaluation of GC-MS spectrum confirmed the presence of five major components in the terpenoid extract of *Baliospermum montanum* at different retention times. [Table 1]. The major constituents were olean-12-ene, 3methoxy,-(3β) [6.778], Alpha Amyrin [7,564], Lanosterol [16.386], Lap-20 (29) -en-3-ol [20.974] and Betulin [23.851] [Figure1].

TABLE I
SUMMARY OF GC-MS WITH THEIR RETENTIOM TIME, MOLECULAR WEIGHT AND MOLECULAR FORMULA

Nameof the compound	Retention time	Molecular Weight	Molecular formula
Olean-12-ene, 3 methoxy, -(3β)	6.778	410.718	$C_{30}H_{50}$
α- Amyrin	7,564	426.729 g/mol	$C_{30}H_{50}O$
Lanosterol	16.386	426.7174	$C_{30}H_{50}O$
Lup-20 (29) - en-3-ol, acetate	20.974	468.7541	$C_{32}H_{52}O_2$
Betulin	23.851	442.728	$C_{30}H_{50}O_2$

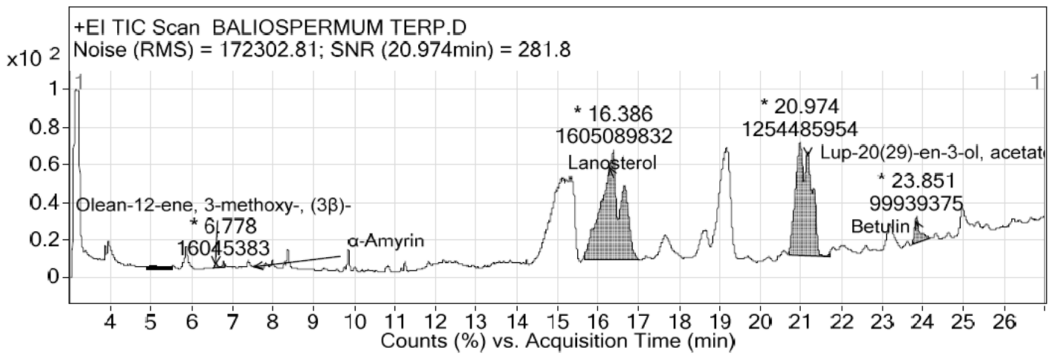
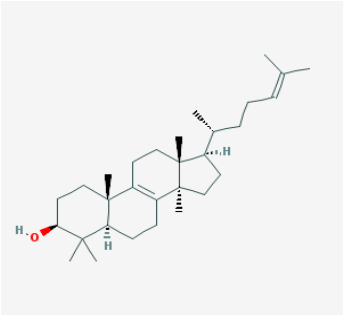
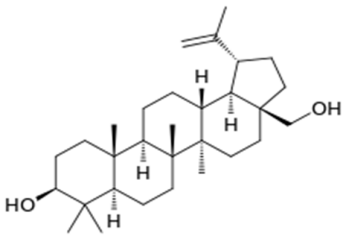


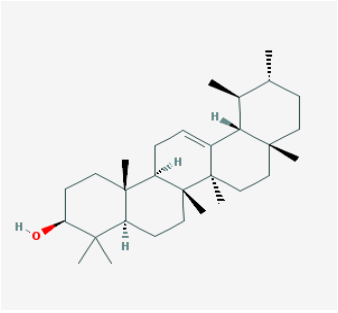
Figure. 1 Chromatogram showing the peaks and their retention time.



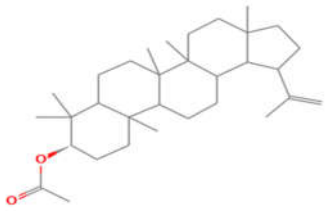
Lanosterol



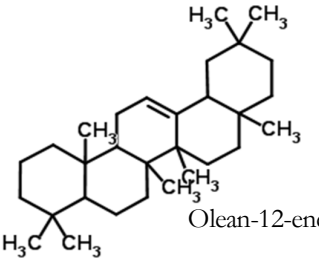
Betulin



α- Amyrin



Lup-20(29)- en-3-ol, acetate



Olean-12-ene

Figure. 2 Chemical structure of probable compounds matched with NIST library.

C. Antibacterial activity:

The study showed significant growth inhibition in all the tested organisms [Figure 3]. The minimum inhibitory concentration was observed to be: *Bacillus cereus* 7.8µg/ml, E-coli 1000µg/ml, *Salmonella typhi* 500µg/ml, *Pseudomonas aeruginosa* 1000 µg/ml, *Staphylococcus aureus* 1000 µg/ml and *Streptococcus mutans* 125 µg/ml [Table2].

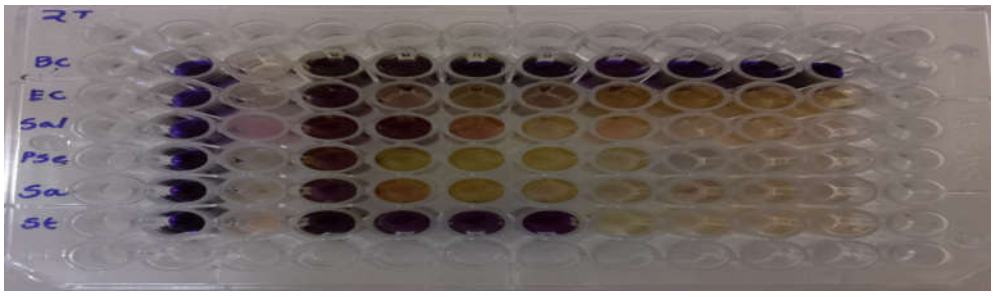


Figure. 3 Antibacterial activity of Terpenoid extract of *Baliospermum montanum* against *Six bacteria. BC- Bacillus cereus, EC- E. coli, Sal - Salmonella typhi, Pse- Pseudomonas aeruginosa, Sa- Staphylococcus aureus and St-Streptococcus mutans*

TABLE 2
MINIMUM INHIBITORY CONCENTRATION (MIC) IN µG.

Test Organism	Minimum Inhibitory Concentration (MIC) in µg Sample
Bacillus cereus	7.8
E-coli	1000
Salmonella typhi	500
Pseudomonas aeruginosa	1000
Staphylococcus aureus	1000
Streptococcus mutans	125

D. Antifungal Activity

The assessment was conducted after 72 h by measuring the diameter of inhibition of the fungal growth. The results showed a remarkable amount of inhibition against all the three tested strains. A clear zone of inhibition was observed around the wells confirming antifungal activity.

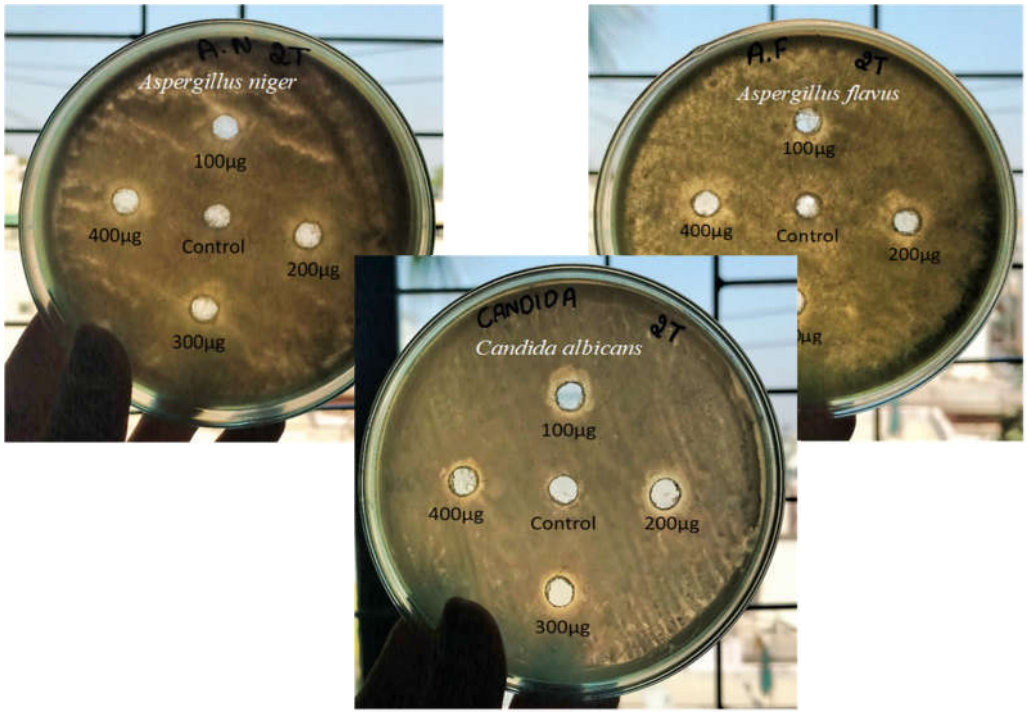


Figure 4. Antifungal activity of leaf extract of *Baliospermum montanum* showing a zone of inhibition in *Aspergillus niger, Aspergillus flavus and Candida albicans with different concentrations.*

TABLE 3
ANTIFUNGAL ACTIVITY IN *BALIOSPERMUM MONTANUM* LEAF EXTRACT SHOWING A ZONE OF INHIBITION.

SAMPLE in µg	FUNGAL ZONE OF INHIBITION IN MM		
	Aspergillus niger	***Aspergillus flavus***	***Candida albicans***
100 µg	9	9	10
200 µg	11	10	10
300 µg	11	10	10
400 µg	11	10	10

IV. DISCUSSION

Current research is carried out to extract the phytotherapeutic active compounds from the plant *Baliospermum montanum*. In the present study, we have concentrated on the extraction of terpenoids from *Baliospermum montanum* leaves. The terpenoid fraction of *Baliospermum montanum* was analyzed by GC-MS for the different types of terpenoids. Evaluation of the extracted fraction confirmed the presence of 5 individual compounds, olean-12-ene, 3 methoxy, Alpha Amyrin, Lanosterol, Lap-20 (29) -en-3-ol and Betulin, most of them were triterpenoids.

Olean-12-en-3-yl acetate is antimicrobial, anti-diabetic, anti-amylase inhibitor. Alpha-Amyrin, a triterpin is anti-diabetic, anti-inflammatory, anti-arthritic, anticancerous and is three times more potent than aspirin [33]. Lanosterol is a tetracyclic triterpenoid and is the compound from which all animal and fungal steroids are derived [34]. Lap-20 (29) -en-3-ol, Lupeol has a complex pharmacology, displaying antiprotozoal, antimicrobial, antiinflammatory, antitumor and chemopreventive properties [35]. Recent clinical studies have shown that betulin was effective against a variety of tumors and causes some types of tumor cells to start a process of self-destruction called apoptosis and can slow the growth of several types of tumor cells [36]. Several reports have also proved that betulin decreased the biosynthesis of cholesterol and fatty acids, amended diet-induced obesity, decreased the lipid contents in serum and tissues, and increased insulin sensitivity. Furthermore, betulin reduced the size and improved the stability of atherosclerotic plaques [37].

The antibacterial activity of extracted terpenoid fraction was determined by resauzine microtitre plate method and the test is based on the fact that resazurin is an oxidation–reduction indicator used for the assessment of cell growth. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydroresorufin (uncolored and nonfluorescent) [38]. In the study, the lowest concentration at which color change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the test material and bacterial strain.

The minimum inhibitory concentration (MIC) against six species of pathogenic microorganisms i.e *Bacillus subtilis, Staphylococcus aureus, Streptococcus mutants, Salmonella typhi, Escherichia coli* and *Pseudomonas aeruginosa* was evaluated using various concentrations (1000, 500, 125, 62.5, 31.2 15.6 and 7.8µg/ ml). Results of antibacterial sensitivity showed that all the six pathogens were susceptible to terpenoid extract. MIC values were found to be 7.8µg/ml, 1000µg/ml, 125 µg/ml, 500µg/ml, 1000µg/ml and 1000µg/ml respectively. Gram positive organisms showed more sensitivity when compared to Gram negative organisms. Among the gram positive organism *Bacillus cereus* showed the highest degree of susceptibility at a concentration of 7.8µg/ml. Whereas *Staphylococcus aureus* showed the least 1000µg/ml. *Streptococcus mutans* was also highly sensitive to the plant extract at a concentration of 125µg/ml. However, Gram negative organism *Salmonella typhi* showed a significant amount of inhibition. *Escherichia coli* and *Pseudomonas aeruginosa* both showed the least amount of inhibition when compared to salmonella. Over all terpenoids extracted from *Baliospermum montanam* leaf showed excellent antibacterial activity.

Antifungal activity was accessed by Plate diffusion method against three fungal pathogens - *Aspergillus niger, Aspergillus flavus and Candida albicans.* A clear zone of inhibition was observed around the wells confirming antifungal activity by the terpenoid fraction. *Candida albicans* showed the highest degree of inhibition when compared to *Aspergillus niger* and *Aspergillus flavus.* This study clearly shows the potential of the terpenoid extract in the treatment of diseases caused by pathogenic fungi.

Above studies confirms that the triterpenes found in the *Baliospermum montanum* leaf extract is of extreme importance and may have been responsible for the antimicrobial activity.

V. CONCLUSION

In conclusion terpenoids widely distributed in the plants has the ability to inhibit microbial infections. The present study clearly indicates that the terpenoids extracted from *Baliospermum montanum* leaves may be developed as functional ingredients in pharmaceutical products as antimicrobial agent.

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