PHYTOCHEMICAL DIVERSITY AND ANTIBACTERIAL ACTIVITY OF SELECTED MEDICINAL PLANTS USED BY KHASI TRIBE IN MEGHALAYA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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MZU REGISTRATION NO: 1600835

PHD REGISTRATION NO: MZU/PHD/1032 OF 26.05.2017

DEPARTMENT OF BOTANY

SCHOOL OF LIFE SCIENCES

MIZORAM UNIVERSITY, TANHRIL-796004

MAY, 2022

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BY

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DEPARTMENT OF BOTANY

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SUBMITTED

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF
DOCTOR OF PHILOSOPHY
IN BOTANY
OF MIZORAM UNIVERSITY, AIZAWL

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ABSTRACT

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ABSTRACT

The use of medicinal plants for the treatment, prevention and cure of diseases accompanies man since the earliest civilizations (Firmo *et al*., 2011). Between 1981 and 2002, of the 877 new molecules introduced into the pharmaceutical market, 49% were substances isolated from natural products (Newman *et al.*, 2003). In 2010, herbal medicines accounted for approximately 15% of the capital of the world pharmaceutical industry (Niero, 2010).

The bioactive compounds present in the medicinal plant are responsible for the medicinal properties of the plant (Bargah, 2015). The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents (Nostro *et al.,* 2000). Indigenous people are generally very knowledgeable about the wild medicinal plants around them, many of which have local names and are important to the people medically or are featured in folklore (Kharkongor and Joseph, 1981).

Meghalaya a small state in North-east India but it is one of the richest states of India in terms of vegetation and flora. This is due to the large variation in the altitude, topographical features, soil characteristics and climatic factors which has favoured the growth and luxuriance of rich flora here. The forests of Meghalaya are rich in biodiversity and endowed with rare species of orchids and medicinal plants.

According to Hynniewta (2010), the use of medicinal plants extract is common among the people of Meghalaya and found to be a significantly higher among rural people due to poor transportation and medical facilities in the rural areas. Modern medicinal facilities are scanty and could not reach these inaccessible areas inspite of government's best efforts.

However, analytical experimental studies like phytochemical screening and antimicrobial activities of medicinal plants of Meghalaya are lacking which is required to establish and confirm the benefit and effectiveness of medicinal plants as a treatment for various diseases. Scientific analysis and evaluation of the ethnomedicinal plants used

by the Khasi tribe in Meghalaya is the need of the hour and research work in this context is very scarce and limited. Hence, efforts are made in this direction in the form of phytochemical screening and antibacterial activity detection. It is a fact that justifies this research of evaluation of the antibacterial activity and phytochemical screening of the selected ethnomedicinal plants used by the Khasi tribe in Meghalaya.

Prior to the field work, all relevant literature of ethno-botanical researches were surveyed and studied, along with interview and consultation of the traditional medicine practitioners and ethnomedicine knowledgeable individuals, to identify a set of 15 plant species having ethnomedicinal value. For the selection of the plant species to study, randomized approach suggested by Albuquerque and Hanazaki (2006) was followed.

For collecting information on ethnomedicinal aspects, the approaches and methodologies suggested by Jain (1989) was followed while for plant collection and herbarium preparation, routine methods suggested by Jain and Mudgal (1999) was followed.

Critical morphological studies were undertaken on the collected plant specimen using various literatures such as Forest Flora of Meghalaya, Vol I & II (Haridasan and Rao, 1985 and 1987), Flora of British India, Vol I – VII (Hooker 1872-1897), Flora of India (Sharma *et al.*, 1993), Check-list of Flora of Meghalaya (Mao *et al.*, 2016).

All the selected potential ethno-medicinal plant were identified and authenticated at the Botanical Survey of India, Eastern Region, Shillong, Meghalaya and voucher specimens (herbarium) were deposited at the Assam Herbarium, BSI, Shillong, Meghalaya.

The medicinal plants selected and documented during the present work are *(i) Achyranthes aspera* L. (Amaranthaceae) (ii) *Acmella paniculata* (Wall.exDC.) R.K. Jansen (Asteraceae) (iii) *Ageratum conyzoides* L. (Asteraceae) (iv) *Bidens pilosa* L. (Asteraceae) (v) *Centella asiatica* (L.) Urb. (Apiaceae) (vi) *Garcinia pedunculata* Roxb. Ex Buch.-Ham. (Clusiaceae) (vii) *Gaultheria fragrantissima* Wall. (Ericaceae) (viii) *Hibiscus sabdariffa* L. (Malvaceae) (ix) *Houttuynia cordata* Thunb. (Saururaceae) (x) *Lantana camara* L. (Verbenaceae) (xi) *Piper attenuatum* Buch.-Ham. ex Miq.

(Piperaceae) (xii) *Potentilla lineata* Trevir. (Rosaceae) (xiii) *Prunella vulgaris* L. (Lamiaceae) (xiv) *Sonchus oleraceus* L. (Asteraceae) (xv) *Sonchus palustris* L. (Asteraceae)

Out of the 15 plants selected, 5 plants belong to the family of Asteraceae, one plant each belong to the family of Amaranthaceae, Apiaceae, Clusiaceae, Ericaceae, Lamiaceae, Malvaceae, Piperaceae, Sauraceae and Verbenaceae.

The specimens collected were as complete as possible and healthy. They are collected from its typical habitat. Flowers and fruit were included, as well as vegetative parts wherever possible. Clearly, in most cases, this is impossible since ripe fruit and flowers do not usually occur at the same time.

The randomly collected plants were brought to the laboratory and thoroughly washed in running water to remove debris and dust particles and then rinsed using distilled water and finally air dried at room temperature before grinding them to powdered form using pestle and mortar / mechanized grinder. The powdered medicinal plants were extracted using water, methanol, ethanol and chloroform. The extracts were then used for antibacterial activity screening and phytochemical analysis.

The test microorganisms used in the study were clinical isolates of *Escherichia coli* AF06, *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24, *Enterobacter cloacae* AM03, *Pseudomonas aeruginosa* CM07 and *Klebsiella pneumoniae* CF09. The microorganisms were isolated from urine samples collected from patients. The collected samples were streak on selective media and characteristic colony morphological study was conducted. The isolated colonies were sub – cultured to obtain pure culture and then the pure culture were then subjected to a biochemical test for identification using Vitek Automated Machine in the laboratory of Department of Microbiology, Woodland Hospital, Shillong.

The pure cultures of the clinical isolates of the test microorganisms were maintained on different agar slants - *Staphylococcus aureus* AM12 (MSA), *Enterococcus faecalis* AF24 (CLED), *Enterobacter cloacae*AM03 (McConkey),

Escherichia coli AF06(EMB), *Pseudomonas aeruginosa* CM07 (Cetrimide), *Klebsiella pneumoniae* CF09 (McConkey).

The susceptibility of the six (6) reference strain of test bacteria to twelve (12) commonly employed different antibiotics viz. Ceftazidime CAZ 30mcg/disc, Gentamicin GEN 10mcg/disc, Piperacillin PI 100mcg/disc, Amikacin AK 30mcg/disc, Cefepime CPM 30mcg/disc, Aztreonam AT 30mcg/disc, Cefoperazone CPZ 75mcg/disc, Ciproflaxacin CIP 5mcg/disc, Levoflaxacin LE 5mcg/disc, Imipenem IPM 10mcg/disc, Meropenem MRP 10mcg/disc and Piperacillin/Tazobactam PIT 100/10mcg/disc was assessed by disc diffusion method using HiMedia's Dodeca Disc for easy and relevant comparision and to ascertain the relative effectiveness of the 15 medicinal plant extracts. The different cultures of the clinical bacterial isolates responded to standard antibiotics in a variable manner resulting in various size of zones of inhibition. The size of zone of inhibition ranges from 10.3±0.5 (Piperacillin/Tazobactam PIT 100/10mcg against *K. pneumoniae* CF09) to 59.7±0.5 (Levoflaxacin LE 5mcg against *S. aureus* AM12).

The test bacteria were grown on Mueller-Hinton Agar (MHA, HiMedia) plate for 16–18 hr. at 37 ± 2°C. Well-isolated colonies were suspended in sterile Mueller-Hinton Broth (MHB, HiMedia) and the turbidity was adjusted against 0.5 McFarland standard to comprise approximately 1.5 x 10^8 CFU/ml.

Antibacterial activity of the methanol extract of the 15 medicinal plants towards different clinical isolates were measured in terms of zone of inhibition using disc diffusion method as stated by Kirby - Bauer *et al*. (1966). It is the recommended method of European Committee on Antimicrobial Susceptibility Testing (EUCAST) and National Committee for Clinical Laboratory Standard (NCCLS), USA.

The susceptibility of different bacterial clinical isolates to the methanol extract of the selected 15 ethnomedicinal plantswere also measured in terms of zone of inhibition using agar well diffusion assay. The plates containing MHA were spread with the inoculum. Wells (8 mm diameter) were cut out from agar plates using a sterilized stainless steel well borer and filled with 250 µl of the plant extracts (200 mg/ml). Wells

filled with 250 µl methanol were used as negative control for methanol plant extract. Disc of ampicillin (10 mcg) and methicillin (5 mcg) antibiotics (HiMedia) were used as reference antibiotics. The plates inoculated with different bacteria were incubated at 37° C for 16 to 24 hours and diameter of any resultant zone of inhibition were measured. For each combination of extract and the bacterial strain, the experiment was repeated thrice. All the tested bacteria were susceptible to *Garcinia pedunculata* while none of the extract of *A. aspera, A. paniculata, A. conzyoides, C. asiatica, H. sabdarifa, H. cordata, P. attenuatum, P. vulgaris, S. oleraceus and S. palustris* were active against any of the tested bacterial species.

Staphylococcus aureus AM12 was susceptible to *B. pilosa, G. pedunculata, G. fragrantissima* and *P. lineata* while both *S. aureus* AM12 and *E. faecalis* AF24 were susceptible to *G. pedunculata, G. fragrantissima* and *P. lineata*. *E. cloacae* AM03, *E. coli* AF05 and *K. pneumoniae* CF09 were inhibited by only *G. pedunculata* while *P. aeruginosa* CM07 was susceptible to only *G. pedunculata* and *L. camara*.

It is noteworthy that *G. pedunculata* shows antibacterial activity against *E. cloacae* AM03, *P. aeruginosa* CM07 and *K. pneumoniae* CF09 which are not susceptible to the standard antibiotics namely Ampicillin and methicillin.

Comparative study of the Antimicrobial Activity of methanol and ethanol extract of *P. lineata* and *G. pedunculata* against the UTI clinical bacterial isolates were also investigated involing MIC and MBC analysis and Time-Kill studies.

The antibacterial activity of the methanol extract and ethanol extract of the selected 2 ethnomedicinal plants viz. *P. lineata* and *G. pedunculata* were determined by the disc and well diffusion method. It was observed that both the organic solvent extract of *P. lineata* produced very effective and notable antimicrobial activities against the two tested Gram-positive bacteria (*Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 but Gram-negative bacteria (*Enterobacter cloacae* AM03, *E. coli* AF05, *Pseudomonas aeruginosa* CM07, *Klebsiella pneumoniae* CF09) were not susceptible to the extract.

On the other hand, both the organic solvent extract of G. *pedunculata* were inhibitory to all the six bacterial isolates and are more potent than that of the extract of *P. lineata*. All the methanol and ethanol negative control discs did not produce any zone of inhibition against any of the tested clinical isolates. The results were compared with those of ampicillin and methicillin as standard antibiotics. The solvent extract (50 mg/ml) of both *P. lineata* and *G. pedunculata* were not as potent as ampicillin (10 mcg) but are quite effective as methicillin (5 mcg) antibiotics or even better. It is noteworthy that *G. pedunculata* shows antibacterial activity against *E. cloacae* AM03, *P. aeruginosa* CM07 and *K. pneumoniae* CF09 which are not susceptible to the standard antibiotics namely Ampicillin and methicillin.

The minimum inhibitory concentrations (MIC) of *P. lineata* plant extracts were determined by using broth microdilution method, as described previously by Wiegand *et al.* (2008) supplemented with Resazurin dye (Elsinkh, 2016) based on Clinical Laboratory Standard Institute. The MICs of only methanol extract of *P. lineata* for the susceptible bacterial isolates was determined, though both the methanol and ethanol extracts showed antimicrobial activities against Gram-positive bacteria, *Staphylococcus aureus* AM12 and *Enterococcus faecalis* AF24. The extract is approximately equally potent against *Enterococcus faecalis* AF24 and *Staphylococcus aureus* AM12 with MICs being 25 mg/ml.

The minimum bactericidal concentration (MBC) of *P. lineata* was determined by plating directly the content of Eppendorf tubes with concentrations higher than the MIC value. The lowest concentration of the extract that did not permit any growth was taken as the MBC. The MBC values of methanol extract is 100 mg/ml for both *Staphylococcus aureus* AM12 and *Enterococcus faecalis* AF24.

The MICs of the methanol extract of *G. pedunculata* for the susceptible bacterial isolates were also determined. The extract is approximately equally potent against *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 *and Pseudomonas aeruginosa* CM07, with MIC being 12.5 mg/ml. Against *Enterobacter cloacae* AM03,

E. coli AF05 and *Klebsiella pneumoniae* CF09, the MIC of the extract was observed to be approximately 25 mg/ml.

The MBC of the methanol extract of *G. pedunculata* was found to be approximately 25 mg/ml against *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 and *Pseudomonas aeruginosa* CM07 while 50 mg/ml was the observed MBC against *Enterobacter cloacae* AM03, *E. coli* AF05 and *K. pneumoniae* CF09.

Time-kill assays were performed by the broth macro-dilution method in accordance with the CLSI guidelines. All the susceptible exponentially growing susceptible test bacteria in mid-logarithmic growth phase were adjusted to the 0.5 McFarland standard. Then, each of the standardized bacterial suspension of all the susceptible test bacteria (50 µl) were inoculated into several Eppendorf tubes (0,1,2,3,4,5,6,7 and 8 h) of MHB containing 450 µl of MHB and 500 µl of the extracts with final concentrations corresponding to 1X MIC (i.e.25 mg/ml of MEGP for *Enterobacter cloacae* AM03, *E. coli* AF05 and *Klebsiella pneumoniae* CF09*;* and 12.5 mg/ml of MEGP for *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 and *Pseudomonas aeruginosa* CM07*;* 50 mg/ml of MEPL for *Staphylococcus aureus* AM12 and *Enterococcus faecalis* AF24) . The final density of bacteria was approximately 4-5x10^5 CFU/mL.

Time-kill assays have been widely used for in vitro investigations of new antimicrobial agents as these provide descriptive (qualitative) information on the pharmacodynamics of antimicrobial agents (Lewis, 2007).

With regards to MEPL, only MEPL with activity towards susceptible bacterial strains was selected for time-kill studies. The kinetic interaction between susceptible bacteria and MEPL was examined at the concentration of 1X MIC (25 mg/ml) and 2X MIC (50 mg/ml). The kill kinetic profiles of MEPL displayed rapid killing rate towards both the susceptible Grampositive bacteria, *S. aureus* AM12 and *E. faecalis* AF24, showing complete destruction after 5 h exposure at 1X MIC and after 4 h exposure at 2X.

In the present study, the kinetic interaction between susceptible bacteria and MEGP was examined at the concentration of 1X MIC (12.5mg/ml for *S. aureus* AM12, *E. faecalis* AF24 & *P. aeruginosa* CM07 *and 25 mg/ml for E. cloacae* AM03, *E. coli* AF05 & *K. pneumonia* CF09), and 2X MIC. The kill kinetic profiles of MEGP displayed rapid bactericidal activity towards all susceptible strains, showing complete destruction after 7 h exposure at 1X MIC and after 5 h exposure at 2X MIC. As expected from the determined MBC/MIC ratios, the time-kill assays for MEGP towards *S. aureus* AM12, *E. faecalis* AF24, *E. cloacae* AM03, *E. coli* AF05, *P. aeruginosa* CM07 and *K. pneumoniae* CF09 were consistent with bactericidal characteristic. The kill kinetic profiles of MEGP exhibited varying degrees of bactericidal activities depending on the tested strains. The killing rate of MEGP was slower against *E. cloacae* AM03 and *K. pneumonia* CF09 (complete killing were only seen after 7 h interaction at 1x MIC) than against *E. coli* AF05 and *P. aeruginosa* CM07 (complete killing were only seen after 6 h interaction at 1x MIC) but the killing rate was found to be similar for all tested Gram negative bacteria - *E. cloacae* AM03, *E. coli* AF05, *P. aeruginosa* CM07 and *K. pneumoniae* CF09 (complete killing were seen after 5 h at 2X MIC). MEGP exhibited a faster killing rate against *S. aureus* AM12 *and E. faecalis* AF24, showing bactericidal activity only after 4 h at both 1X MIC and 2X MIC indicating that increase concentration does not have any significance.

According to the 31st edition of CLSI performance standard for antimicrobial susceptibility testing, *E. coli* AF05 was susceptible to all the 12 standard antibiotics tested; *E. cloacae* AM03 was resistant to CAZ, PI, CPM, AT, CPZ and PIT but susceptible to GEN, AK, CIP and LE; *K. pneumoniae* CF09 was susceptible to GEN, AK, CIP, LE, IPM and MRP but resistant to CAZ, PI, CPM, AT, CPZ and PIT; *P. aeruginosa* CM07 was susceptible to CAZ, GEN, AK, CPM, AT, CIP, LE and IPM; *S. aureus* AM12 was susceptible to GEN, CIP and LE; *E. faecalis* AF24 was susceptible to CIP and LE. All the five (5) test bacteria viz. *S. aureus* AM12, *E. faecalis* AF24, *E. coli* AF05, *P. aeruginosa* CM07 and *K. pneumoniae* CF09, were susceptible to the twelve

(12) different antibiotics except *E. cloacae* AM03 which showed resistance towards Ceftazidime, Cefepime and Aztreonam.

The antibacterial activity of the selected 15 ethnomedicinal plants were evaluated by disc and well diffusion method measuring the diameter of zone of inhibition. *Potentilla lineata* and *Garcinia pedunculata* extract were observed to exhibit largest zone of inhibition and therefore, were chosen for comparative study. It was observed that both the organic solvent extract of *P. lineata* produced very effective and notable antimicrobial activities against the two tested Gram positive bacteria (*S. aureus* AM12 and *E. faecalis* AF24) but Gram negative bacteria (*E. cloacae* AM03, *E. coli* AF05, *P. aeruginosa* CM07 and *K. pneumoniae* CF09) were not susceptible to the extract. Tomczyk *et al*. (2007) reported that the aqueous extract of nine *Potentilla* species (*P. rupestris, P. argentea, P. fruticoa, P. recta* L, *P. erecta, P. anserine, P. nepalensis* HOOK var Miss Willmott, *P. thuringiaca* BERNH ex LINK, *P. grandiflora* L.) show inhibitory effect on the various species of bacteria. In the present study, one more species of *Potentilla*, named *P. lineata* antibacterial activity and bioactive component was studied. The methanol and ethanol extract of *P. lineata* show moderate antibacterial activity against Gram positive bacteria (*S. aureus* and *E. faecalis*) but no activity was observed against Gram negative bacteria (*E. cloacae, E. coli, P. aeruginosa, K. pneumoniae*). This observation is in good agreement with the findings of Tomczyk *et al.* (2007) since the plant under investigation also belongs to the same genus.

The investigation also revealed the potency of *G. pedunculata* extract as an effective antibacterial agent against both Gram positive bacteria (GPB) and Gram negative bacteria (GNB). *G. pedunculata* are evaluated to be bactericidal against the tested bacteria. The antibacterial activity may be due to an individual compound, mangostin or synergistic effect of more than one compound present in the medicinal plant extract detected in LC-ESI-MS study (Priscilla *et al*., 2007).

The antibacterial activity may be due to an individual compound or synergistic effect of more than one compound present in the medicinal plant extract. Heymsfield *et*

al. (1998), Kumar *et al.* (2013) and Aravindakshanpillai *et al.* (2016) reported xanthones, biflavonoids, benzophenones, benzoquinones, and triterpenes are bioactive chemicals found in *Garcinia* species that exhibit antibacterial, antifungal, antioxidant, and cytotoxic properties (Heymsfield *et al*., 1998; Kumar *et al*., 2013; Aravindakshanpillai *et al*., 2016).

The antibacterial activity of MEGP was quantitatively evaluated by determining its minimum inhibitory concentration (MIC) values and minimum bactericidal concentration (MBC). As the bigger zone of inhibition was observed with methanol extract of *G. pedunculata* (MEGP) than that of ethanol extract of *G. pedunculata* (EEGP), MEGP was preferred to EEGP for determination of MIC and MBC. The MEGP is considered to be bactericidal rather than bacteriostatic since its MBC/MIC ratio is ≤ 4. An antimicrobial agent is considered bactericidal if the MBC is not more than fourfold higher than the MIC (Levison, 2004).

The present investigation revealed the potency of both methanol and ethanol extracts of *G. pedunculata* as an antibacterial agent. *G. pedunculata* is effective as an inhibitory agent against both GPB as well as GNB. The antibacterial activity can be attributed to its phytochemical constituents (Priscilla *et al*., 2007).

It can be concluded that MEGP possess potent and differential activity against Gram-positive and Gram-negative bacteria pathogens while MEPL shows antibacterial activity towards only Gram-positive bacteria. As the antibacterial activities and bacterial killing rates of MEPL and MEPL were different from each other, it is likely that different mechanisms are involved. Further investigation is needed to determine the mechanism(s) of action of these extracts in order to strengthen their potential as therapeutic antibiotics. MEGP, in particular, with its potent and specific antibacterial profile, deserve further investigation.

Time-kill studies have provided valuable information on the rate, concentration and potential action of MEGP and MEPL in vitro. Time-kill assays have been widely used for in vitro investigations of new antimicrobial agents as these provide descriptive

(qualitative) information on the pharmacodynamics of antimicrobial agents (Lewis, 2007). Time-kill curves that monitor bacterial growth and death over a wide range of antimicrobial concentrations have been frequently used to evaluate the effect of antimicrobials over time. Information about the effects of antimicrobials covering a wide range of antimicrobial concentrations below and above the MIC is particularly valuable for pathogens where data about PK/PD effects are limited.

In summary and consistent with the MBC/MIC ratios, at its MIC value, MEGP were found to be bactericidal towards all the tested bacteria after 7 hours exposure, whereas MEPL was observed to be bactericidal towards only tested Gram-positive bacteria after 5 hours exposure. MEGP possess potent and differential activity against Gram-positive and Gram-negative bacteria pathogens while MEPL shows antibacterial activity towards only Gram-positive bacteria. The time-kill studies have provided valuable information on the rate, concentration and potential action of MEGP and MEPL in vitro. As the antibacterial activities and bacterial killing rates of MEPL and MEPL were different from each other, it is likely that different mechanisms are involved. Further investigation is needed to determine the mechanism(s) of action of these extracts in order to strengthen their potential as therapeutic antibiotics. In particular, MEGP, with its potent and specific antibacterial profile, is deserving of further investigation.

Preliminary qualitative phytochemical analysis of the crude extract of the 15 plants collected was determined as as per standard methods described by Brain and Turner (1975) and Evans (1996). The result of the phytochemical group test revealed that there was great diversity in the phytochemical content of various species of plants. Phytochemical screening revealed variations and diversity in the content of phytochemical compounds, qualitatively and quantitatively, for the 15 selected medicinal plants investigated. The variations and diversity were observed not only between the family and genus, but also between the species.

Quantitative phytochemical analysis was also persued. The alkaloid content was determined gravimetrically by following Harborne (1973) Method. The flavonoids were estimated spectrophotometrically by the method of Zhishen *et al*. (2010) whereas saponins and tannins were estimated by the method of Hiai *et al.* (1976) and Van-Burden and Robinson (1981) respectively. The result of the phytochemical group test revealed that there was great diversity in the phytochemical content of various species of plants. *S. palustris* contain highest total alkaloid content viz.13.2 µg/ml while the least alkaloid content (2.6 µg/ml) was estimated for *A. aspera. S. oleraceus* contain highest total flavonoid content viz.155.97 µg/ml while the least flavanoid content (50.135 µg/ml) was estimated for *S. palustris. B. pilosa* contain highest total saponin content viz.74.73 µg/ml while the least saponin content (47.36 µg/ml) was estimated for *P. vulgaris*. *C. asiatica* contain highest total tannin content viz.71.77 µg/ml while the least tannin content (15.04 µg/ml) was estimated for *S. oleraceus*.

Phytochemical analysis by TLC were carried out by following the method of Harborne (Harborne, 1998). TLC is a quick, sensitive, and inexpensive technique, which separates the number of components present in any non-volatile complex mixture or plant sample using a suitable solvent for separation of different components.

Clear separated resolved bands/spots were observed in three solvent systems i.e., Chloroform: Methanol (6:1), Hexane : Ethyl acetate (4:1) and Ethyl acetate:Methanol:Water:Glacial acetic Acid (1.35:0.5:0.5:0.5) after being sprayed with reagent. Therefore, they are recommendable as a solvent system for further analysis.

The evaluations of various plants extract showed presence of different bioactive compounds as indicated by varying number of spots on a TLC plate and different Rf values (Table 8). The TLC profiles are depicted in Plate 41 (a-f).

Alkaloid were found to be present in eleven (11) of the medicinal plants Viz. *Achyranthes aspera, Acmella paniculata, Ageratum conzyoides, Centella asiatica, Houttuynia cordata, Lantana camara, Piper attenuatum, Potentilla lineata, Prunella vulgaris, Sonchus oleraceus* and *Sonchus palustris. S. palustris* contain highest total

alkaloid content viz.13.2 µg/ml while the least alkaloid content (2.6 µg/ml) was estimated for *A. aspera*.

Flavonoid were detected in ten (10) of the medicinal plants Viz. *Bidens pilosa, C. asiatica, Garcinia pedunculata, Hibiscus sabdarifa, H. cordata, L. camara, P. lineata, P. vulgaris* and *S. oleraceus*. *S. oleraceus* contain highest total flavonoid content viz.155.97 µg/ml while the least flavanoid content (50.135 µg/ml) was estimated for *S. palustris*.

In this research, appreciable quantities of saponin were present in five (5) of the medicinal plants Viz. *A. aspera, B. pilosa, L. camara, P. vulgaris* and *S. oleraceus*. *B. pilosa* contain highest total saponin content viz. 74.73 µg/ml while the least saponin content (47.36 µg/ml) was estimated for *P. vulgaris*.

Tannin was detected in eight (8) of the medicinal plants viz. *A. aspera, C. asiatica, Gaultheria fragrantissima, L. camara, P. lineata, P. vulgaris, S. oleraceus* and *S. palustris*. *C. asiatica* contain highest total tannin content viz.71.77 µg/ml while the least tannin content (15.04 µg/ml) was estimated for *S. oleraceus*. Thus, from the result of this research, the leaves of *A. aspera, C. asiatica, G. fragrantissima, L. camara, P. vulgaris, S. oleraceus* and *S. palustris* and root of *P. lineata* may be an ideal source for tannin extraction.

Steroid was found to be present in all the selected medicinal plants except *G. pedunculata* while Cardiac glycoside were detected in five (5) of the medicinal plants viz. *A. aspera, A. conzyoides, C. asiatica, Hibiscus sabdarifa* and *L. camara*.

The methanol extracts of *P. lineata* and *G. pedunculata* (MEGP) were subjected to LCMS analysis. Methanol extract of *P. lineata* (MEPL) and *G. pedunculata* (MEGP) was selected for LCMS analysis over ethanol extract of *P. lineata* (EEPL) and *G. pedunculata* (EEGP) since it was observed that it gives larger zone of inhibition against the bacteria tested. It implies that methanol may be a better organic solvent for extraction of antibacterial compounds. A continuous gradient system was followed rather than isocratic system using mobile phase composed of acetonitrile (ACN) and formic acid (0.1 % FA, v/v in water).

All analyses were carried out in the full scan mode from 150.0 to 2000.0 m/z using an ESI source in both positive-ion mode and negative-ion mode for MEPL while the analysis for MEGP was carried out in negative ion mode over a mass range of 150.0-750.0 m/z.

LC-MS of the methanol extract of *G. pedunculata* fruit revealed presence of several compounds. A total of 23 compounds (ESI-ve) and 17 compounds (ESI+ve) were characterized. Among them, 5 compounds viz. Hydroxy Citric Acid Lactone (MW-190), Garcinone E (MW-464), α-Mangostin (MW-410), β–Mangostin (MW-424), and γ-Mangostin (MW-396) were tentatively identified (peaks 2, 36, 46, 69 and 72). On the other hand, gallic acid, catechin/epicatechin, epiafz/afz-epicat/cat dimer, epicat/cat-epicat/cat dimer, epiafz/afz-epiafz/afz-epicat/cat trimer, epiafz/afz-epicat/cat-epicat/cat trimer, and epiafz/afz-epiafz/afz-epiafz/afz-epicat/cat tetramer were identified in the methanolic extract of *P. lineata*.

Identification was carried out based on molecular weight, using the mass spectra of the LC-MS. Retention times (tR), UV and mass spectral data of compounds were compared to literature data and to those of authentic standards, where available, for unambiguous identification.

Publication

Zoliansanga & Lalfakzuala (2021). Antibacterial Activity and Phytochemical Screeningof *Garcinia pedunculata* Roxb. ex Buch. - Ham. Fruit extract by HPLC–ESI- MS. J Pure Appl Microbiol |Article 7233 |

Paper Presentation in National Seminars / Conferences

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1. National Seminar on Conservation of Biodiversity, Microbial Diversity with potential for application in Agriculture and Sericulture in North East India. Organised by Department of Botany, University of Science & Technology, Meghalaya in collaboration with Central Silk Board, Assam. (25^{th} to 26^{th} March, 2022). **"Antibacterial Activity and Phytochemical Screeningof *Garcinia pedunculata* Roxb . ex Buch. - Ham. Fruitextract by HPLC–ESI- MS."**

2. National Seminar on Conservation on Emerging Trends in Plant Sciences (ETPS). Organised by Department of Botany, North Eastern Hill University, Shillong, Meghalaya in collaboration with CSIR – National Botanical Research Institute, Lucknow, Uttar Pradesh. ($29^{th}$to 30^{th} March, 2022).
   "Comparative study of the Antibacterial Activity of *Garcinia pedunculata* Roxb. ex Buch.- Ham and *Potentilla lineata* against the UTI clinical bacterial isolates – Disc Diffusion and Well Diffusion Assay."

Chapter 1
Introduction

In order to survive and thrive, mankind has been depending upon nature for meeting its various needs. Be it, food, shelter or clothing, nature never failed in providing the need of man. The various forms of life on earth do suffer from various kinds of illness in order to check and balance population growth. Since there are no medicine or drugs in the primitive world, mankind is compel to find and derive their cure from the naturally available source in their surounding. These led to their exploration of natural products with trials and errors. Medicinal plants lie at the center of this persistence war between man and diseases.

"Modern drugs era" makes its beginning at the beginning of the nineteenth century when pharmacologically active compound morphine was isolated from opium plant for the first time in 1805 by Friedrich Serturner (Joo, 2014; Hamilton and Baskett, 2000). Subsequently, countless active compounds have been separated from natural products. Later, the development of synthetic techniques led to a significant reduction in the importance of natural products. However, natural products are important for the development of new drugs, and these products have been in constant use (Joo, 2014; Newman *et al*., 2003).

Even after lapse of 200 years since the beginning of modern drugs era, many people especially the tribals in rural areas still do practice traditional medicine for various reasons. The Khasi, the inhabitants of Meghalaya in the remote north eastern corner of India, are no exception and many traditional healers have their own clinic where they practice and prescribe ethnomedicinal formulation to their patient.

The bioactive compounds present in the medicinal plant are responsible for the medicinal properties of the plant (Bargah, 2015). The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents (Nostro *et al.,* 2000).

The phytochemical constituents may exert a definite metabolical and physiological action on the human body or kill the pathogens that enter the body

(Edeoga, 2005). Medicinal plants possess a variety of compounds of known potent therapeutic property. Worldwide trend towards the utilization of natural plant remedies has created an enormous need for the use of medicinal plants. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds. Now- a-days, these phytochemicals become more popular due to their countless medicinal uses. Plants are recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities.

Natural products from plants called secondary metabolites are the end products of primary metabolites such as carbohydrates, amino acid, chlorophyll lipid, etc. Secondary metabolites include alkaloids, steroids, flavonoids, terpenoids, glycoside, saponin, tannins, phenolic compounds, etc. (Ghahi *et al.*, 1990). The active principle of many drugs found in plants are secondary metabolites (Dobelis, 1993). Therefore, basic phytochemical investigation is vital. The identification and isolation of such active compounds makes it more effective for therapeutic application.

Secondary metabolites are the classes of compounds which are known to show curative activity against several ailments in man, and therefore, could explain the traditional use of medicinal plant for the treatment of some illnesses. There are chemical compounds (phenolic compounds, alkaloids, terpenoids, steroids, quinones, saponins etc.) with complex structures and with more restricted distribution than primary metabolites. They are not indispensable for the plant that contains them; at least their metabolic functions have not been discovered yet (Zohra, 2012).

Ethnobotany is important in drug research. Recent advances in technology have made natural drug prospecting a viable solution to the search for new cures for diseases. Taking clues from folk uses followed by critical scientific evaluation have given to the world newer resources not only to fight diseases but also to other aspects of health care system. In view of fast-growing demand for the medicinal plants for the use in pharmaceuticals, nutraceuticals, cosmetologicals, dietary supplements etc. both at national and international level, implementation of the projects and the schemes meant for cultivation, collection, awareness, commercial supply of the medicinal plants for generation of income and employment while ensuring

conservation of endemic and threatened species through the sustainable use is the urgent need of the hour.

There has been an increasing interest worldwide on therapeutic values of natural products. Nature provides mankind vast therapeutic flora with a wide variety of medicinal potential. The revival of interest in plant derived drugs is mainly due to the current widespread belief that “green medicine” is safe and more dependable than the costly synthetic drugs many of which have adverse side effects. The need of the hour is to screen a number of medicinal plants of promising biological activity (Parekh *et al.*, 2006). The future of ethnobotany lies not simply in adding to the observational foundation, but rather in using the knowledge base to effectively address the current concerns of mankind (Bates, 2001).

India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants (Saxena, 2008). Therefore, investigation of plant chemical compounds has become desirable (Ahmad *et al.*, 1998). Even though the effect of certain plants on pathogenic bacteria have been demonstrated (Voravuthikunchai *et al.*, 2004), a number of them have not yet been investigated for their antimicrobial activities. Hence, it is essential to establish the scientific basis for their therapeutic actions, which may serve as the source for the development of effective drugs (Kader *et al.*, 2011). Keeping this in view, efforts are underway to search for better understanding of qualitative, quantitative chemical composition and to explore the antibacterial activity of extracts of various medicinal plants. The traditional system of herbal medicine has become a topic of global importance since they are considered as rich sources of lead compounds and quietly safe for both human use and environment-friendly (Pradeepa *et al.*, 2016).

In India, there are 227 ethnic groups and comprising 573 tribal communities derived from six racial stocks viz. Negroid, Proto-Australoid, Mongoloid, Mediterranean, West Breachy and Nordic exists in different part of the country. Nearly 80% people in India and the developing countries of the world are depending on forest resources for meeting their health needs, they live in the vicinity of forests and have managed and conserved the biodiversity of their localities since long time.

India, a megadiverse country with only 2.4% of the world's land area, harbours 7 – 8 % of all recorded species, including over 45,000 species of plants and

91,000 species of animals. It is also amongst the few countries that have developed a biogeographic classification for conservation planning, and has mapped biodiversity-rich areas in the country. Of the 34 global biodiversity hotspots, four are present in India, represented by the Himalaya, the Western Ghats, the North-east, and the Nicobar Islands (India's Fifth National Report to the Convention on Biological Diversity, 2014).

North-east India comprises of eight states viz. Meghalaya, Manipur, Nagaland, Assam, Mizoram, Tripura, Arunachal Pradesh, Sikkim. It comprises 7.7% of India's total geographical area supporting 50% (8000 species) of the country's total flora (Rao, 1994), of which 31.58% (2526 species) are endemic (Nayar, 1996). North-east region has 4 biosphere reserves, 48 sanctuaries, 14 national parks and 2 world heritage sites. With more than 150 tribes speaking as many languages, this region is a melting pot of variegated cultural mosaic of people and races and ethnic tapestry of many hues and shades. The North Eastern region has still remained unexplored because of various reasons particularly difficult terrain, poor communication, hostility of the inhabitants and insurgency conditions in the region.

Meghalaya a small state in North-east India but it is one of the richest states of India in terms of vegetation and flora. This is due to the large variation in the altitude, topographical features, soil characteristics and climatic factors which has favoured the growth and luxuriance of rich flora here. The forests of Meghalaya are rich in biodiversity and endowed with rare species of orchids and medicinal plants.

The traditions of collecting, processing and applying and consuming plants and plant based medications, long and carefully maintained by individuals with a profound knowledge, have been handed down from generation to generation among the indigenous people. The value of such ethno-medicine and traditional pharmacology is now increasingly recognised in modern human and veterinary medicine (Maydell, 1990). These plants mostly grow naturally and are collected by local people and sold to traders who, in turn, sell to the pharmaceutical and cosmetic industry and to exporters. There is no scientific system of collection or regeneration as a result of which many of these plants have become endangered and extinct (Kayang *et al.*, 2005).

Indigenous people are generally very knowledgeable about the wild medicinal plants around them, many of which have local names and are important to the people medically or are featured in folklore (Kharkongor and Joseph, 1981). Knowledge of herbal remedies for treatment of various diseases rest with the traditional healers, which belong to a family of indigenous practitioners and skills have been passed on from one generation to the other only by word of mouth. Each village has one or two traditional healers or Nongaidawaikynbat as they are called locally. Each of them practices at home or has a place in the weekly market of the villages, where people come to consult him (Hynniewta and Kumar, 2007). Inspite of the development of advanced/modern medicine, tribal people in Meghalaya continue to use medicinal plants extract to cure infectious diseases amongst other diseases (Kayang *et al.*, 2005; Sajem and Gosai, 2006; Hynniewta, 2010).

According to Hynniewta (2010), the use of medicinal plants extract is common among the people of Meghalaya and found to be a significantly higher among rural people due to poor transportation and medical facilities in the rural areas. Modern medicinal facilities are scanty and could not reach these inaccessible areas inspite of government's best efforts.

Ethnomedicines and medicinal plants of Meghalaya have attracted attention of various researchers. Ethnobotanical research work in Meghalaya had been done in Jaintia Hills district of Meghalaya by Samati (2007) and in Khasi Hills of Meghalaya by Hynniewta (2010). However, most of these studies are restricted to documentation of uses of plants by tribal people. Ethnobotanical knowledge of Khasi tribe in Meghalaya is very now well documented. Enormous work on the distribution of medicinal plants in various agro-ecological regions of Meghalaya, analysis of the status of medicinal plant sector in the state and the strategies for equitable utilisation and management of this vital resource had been accomplished (Mishra and Dutta, 2003; Tynsong *et al*., 2006). However, analytical experimental studies like phytochemical screening and antimicrobial activities of medicinal plants of Meghalaya are lacking which is required to establish and confirm the benefit and effectiveness of medicinal plants as a treatment for various diseases. Scientific analysis and evaluation of the ethnomedicinal plants used by the Khasi tribe in Meghalaya is the need of the hour and research work in this context is very scarce

and limited. Hence, efforts are made in this direction in the form of phytochemical screening and antibacterial activity detection. It is a fact that justifies this research of evaluation of the antibacterial activity and phytochemical screening of the selected ethnomedicinal plants used by the Khasi tribe in Meghalaya.

In view of this gap, the present study focuses on the following objectives:

1. To collect and identify the Khasi medicinal plants growing in various sites of study area.
2. To carry out the phytochemical analysis of selected plant species.
3. To study the antibacterial potential of the selected plants.

Chapter 2

Review of Literature

Nature has been a source of medicinal agents for thousands of years and since the beginning of mankind. Medicinal plant is an integral part of human life to combat the sufferings from the dawn of civilization. It is estimated that more than 80,000 of total plant species have been identified and used as medicinal plants around the world (Chaudhary *et al*., 2010). Over the past twenty years, interest in medicinal plants has grown enormously from the use of herbal products as natural cosmetics and for self-medication by the general public to the scientific investigations of plants for their biological effects in human beings (Joy *et al*., 1998). Therefore, people are encouraging indigenous production and processing of these medicinal plants to use in different cultures and religion for the treatment of various diseases (Burkill *et al*., 1966). The demand for plant-based medicines, health products, pharmaceuticals, food supplement, cosmetics etc. are increasing in both developing and developed countries, due to the growing recognition that the natural products are nontoxic, have less side effects and easily available at affordable prices (Kalia, 2005). Nowadays, there is a revival of interest with herbal-based medicine due to the increasing realization of the health hazards associated with the indiscriminate use of modern medicine and the herbal drug industries is now very fast-growing sector in the international market (Sharma *et al.*, 2008). There is great demand for herbal medicine in the developed as well as developing countries like India, because of their wide biological activities, higher safety of margin than the synthetic drugs and lesser costs (Gadre *et al*., 2001; Akbar, 2011).

Ethnomedicinal Used

When reviewing the literature available on ethnomedicinal plants studies conducted especially in the North-Eastem part of India, it is obvious that there are few doctoral theses and ethnobotanical studies on some of its parts. Borthakur (1980)

elaborated the regional perspective of ethnobotanical studies in Mikir Hills (Assam), Sinha (1986) on Manipur, Sapu (1991) on Mokokchung and Tuengsang districts ofNagaland, Ethnobotany of Jaintia Hills (Samati, 2007) on ethnobotany of Jaintia Hills and Hynniewta (2010) on ethnobotany of Khasi Hills.

Arora (1981) gave a list of 300 native food plant species of Northeastern tribals. Islam (1996) noted the ethnobotany of some underground parts of plants of North-Eastem India. Tripathi and Goel (2001) studied the Ethnobotanical use of 43 taxa of Zingibers in North- Eastern India. Dutta and Dutta (2005) in their paper gave an overview on the potential of Ethnobotanical studies in North East India.

An ethnobotanical study on medicinal and aromatic plants of Mizoram was done by Lalramnghinglova (1991, 1996), ethnomedicine from Mizoram – North East India (Lalramnghinglova and L.K. Jha, 1997) and medicinal plants in tropical, sub-tropical and semievergreen forests of Mizoram (Lalnundanga, 2000). Bhardwaj and Gakhar (2003) gave the usage of wild plants by the native people of Mizoram for the cure of dysentery while Lalhriatpuii (2010) had studied the phytochemistry and pharmacology of selected medicinal plants of Mizoram.

Devi (1989) also contributed to ethnobiological studies of Manipur Valley with reference to Museological aspects. Singh *et al.* (2003) studied 25 plants belonging to 13 families ranging from mushrooms to higher angiospermic plants, used by the traditional Meitei singers of Manipur to enhance vocalism. Sharma *et al.* (2003) recorded 2153 species of edible fruits of Manipur. Jain *et al.* (2007) worked on the aquatic and semi-aquatic plants used as herbal remedies by the people living in the wetlands of Manipur.

Medicinal flora of Lohit District with special reference to ethnobotany (Bhuyan, 1989), ethnobotany of wild edible plants (Haridasan *et al.*, 1990), ethnobotany of Nishis, Karbis, Kacharis and Chakma (Maikhuri and Ramakrishnan, 1992) and on the edible mushrooms of East Siang district of Arunachal Pradesh (Das, 2001) are important contributions in the field of ethnobotany in Arunachal Pradesh. An ethnobotanical

studywas conducted by Singh *et al.* (2007) on the traditional foods of Monpa tribe of West Kameng. Sarmah *et al.* (2008) documented 63 medicinal plant species used by Chakmas of Arunachal Pradesh.

Deb (1968) has reported on medicinal plants of Tripuri tribe while Singh *et al.* (1999) gave an account of 37 wild edible plants used by the Tripuri tribe of Tripura state. Wild plants used and sold in markets of Sikkim have been documented by Bennet (1983), Hajra and Chakraborty (1982) respectively. Hussain and Hore (2007) worked on the collection and conservation of major medicinal plants of Darjeeling and Sikkim Himalayas.

Some good publications have appeared on ethnobotany of Assam during the lasttwo decades by Borthakur (1976, 1996); Baruah and Sarma (1984 and 1987); Sarma *et al.* (2001). Food adjunct, beverages and masticatories used by Karbis have been studied by Borthakur (1997). Borthakur (1998 and 1999) also studied on the house gardens of Assam. Gogoi and Borthakur (2001) recorded 69 herbal recipes for 27 ailments involving 68 plant species prevalent among the Bodo tribe inhabiting Kamrup district of Assam. Anaccount of the folklore medicinal uses of 71 plant species used among the Tai Ahoms, one of the ethnic groups predominant in Assam is reported by Dutta and Nath (2003). Barua *et al.* (2003) documented 58 plant species used as folk medicine among the Rajbanshis of Assam.

A list of medicinal plants of Nagaland have been given by Rao and Jamir (1982a and b), medico-botany used by Ao Naga tribe by Jamir (1989), fifty medicinal plantsused by Zeliangs of Nagaland have been reported by Jamir and Rao (1990). Ethno-botanical folk practices and beliefs of the Ao-Nagas have been reported by Sapu and Yogendra (1996). Mao (2003) worked on the symbolic uses and superstitions in botanical folklore about Mao Naga tribe of Manipur. Five plants with symbolic uses andsix plants with superstition beliefs in the Mao Naga tribes have been listed. Traditional fermented foods of the Naga tribes of North east India were reported by Mao and Odyuo (2007).

Jain *et al.* (1977) made a survey of the edible plants in the bazaars of Meghalaya. Joseph and Kharkongor (1981) brought forward two papers based on the Ethnobotanical studies of the Khasis and Jaintia tribes of Meghalaya. One paper deal with over 100 plants of Ethnobotanical importance used as medicines, subsidiary food, making implements and musical instruments, religious ceremonies etc. The other paper presents an enimieration of 100 species of plants belonging to 81 genera and 46 families having medicinal virtues. Rao (1981) gave a list of 33 medicinal plants belonging to 27 families used by Khasis and Garos. The work of Hajra (1981) on nature conservation in Khasi folk beliefs and taboos deals with the religious beliefs of the tribe that helps in thepreservation of forests in their natural condition. Shanpru and Vasudeva (1981) investigated the Garos and reported 25 plant species used by them for food, 24 for medicine, 5 for fish poison, 7 for fibres, 3 for dyes, 4 for magico-religious beliefs and 10 for miscellaneous purposes.

Kumar *et al.* (1987) recorded 74 species used by the Khasiand Jaintia tribes. Rao *et al.* (1989) worked on the Ethnobotany of some weeds of Khasi and Garo Hills of Meghalaya. The paper deals with the ethnobotany of 65 taxa belonging to 26 families of angiosperms. Maikhuri and Gangwar (1993) recorded a total of 105 plants used by Khasis and Garos of Meghalaya. Choudhury and Neogi (2003) reportedon the ethnobotany of Khasi and Chakma tribes of North-east India. In the paper, 37 such taxa belonging to 34 genera and 15 families have been discussed. Samati (2004) reported 55 taxa of kitchen garden plants of Pnar tribe in Jaintia Hills. Fourteen wild edible berries, fruits, roots and nuts consumed by the Khasi tribe of Meghalaya were identified and analyzed for their nutrient content (Murugkar and Subbulakshmi, 2005).

Ahmed and Borthakur (2005) documented a total of 577 species of plants belonging to 375 genera under 146 families used by the Hynniewtreps of Meghalaya as edibles, plant masticatories, ethnoiatrical plants etc. Baruah *et al.* (2006) recorded 45 ethnomedicinal plants used by the Khasi tribe of Cachar district, Assam. Kayang (2007) documented 110 wild growing plants, which are eaten by the local people of Meghalaya.

Hynniewta (2010) documented 172 ethnomedicinal plants used by the Khasi tribals of Meghalaya.

Ratra *et al*. (1970) reported that *Achyranthes aspera* L. is use as folk medicine since time immemorial in different parts of India. It holds a reputed position as medicinal herb in different systems of medicine in India. Traditionally, the plant is used in asthma and cough. It is pungent, antiphlegmatic, antiperiodic, diuretic, purgative and laxative, useful in oedema, dropsy and piles, boils and eruptions of skin, etc. *A. aspera* is used in indigenous system of medicine as emenagogue, antiarthritic, antifertility, laxative, ecbolic, abentifacient, anti-helminthic, aphrodisiac, antiviral, anti-plasmodic, anti-hypertension, anti-coagulant, diuretic and anti-tumor.

Kamble (2018) stated that *A. aspera* is useful to treat cough, renal dropsy, fistula, scrofula, skin rash, nasal, infection, chronic malaria, impotence, fever, asthma, piles and snake bites. Crushed plant is boiled in water and is used in pneumonia. Infusion of the root is a mild astringent in bowel complaints. The flowering spikes or seeds, ground and made into a paste with water, are used as external application for bites of poisonous snakes and reptiles, used in night blindness and cutaneous diseases (Nadkarni, 2009).

For snake bites, the ground root of *A. aspera* is given with water until the patient vomits and regains consciousness. Inhaling the fume of *A. aspera* mixed with *Smilax ovalifolia* roots is suggested to improve appetite and to cure various types of gastric disorders (Bhattaraj, 1992). It is useful in haemorrhoids, leaves and seeds are emetic, hydrophobia, carminative, resolve swelling, digestive and expel phlegm. Ash of the plant is applied externally for ulcers and warts. The crushed leaves rubbed on aching back to cure strained back (Singh, 1996).

Gupta (2010) reported that a fresh piece of root or dried twigs of *A. aspera* is used as tooth brush. In Western India, the juice is applied to relieve toothache. Paste of the roots in water is used in ophthalmia and opacities of the cornea. Paste of fresh leaves

is used for allaying pain from bite of wasps. The plant is useful in liver complaints, rheumatism, scabies and other skin diseases. It also possesses tranquillizing properties (Khare, 2007; Anonymous, 2007).

A. aspera is widely studied for its medicinal properties and reported to have immunostimulatory properties (Rao *et al*., 2002), wound healing activity (Edwin *et al*., 2008), antioxidant activity, hemolytic activity (Priya *et al*., 2010), anti-inflammatory (Kumar *et al*., 2009), antibacterial activity (Alam *et al*., 2009) and antifungal activity (Elumalai *et al*., 2009). Seeds, roots and shoots are the most important parts of *A. aspera* that are used in traditional systems of medicines. The major chemical constituents are carbohydrates, protein, glycosides, alkaloids, tannins, saponins, flavonoids, lignin, etc.

The flowers of *Acmella paniculata* (Wall. ex DC.) R.K. Jansen [syn: *Spilanthes acmella var. paniculata* (Wall. ex DC.)] are chewed to reduce toothache and the crushed plant is used in rheumatism. The leaves are also eaten raw or as a vegetable by many tribes of India. This species is well-known as a folklore solution for toothache and for throat and gum infections (Wongsawatkul *et al*., 2008).

Leaf paste of *Ageratina conzyoides* is applied on cuts and wounds as antiseptic. Whole plant together with fruit of *Piper peepuloides* and rhizome of *Acorus calamus* are ground and the content is taken orally for treatment of cancer and ulcer. The herb is not eaten by humans except for medicinal purposes, but in some cultures, it is a delicacy for domestic guinea-pigs, horses, cattle and also used to feed fish (Menut *et al*., 1993).

Leaf paste of *Bidens pilosa* is applied on cuts and wounds as antiseptic. The leaves are ground and the extracted juice is taken orally for treatment of gastric disorder.

Centella asiatica is used as a culinary vegetable and as medicinal herb. The whole plant is used for medicinal purposes. It is widely used as a blood purifier as well as for treating high blood pressure, for memory enhancement and promoting longevity. In Ayurveda, *C. asiatica* is one of the main herbs for revitalizing the nerves and brain cells. Eastern healers relied on *C. asiatica* to treat emotional disorders, such as

depression that were thought to be rooted in physical problems (PDR for herbal medicine, 1999; Hagemann *et al.,* 1996). In the Western medicine, during the middle of the twentieth century, *C. asiatica* and its alcohol extracts reported to have shown positive results in the treatment of leprosy (Baily, 1945). *C. asiatica* stem, leaves, and aerial parts are used in the traditional drug formulation to decrease blood pressure, cure the fresh wound, heal bruised and diuretic.

In 'Malabar' and 'Konkan' region of southern India, *Garcinia pedunculata* are used in garnishing curries and also as a replacement for tamarind. In North Eastern India, the sundried slices of the fruits are used for culinary purposes and as folk medicine. Some species, like *G. cambogia, G. indica* and *G. cowa*, are cultivated in certain parts of India. *G. pedunculata, G. kydia, G. cowa and G. lancifolia* are the most important species in North Eastern parts of India. Many species of Garcinia have fruit with edible arils and are eaten locally. The best-known species is the mangosteen (*G. mangostana*), which is now cultivated throughout Southeast Asia and other tropical countries. The seeds of *G. indica* fruits yield valuable edible fat known as 'kokum butter'. The fruits of *Garcinia* are a food source for several animals (CSIR, 1956). Most species in *Garcinia* are known for their gum resin which is used as purgative or cathartic. Fruits of some *Garcinia* species are also one of the richest sources of red pigments in the plant kingdom.

Infusions of *Hibiscus sabdarifa* leaves or calyces are regarded as diuretic, febrifugal and hypotensive, decreasing the viscosity of the blood and stimulating intestinal peristalsis. The calyx infusion is also taken to relieve coughs.

In China, the seeds of *H. sabdarifa* are used for their oil and the plant is used for its medicinal properties, while in West Africa the leaves and powdered seeds are used in meals. Additionally, it is used in the pharmaceutical and food industries. *H. sabdarifa* is reported to be useful in the treatment of hypertension (Wahabi *et al*., 2010) and hyperlipidemia (Hopkins *et al.*, 2013).

The whole plant of *Houttuynia cordata* is used against dysentery, controlling blood sugar level and as a blood purifier.

Lantana camara uninfected leaves are crush into watery – moist soften appearance structure and applied on fresh wound and bruise. They believe that it has antiseptic property preventing infection of wounds.

L. camara is regularly used as herbal medicine and in some areas as firewood and mulch (Saraf *et al*., 2011). Especially in India, there has been to a great extent work conducted on the chemical constituents of *L. camara*. The leaf oil is employed as an antiseptic for scars; the roots are used for the treatment of a toothache and the flowers for chest complaints in children (Kirtikar and Basu, 1981). *L. camara* leaves extract exhibited anti-proliferative, antimicrobial, fungicidal, insecticidal and nematicidal activities (Saxena *et al*., 1992; Begum *et al*., 1995; Sharma *et al*., 1999; Day *et al*., 2003.). *L. camara* shoots extract exhibited significant antioxidant activity (Basu and Hazra, 2006). The berries fruits are useful in fistula, pocks, tumors and rheumatism (Kirtikar and Basu, 1935; Chopra *et al*., 1956; The Wealth of India, 1962; The useful plant of India, 1992).

L. camara has several uses, mainly as a herbal medicine and in some areas as firewood and mulch. It is also used for the treatment of cancers, chicken pox, measles, asthma, ulcers, swellings, eczema, tumors, high blood pressure, bilious fevers, catarrhal infections, tetanus, rheumatism, malaria and atoxy of abdominal viscera (Abu-Shanab *et al.*, 2006).

Lantana camara oil is sometimes used for the treatment of skin itches, antiseptic for wounds, leprosy and scabies.

Different parts of *P. attenuatum* have been used as herbal medicine for the treatment of headache, muscular pain and have been used as a rubefacient. The wood of plant has been used to treat throat pain. The root part possesses diuretic activity (Khare, 2007). Leaves have been used for their wound healing property. The whole plant is

reported to contain a rare phytoconstituent crotepoxide, which has been reported to exhibit significant antitumor activity against Lewis lung carcinoma cell lines (Morris *et al*., 1969).

Potentila lineata Trevir (Syn: *Potentilla fulgens* Wall. ex Hook) roots are thoroughly wash with water and the bark are peeled off. The inner part of the root is then chew alone or chew with arecanut. Traditionally, it is an important ingredient for the local traditional medicine used to relieve dental problems. It is also used for the treatment of diarrhea, stomach ailments, high blood pressure, diabetes and cancer.

Prunella vulgaris tender leaf paste is applied on cuts and wounds for quick healing. Leaves are taken raw for cure of blood dysentery. *P. vulgaris* is a known plant with traditional uses for treatment of infectious diseases such as wounds and sore throat, minor injuries, sores, burns, bruises and can also be used as a mouthwash to treat mouth ulcers. It is known for its antiviral activity against HSV and HIV. The antiviral activity is related to an anionic polysaccharide (Baba *et al*., 1988).

Sonchus oleraceus young leaves taken raw to stop white discharge in women. Whole plant taken raw to cure anaemia. Baruah and Sarma (1984) reported that the whole plant is applied on wounds to relieve pain by the Bodo tribals of Assam. *Sonchus palustris* are mainly eaten as vegetable leaves but are claim to be useful for treatment of high blood pressure and diabetis (Hynniewta, 2010).

Phytochemistry

Kamble (2018) reported that the preliminary phytochemical screening of leaf and flower extracts of *A. aspera* revealed the presence of phytochemicals such as alkaloids, flavonoids, saponin, tannins, saponins, glycoside and steroids. All these, compounds may confirm the clinical affect associated with *A. aspera*.

A. aspera contains triterpenoid saponins which possess oleanolic acid as the aglycone. Ecdysterone, an insect moulting hormone, and long chain alcohols are also found (Indian Herbal Pharmacopoeia).

Misra *et al*. (1966) isolated various compounds like tetracontanol-2, 4-methoxyheptatriacont-1-en-10-ol and β-sitosterol. Banerji *et al*. (1971) isolated ecdysterone from the whole plant. Laddha *et al*. (2005) reported extraction, isolation and purification of 20-hydroxyecdysone from *A. aspera*. Kapoor and Singh (1966) reported betaine from the whole plant which is also a water soluble alkaloid. Seshadri *et al*. (1981) isolated two constituents from the fruits and were identified as Saponin C and D.

Ali (1993) isolated various compounds from the stem, viz. Pentatriaontane, 6-pentatriacontanone, Hexatriacontane and Tritriacontane. Kunert *et al*. (2000) reported three bisdesmosidic saponins (I-III), 20-hydroxyecdysone, and quercetin-3-O-β-D-galactoside and they were isolated from the methanol extract of the aerial parts of *A. aspera*. Other chemical constituents such as achyranthine, betaine, pentatriaontane, 6-pentatriacontanone, hexatriacontane, and tritriacontane are also present (Srivastav, 2011).

The major pungent constituent reported in *A. paniculata* is "spilanthol," which is an isobutylamide and is well known for its insecticidal properties (Jondiko, 1986; Kadir *et al*., 1989). Spilanthol is chemically N-isobutylamide which is bitter in taste and could stimulate salivation. The molecular formula of spilanthol was determined as (2E,6Z,8E)-N-isobutylamide-2,6,8-decatrienamide (Yasuda *et al*., 1980). Spilanthol has a strong pungent taste. It may produce local astringency and anaesthetic effects.

Triterpenoids have also been found in the plant (Mukharya and Ansari, 1987). Ramsewak (1999) also reported that *Acmella* contain Spilanthol, Undeca - 2E, 7Z, 9E - trienoic acid and Undeca - 2E – en - 8, 10 - diyonic acid isobutylamide while Nakatani (1992) detected the presence of 2E-N-(2-Methylbutyl)-2-undecene-8,10-diynamide, 2E,7Z-N-Isobutyl-2,7-tridecadiene-10,12-diynamide and 7Z-N-Isobutyl-7-tridecene-

10,12-diynamide. β-Sitosterol, α-Amyrin, β-Amyrin and Germacrene-D were reported by Krishnaswamy (1975).

Stigmasterol and Myrecene were reported by Tiwari (1990) while 3-Acetylaleuritolic acid, Vanillic acid, β - Sitostenone, O Scopoletin, trans-Ferulic acid and trans - Isoferulic acid are the other various secondary metabolites which have been found to be present (Prachayasittikul, 2009). Limonene (Baruah, 1993), β-Caryophyllene (Boonen, 2010) and (Z)-β-Ocimene (Baruah,1993) were also reported to be present.

The essential oil obtained from *A. conzyoides* has been reported to have a powerful nauseating odour (Sood, 1973) and found to be poisonous to rabbits due to the presence of HCN and coumarin (Abbiw, 1990).

Presence of pyrrolidine alkaloids are known to hepatotoxic and to cause lung cancer and other ailments (Couett *et al.,* 1996) but of biological interest due to their association with Leptidoptera (Vera, 1993).

The GC-MS analysis of the essential oil showed that it is a complex mixture of 213 compounds of which 43-51 constituents have been reported. The constituents identified include twenty monoterpenes (6.4%), thirteen monoterpenoid hydrocarbons (5.0%), seven oxygenated monoterpenoids (0.08-1.4%), twenty sequiterpenes (5.1%), sixteen sesquiterpenoid hydrocarbons (4.3%), four oxygenated sesquiterpenoids (0.8%), three phenylpropanoids and benzenoids (2.33%) (Ekundayo *et al*., 2003; Rao *et al*., 1973).

The ethanolic extract of the plant was found to be devoid of tannins. (Srivastava *et al*., 1985). *A. conyzoides* is very rich in polyoxygenated flavonoids. A total of twenty-one polyoxygenated flavonoids have been reported (Vyas *et al*., 1984). The triterpenes isolated from plant are friedelin, and the major sterols are sterols-β-sitosterol (26.7%) and stigmasterol (59.9%) (Dubey *et al*., 1989; Horng *et al.,* 1976; Hui *et al*., 1971; Wiedenfeld *et al*., 1991; Sur *et al*., 1997).

Phytochemicals interest in basic research and application of *B. pilosa* has increased since its first identification in 1753. This is mainly due to its wide application in medicines, foods, and drinks. Around 116 publications have documented the exploitation and medical use of *B. pilosa*. To date, 201 compounds comprising 70 aliphatics, 60 flavonoids, 25 terpenoids, 19 phenylpropanoids, 13 aromatics, 8 porphyrins, and 6 other compounds, have been identified from this plant as compiled previously (Silva *et al.,* 2011). However, the association between *B. pilosa* phytochemicals and their bioactivities is not yet fully established and should become a future research focus. Compelling evidence suggests that the various diverse bioactivities reported for *B. pilosa* reflect its phytochemical complexity.

B. pilosa is an extraordinary source of phytochemicals, particularly flavonoids and polyenes. Plant flavonoids are commonly reported to possess anticancer, antiinflammatory, antioxidant, and other bioactivities. However, the bioactivities of only seven of the 60 flavonoids present in *B. pilosa* have been studied. The bioactivities of the remaining 53 flavonoids are poorly understood and deserve further investigation.

The primary active constituents of *C. asiatica* are saponins (also called triterpenoids), which include asiaticosides, in which a trisaccharide moiety is linked to the aglycone asiatic acid, madecassoside and madasiatic acid (Singh, 1969). These triterpene saponins and their sapogenins are mainly responsible for the wound healing and vascular effects by inhibiting the production of collagen at the wound site. Other components isolated from *C. asiatica*, such as brahmoside and brahminoside, may be responsible for CNS and uterorelaxant actions, but are yet to be confirmed by clinical studies. Crude extract containing glycosides isothankuniside and thankuniside showed antifertility action in mice (Heidari *et al.*, 2007; Boca Raton, 1985).

Centelloside and its derivatives are found to be effective in the treatment of venous hypertension. In addition, the total extract contains plant sterols, flavonoids, and other components with no known pharmacological activity (Srivastava *et al*., 1997), namely, abundant tannins (20-25%), essential acid (0.1% with beta-chariophylen, trans-

beta-pharnesen and germachrene D), phytosterols (campesterol, sitosterol, stigmasterol), mucilages, resins, free aminoacids (alanine, serine, aminobutyrate, aspartate, glutamate, lysine and treonine), flavonoids (derivates of chercetin and kempferol), an alkaloid (hydrochotine), a bitter component (vallerine), fatty acids (linoleic acids, linolnelic, oleic, palmitic and stearic acids. The herb contains many types of active compounds, Terpenes or Terpenoids.

Garcinia also contains a high amount of Vitamin C and is used as a heart tonic. *Garcinia* is the source for a natural diet ingredient (-) hydroxycitric acid. HCA, (1,2-dihydroxypropane-1,2,3-tricarboxylic acid) which is an anti-obesity compound present in the fruit rind and leaves of *Garcinia* and is known to inhibit lipid and fatty acid synthesis in living systems (Lewis and Neelakandan, 1965). HCA is also hypocholesterolamic agent (Lowenstein, 1971; Sullivan *et al*., 1972; Sullivan and Triscari, 1977). On a dry weight basis, HCA constitutes about 20-30% of the fruit (Cheek, 2004.).

Mukherjee *et al*. (1995) reported that *Garcinia* contain bioactive compounds such as xanthones, biflavonoids, benzophenones, benzoquinones, and triterpenes which have antibacterial, antifungal, antioxidant, and cytotoxic effects.

Extensive investigations on the chemical constituents from 34 species in the genus *Gaultheria* have led to the identification of 110 compounds, mostly assigned to five structural types, including methyl salicylate derivatives, C6-C3 constituents, terpenoids, steroids and other compounds. From an overall perspective, a majority of these compounds were found in two plants, *G. yunnanensis and G. nummularioides*.

To the best of our knowledge, about 109 compounds were reported from this genus, including methyl salicylate derivatives, C6-C3 constituents, terpenoids, and steroids. In particular methyl salicylate and three methyl salicylate glycosides, methyl salicylate 2-O-β-D-glucopyranoside, gaultherin and methyl benzoate-2-O-β-D-xylopyranosyl(1-2)[O-β-Dxylopyranosyl(1-6)]-O-β-D-glucopyranoside, from the aerial

parts of *G. yunnanensis*, display notable analgesic and anti-inflammatory activities, which has impelled a number of studies on the phytochemistry and biology of this genus (Zhang, 2007).

Meanwhile, it's worth mentioning that methyl salicylate glycoside when used in animal experiments to explore its pharmacological effects could overcome the side-effects caused by aspirin in the clinic (Zhang, 2007; Hirsh, 1985; Levy, 1974).

Ursolic acid (Murthy, 1972), p-Coumaric acid (Towers, 1966), Caffeic acid (Towers, 1966), Quercetin 3-galactoside (Murthy, 1972) and o-Pyrocatechuic acid (Towers, 1966) were isolated from the leaf of *G. fragrantissima.*

Protocatechuic acid and ferulic acid were found in the root while 4-hydroxybenzoic acid, salicylic acid and vanillic acid were found in the whole plant of *G. fragrantissima* (Towers, 1966).

(-) – Isolariciresinol-2α – O – β – D-xylopyranoside (Ma, 2002), (+) – Lyoniresinol – 2α – O – β – L-arabinopyranoside (Ma, 2002), (+) – Lyoniresinol – 2α – O – β – D-glucopyranoside (Ma, 2002) and Gentistic acid (Towers, 1966) were obtained from the root, stem and leaf of *G. fragrantissima*.

The main constituents of *H. sabdariffa* relevant in the context of its pharmacological activity are organic acids, anthocyanins, polysaccharides and flavonoids (Eggensperger and Wilker, 1996; Müller and Franz, 1990). *H. sabdarifa* extracts contain a high percentage of organic acids (including citric acid, hydroxycitric acid, hibiscus acid, malic and tartaric acids) as major compounds, and oxalic and ascorbic acid as minor compounds. Based on previous studies, the percentage of organic acids in *H. sabdarifa* varies; hibiscus acid accounts for 13–24%, citric acid 12–20%, malic acid 2–9%, tartaric acid 8% and 0.02– 0.05% of ascorbic acid (vitamin C) (Eggensperger and Wilker, 1996; Schilcher, 1976).

The anthocyanins are a group of flavonoid derivatives and natural pigments present in the dried flowers of *H. sabdarifa* and their colour varies with pH. Delphinidin and cyanidin-based anthocynins, include delphinidin-3-sambubioside (hibiscin), cyanidin-3-sambubioside (gossypicyanin), cyanidin-3,5-diglucoside, delphinidin (anthocyanidin) and others (Williamson *et al.*, 2009).

H. sabdarifa contain polyphenols of the flavonol and flavanol type in simple or polymerised form. The following flavonoids have been described in *H. sabdarifa* extracts: hibiscitrin (hibiscetin-3-glucoside), sabdaritrin, gossypitrin, gossytrin and other gossypetin glucosides, quercetin and luteolin (McKay, 2009; Williamson *et al*., 2009); as well as chlorogenic acid, protocatechuic acid, pelargonidic acid, eugenol, quercetin, luteolin and the sterols (b-sitosterol and ergosterol) (McKay, 2009; Williamson *et al*., 2009).

Volatile compounds are responsible for the aroma of *H. sabdarifa*. Jirovetz *et al*. (1992) reported that there are more than twenty-five volatile compounds (accounting for less than 8% of total *H. sabdarifa* seeds composition) in the seed oil of *H. sabdarifa*. They were mainly unsaturated hydrocarbons, alcohols and aldehydes from C8 to C13.

Rodrigues *et al*. (2011) revealed presence of thirty-two compounds in the volatile profile of aqueous extracts from fresh and dried calyx of *H. sabdarifa* using two different time–temperature extraction conditions by GC–MS and could be divided into five chemical groups: aldehydes (fourteen compounds), alcohols (ten compounds), ketones (five compounds), terpenes (two compounds) and acids (one compound).

A total of 346 volatile components were reported from *H. cordata*. They were composed mainly of terpenoids (27.0%), hydrocarbons (16.8%), esters (11.9%), alcohols (11.6%), ketones (7.2%), aldehydes (4.9%), acids (3.8%), phenols (1.7%), aethers (0.9%) and mixed compounds (14.2%). Among these, which were found with higher frequency are: methyl n-nonyl ketone, β-myrcene, houttuynin, decanal, trans-caryophyllene, decanoic acid, camphene, β-pinene, lauraldehyde, bornyl acetate, α-

pinene, limonene, 4-terpineol, caryophyllene oxide, nonanol and linalool so on. A number of flavonoids and other polyphenols have been isolated and identified from *H. cordata* (Zheng *et al*., 1998).

The quercetin is the first flavonoids extracted from the leaves and stems of *H. cordata* (Nakamura, 1936). Meng *et al*. (2007) reported that three flavonoid glycosides viz. Quercetin-3-O-β-D-galactoside-7-O-β-D-glucoside, kaempferol 3-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside], quercetin 3-O-α-L-rhamnopyranosyl-7-O-β-D-glucopyranoside and five polyphenols viz. chlorogenic acid methyl ester, 4-[(2E)-3-(β-D-glucopyranosyloxy)-2-buten-1-yl]-4-hydroxy-3,5,5-trimethyl-2 cyclohexen-1-one,2-(4-hydroxyphenyl) ethyl-β-D-glucopyranoside, 2-(3,4-dihydroxyphenyl) ethyl-β-D-glucopyranoside, 4-(β-D-glucopyrano-syloxy)-3-hydroxybenzoic acid. Nuengchamnong and Chou (2009) also revealed that catechin, procyanidin B, houttuynamide A and houttuynoside A were also isolated from *H. cordata*.

Many kinds of alkaloid have been isolated from *H. cordata*, including aporphine, pyridine and the others. C is -N-(4-Hydroxystyryl) benzamide and trans-N-(4-Hydroxystyryl) benzamide were isolated from the $CHCl_3$ extraction of this herb by a combination of HPLC and other techniques (Nishiya, 1988).

A number of common sterols have been isolated from *H. cordata*. Stigmast-4-en-3-one, 3β-hydroxystigmast-5-en7-one, 5α-stigmastane-3,6-dione and stigmast-4-ene-3,6-dione were isolated from this plant (Jong and Jean,1993).

Stigmasterol-3,6-dione, sitoindoside I and daucosterol were isolated and purified from dried rhizome of *H. cordata* by solvent extraction, silica gel and Sephadex LH-20 column chromatographs (Wang *et al*., 2007).

β-Sitosterol (Zheng *et al*., 1998; Takagi*et al*., 1978; Chou, 2005), β-sitosterol glucoside (Chou, 2005.), brassicasterol (Zheng, 1998), stigmasterol (Zheng, 1998),

spinasterol (Zheng, 1998) and stigmast-4-ene-3,6-dione (Bauer,1996) were also found in *H. cordata*.

In *L. camara*, triterpenes like lantadenes A, B, C, and D (Venkatachalam *et al*., 2011), alkaloids, flavonoids (Thamotharan *et al*., 2010), saponins, tannins (Mariyajancyrani *et al*., 2009), germacrene A, B and D are present. Ghisalberti (2000) reported the presence of valencene and γ- gurjunene as the chief compounds in *L. camara.*

Phytochemical studies carried out by different group of workers on different parts of *L. camara* have resulted in the isolation of various terpenoids, steroids and flavonoids. Camaryolic acid, methylcamaralate, camangeloyl acid, 22 betaacetoxylantic acid, lantic acid, 22 betadimethylacryloyloxylantanolic acids, lantanolic acid, β – sitosterol, 3-O- β -bglucopyranoside, octadececanoic acid, docosanoic acid, palmitic acid, camaric acid, lantanolic acid, lantanoside, linaroside and camarinic acid were isolated from *L. camara*. 22 beta-Acetoxylantic acid showed antimicrobial activity against *Staphylococcus aureus* and *Salmonella typhi*. 22 beta-Acetoxylantic acid and 22 beta-dimethylacryloyloxylantonolic acid also showed antimutagenic activity (Agarwal *et al*., 1986).

The valuable medicinal properties of different plants of *P. attenuatum*are due to the presence of several constituent i.e., saponin, tannin, alkaloid, alkenyl phenols, glycoalkaloid, flavonoid, sesquiterpenes lactones and terpenoid (Tiwar and Singh, 2004). Phytochemical studies have shown that the plant contains pipoxide and chlorohydrins which are major chemical components. (−) – galbelginand a new aliphatic alcohol; 8-hentriacontanol have also been isolated from the leaves of *P. attenuatum*. Several aristolactams have been reported from the aerial parts of the plant. Roots have been reported to contain alkamides including piperine, piper longuminine and guineensine. Piperine and piperlonguminine are the main alkaloids/amides present in *P. attenuatum*. The petroleum extract of stems and leaves of *P. attenuatum* have been reported to contain a novel long chain alcohol, 14-benzo (1, 3) dioxol-5-yl-tetradecan-2-

ol (Parmar *et al*., 1998). Recently methanol extract of dry fruits has also been reported to contain antioxidant components with promising activity (Ohlyan *et al*., 2012).

In addition, many substances such as cepharanone B, piperolactam A, piperolactam D, and cepharadione A have been isolated from *P. attenuatum*. Cepharanone and piperolactam exhibit anti-inflammatory effects (Kumar *et al*., 2013).

Potentilla lineata syn *P. fulgens* is rich in polyphenolic and triterpene constituents (Laloo *et al*., 2017; Tomczyk and Latte, 2009; Jaitak *et al*., 2010; Kumar *et al*., 2013).

Phytochemical investigation of the root parts of *P. fulgens* led to the isolation of a novel bioflavonoid potifulgene (Epiafzelchin-6-o-8´´ epiafzelchin) along with epicatechin. Two new ursane type triterpenoids, Fulgic acid A (Bhattari, 1993) and Fulgic acid B were identified and characterized (Farooqui, 1998).

Choudhary *et al.* (2017) had undertaken extensive studies to evaluate the anticariogenic effects of the plant and search for potent anticariogenic phenolic molecules. Polyphenolic compounds viz. Afzelechin, Epiafzelechin, Epigallocetechin, Epigallocetechin gallate, Epicatechin, Catechin, Aafzelechin(4β→8)epicatechin, Epiafzelechin (4β→8) epicatechin, Catechin (4α→8) epicatechin, Afzelechin(4α→8)catechin and Afzelechin (4α→8) epiafzelechin (Choudhary *et al*., 2017) were isolated from the roots of *P. fulgens* using semi-preparative HPLC. Ursolic acid and epicatechin were the major compounds in the *Potentilla* root extract.

In another study, Choudhary *et al*. (2013) isolated 2α,3α,20β-trihydroxyrus-13-en-28-oic acid, 2α,3β,20-β-trihydroxyurs-13en-28-oic, p-hydroxy benzaldehyde and gallic acid from ethyl acetate extract *of P. fulgens* and they exhibited good antioxidant activity.

Phenolic compounds, quercetin, ellagic acid and kaempferol were reported for the first time in n-butanol fraction of *P. fulgens* and act as potential antioxidative and cancer chemopreventive agents (Choudhary *et al*., 2014).

Prunella vulgaris is reported to be rich in bioactive chemicals, including polysaccharides, flavonoids, triterpenes and phenolic acids. Thus, *P. vulgaris* has many notable pharmacological activities, such as anticolitic, antioxidant, anti-inflammatory, anticancer, neuroprotective (Ru *et al*., 2017), antiestrogenic (Collins *et al*., 2009) and anti-metastatic effects (Choi *et al*., 2010).

Many bioactive constituents from *P. vulgaris* have been identified, including phenolic constituents, complex carbohydrates and hydrophobic metabolites such as triterpenes. The aqueous extract contains abundant polyphenols, rosmarinic acid and complex carbohydrates, whereas more hydrophobic metabolites, such as triterpenes and flavonoids along with some polysaccharides and polyphenols, are also found in the ethanol extract (Oh *et al.*, 2011; Brindley *et al*., 2009). Several of the triterpenes display significant anti-inflammatory activity. Rosmarinic acid has also shown to be an anti-inflammatory compound due to its specific inhibition of T cell signaling and its impact on glucose metabolism (Brindley *et al*., 2009).

The triterpenoids isolated from *P. vulgaris* are mainly oleanane, lupinane and ursane. At present, a total of 28 triterpenoids have been isolated: 20 triterpenoids (freestate), 8 saponins (binding state). The two compounds with highest content, oleanolic acid and ursolic acid, are mainly responsible for the pharmacological activity of *P. vulgaris*. Many compounds have also been isolated from methylated *P. vulgaris* extracts such as methyl oleanolate, methyl ursolate, methyl maslinate and other related compounds (Kajima and Ogura,1986; Kajima *et al*., 1987; Zhang and Yang, 1995).

Pravuloside A and Pravuloside B along with two ursane-type specific triterpenoid saponins have also been isolated from *P. vulgaris* (Zhang and Yang, 1995;

Meng *et al*., 2007). Some other compounds present in *P. vulgaris* are vulgar saponin A (Gu *et al*., 2007) and vulgar saponin B (Wang *et al*., 1999).

The main sterols present in *P. vulgaris* include: β-sitosterol (Chen *et al*., 2008), stigmasterol (Jung *et al*., 2001), α-spinolol and stigmast-7-en-3β-ol (Yue *et al*., 2012).

In addition to rutin and hyperoside, three other flavonoids have been isolated and identified from *P. vulgaris*, namely luteolin, homoorinetin and cinaroside. Besides these compounds, *P. vulgaris* also contains quercetin, quercetin-3-O-β-D - galactoside, quercetin-3-O-β-D-glucoside, kaempferol-3-O-β-D-glucoside and other related components. Alkaloids, inorganic salts, vitamins, resins, bitter taste, tannic acid, proteins and lipids are also present in *P. vulgaris* (Su-Juan Wang *et al*., 2019). The main phenolic acid component of *P. vulgaris* is rosmarinic acid (Lamaison *et al*., 1991; Psotova *et al*., 1998).

S. oleraceus contains variety of phytochemical compounds such as sesquiterpene lactones of the eudesmanolides and guaianolide structures. It also contains flavonoids, flavonols, proanthocyanidins, total phenols, saponins, and alkaloids. High concentration of fatty acids, vitamin C, carotenoids, oxalic acid, and high mineral contents is found in this plant which gave high value in as nutritional supplements (Alothman, 2018).

Oleksandr Shulhaa *et al*. (2020) isolatedtwelve natural products from the roots of *S. palustris*. Seven previously undescribed sesquiterpene lactones (including three lactucin derivatives esterified with a eudesmanic acid moiety), three known sesquiterpene lactones (ixerin D, 15-p-hydroxyphenylacetyllactucin, and 15-p-hydroxyphenylacetyllactucin-8-sulfate), and two known quinic acid derivatives (3-O-feruloylquinic acid and 3,5-di-O-caffeoylquinic acid) were isolated from *S. palustris* L. roots.

Chemophenetically, the genus *Sonchus* is in general characterized by a prevalence of eudesmanolides in its sesquiterpene lactone profiles (Zidorn, 2008 and 2019b; Shulha and Zidorn, 2019).

Antibacterial activity

Londonkar *et al*. (2011) reported that the methanol extract of *A. aspera* leaves obtained by maceration did not show any effect against *E. coli, Pseudomonas aeruginosa, K. pneumoniae, S. aureus*and *Staphylococcus epidermidis*, but the methanolic extracts of *A. aspera* leaves obtained by infusion has shown an antibacterial activity against *S. aureus*.

Alam *et al*. (2009) contributed that none of the organic solvents (namely methanol, ethanol, ethyl acetate and chloroform) leaf and stem extracts of *A. aspera* exhibited antibacterial activities against *E. coli, Bacillus subtilis, Vibrio cholerae, Salmonella typhi* and *S. aureus*. Evaluation of antibacterial activity of *A. aspera* extract against *Streptococcus mutans* was done by Yadav *et al*. (2016). They concluded that *A. aspera* showed marked antibacterial activity against *S. mutans*.

Estari and Rajendra (2013) showed that chloroform, petroleum ether, and methanol extracts of *A. paniculata* completely inhibited the growth of *Enterobactum aerogenusa*. The chloroform extract also completely exhibited anti growth activity against *Bacillus megaterium* and *P. aeruginosa*. Petroleum ether and chloroform extracts does not show activity towards *Proteus merabitus*. Pet ether, chloroform extracts does not show activity towards *K. pneumoniae*. All extracts do not show activity against *Bacillus subtilis* and *Salmonella paratyphi.*

Krishna *et al*. (2014) showed the aqueous extracts of *A. paniculata* flower and leaf showed relatively high antibacterial activity against the tested pathogens, namely *Bacillus subtilis, E. coli, Salmonella typhi, S. aureus, Vibrio cholerae* and *Vibrio parahaemolyticus* while the root exhibited comparatively low antibacterial activity. *Vibrio cholera* and *Vibrio parahaemolyticus* were the most resistant strains on *Acmella paniculata* extracts and it is suggested that these bacterial strains may possess resistant mechanism and require high concentration of compound to inactivate the bacterial activity.

Salehuddin *et al*. (2020) reported that n-hexane leaves extract, methanol leaves extract, n-hexane flowers extract, and Dichloromethane flowers extract of *A. paniculata* demonstrated bactericidal properties and exhibited anti-biofilm activities against *S. mutans*. All the antibacterial tests and anti-biofilm effects were concentration-dependent.

The antibacterial properties of methanolic extract of *Ageratina conzyoides*were studied against 11 wound isolates *S. aureus* (four strains), *E. coli* (two strains), *P. aeruginosa* (one strain), *Proteus* spp. (three strains) and *Shigella* spp. (one strain) using the well diff usion method and observed variable results (Almagboul *et al*., 1985; Chah *et al*., 2006).

The studies by Osho and Adetunji, (2011) have reported antibacterial activity of essential oils of leaf extracts of *A. conyzoides* while Mitra *et al.* (2013) has reported positive antibacterial activity of different fractions of leaf extracts of this plant.

Onuoha *et al*. (2013) have reported that methanolic extracts of *A. conyzoides* possess high bacteriacidal activity. Phadungkit *et al*. (2012) states that the antibacterial activity of *A. conyzoides* can be attributed to the presence of various bioactive constituents such as alkaloids, flavonoids, tannins, saponins and phenols.

Silva *et al*. (2014) reported that *B. pilosa* possessed antibactericidal potential against oxacillin resistant *S. aureus* (ORSA).

The results obtained from the study conducted by Rabe and Van Staden (1997) showed that the leaf extracts from *B. pilosa* inhibited Gram-positive bacteria but could not inhibit Gram-negative strains *E. coli* and *K. pneumoniae* while the whole plant extracts inhibited both Gram-positive and Gram-negative bacteria (Khan *et al.,* 2001).

Ashafa *et al.* (2009) reported that the root extracts of *B. pilosa* could inhibit both Gram-positive (*S. aureus, Staphylococcus epidermidus, Bacillus cereus, Micrococcus*

kristinae, Streptococcus faecalis) and Gram-negative bacteria (*E. coli, P. aeruginosa, Shigelia flexneri, K. pneumoniae*).

Several reports showed that the crude extraction of *C. asiatica* by 95% ethanol exhibited antimicrobial activity against *B. cereus* and *Listeria monocytogenes* under normal, osmotic stress, and low pH (Pitinidhipat and Yasurin,2012; Utami *et al*., 2012).

Mamta *et al*. (2004) tested the ethanolic *C. asiatica* extract against enteric pathogens. Lee and Vairappan (2011) studied antibacterial activities of essential oils and ethanol extracts of *C. asiatica* and concluded that ethanolic extract of *C. asiatica* showed antibacterial activity against *Salmonella enteritidis* and *Salmonella typhimurium* while essential oil of *C. asiatica* showed no inhibition at same concentration.

Samy and Ignacimuthu (2000) studied folklore medicinal plants used by tribals in Western Ghats of India. Various fraction of solvent (hexane, dichloromethane, ethylacetate, diethyl ether and methanol) extract of *C. asiatica* showed antibacterial activity against *B. subtilis*. However, methanol and diethyl ether showed higher antibacterial activity against *B. subtilis* compare to hexane, dichloromethane and ethylacetate.

Das *et al*. (2011) studied antibacterial and antifungal activities of petroleum ether, ethanol, chloroform, n-hexane and aqueous extracts of *C. asiatica* against some human pathogenic microbes. The results showed that ethanol extracts gave highest antibacterial activity against *B. subtilis* followed by petroleum ether, chloroform, n-hexane and aqueous; respectively.

Jagtap *et al*. (2009) also studied antimicrobial and antifungal activity of *C. asiatica*. They used three different solvents and concentration of petroleum ether, ethanol and water extracts. They found out that ethanolic extracts gave highest antibacterial activity against *B. subtilis* followed by petroleum ether and water, respectively.

Oyedeji and Afolayan (2005) reported that the essential oil of *C. asiatica* had a broad-spectrum antimicrobial activity and the oil was more active against the Gram-positive bacteria than the Gram-negative ones.

Fruit and syrup of *G. indica* is very popular in 'Konkan' region and has antioxidant (Mishra *et al*., 2006) and antibacterial (Negi *et al*., 2008.) activities.

Priya *et al.* (2010) study revealed the anti bacterial activity of Pericarp extract of *G. mangostana* against *S. aureus, Staphylococcus albus* and *Micrococcus luteus*. The extract from mangosteen pericarp has been known for its broad-spectrum antibacterial activity against several Gram-positive and Gram-negative bacteria, especially those associated with skin infection, diarrhea, tuberculosis or acne. Torrungruang *et al*. (2007) showed the antibacterial activity of mangosteen pericarp extract against cariogenic *Streptococcus mutans*.

Among xanthone derivatives from mangosteen extract, α-mangostin has been known to exert the most potent antimicrobial activity (Iinuma *et al*., 1996a and b).

The information and scientific report with reference to *G. fragranttissama* antibacterial activity is limited and scarce though there are reports about the antibacterial activity of its related *Garcinia* species.

Nikolic *et al*. (2013) noticed that essential oil of *G. procumbens* has shown antimicrobial activity against all the bacterial strains tested, but in variable degree. Essential oil of *G. procumbens* exhibited antibacterial activity against oral bacteria, *S. mutans*. The most sensitive bacteria to *G. procumbens* essential oil were *P. aeruginosa*, while the most resistance was *S. aureus*.

Hammer *et al.* (1999) examined antimicrobial activity of essential oils of *G. procumbens* against bacteria and fungi using agar dilution method. They reported good antibacterial activity of this oil against *E. coli, S. typhimurium, Serrati amercescens, Aeromonas sobria, Acinetobacter baumanii, P. aeruginosa and S. aureus.* The inhibition

activity of *G. procumbens* essential oil can be explained by the presence of its major component, methyl salicylate (96.90% of the total Essential Oil content). Previous reports have showed antimicrobial, antioxidant (Zhang *et al*., 2011) and anti inflammatory (Oloyede, 2011) activities of methyl salicylate. The strongest antimicrobial activity expressed by the mouthrinse is directly linked to its chemical ingredients, including methyl salicylate, the main constituent of *G. procumbens* essential oil.

The water extract of calyxs of *H. sabdarifa* and protocatechuic acid (5 mg/ml) inhibited the growth of methicillin-resistant *S. aureus, K. pneumoniae, P. aeruginosa* and *Acinetobacter baumannii* (Liu *et al*., 2005). Moreover, protocatechuic acid (in a dose dependent manner) showed greater antimicrobial activity against these pathogens in broth than in human plasma. The study also revealed that the antibacterial effect was independent from temperature, as shown by a heat treatment.

Hibiscus extract also demonstrated antibacterial effect against *S. mutans*, cariogenic bacteria from the oral cavity (Afolabi*et al*., 2008) and *Campylobacter jejuni* that contaminates meat like poultry, beef and pork (Yin and Chao, 2008).

Olaleye (2007) reported that the aqueous-methanol extract of dried calyx of *H. sabdarifa* also showed an in vitro inhibitory effect against several bacterial strains, such as *S. aureus, Bacillus stearothermophilus, Micrococcus luteus, Serratia mascences, Clostridium sporogenes, E. coli, K. pneumoniae, Bacillus cereus* and *Pseudomonas fluorescence,* but did not affect the growth of fungus *Candida albicans*.

Chao and Yin (2009 and 2008) revealed that the fresh calyx of *H. sabdarifa* water extract, ethanol extract and protocatechuic acid (20 mg/ml) was effective in inhibiting the growth of food spoilage bacteria such as *S. typhimurium DT104, E. coli O157:H7, Listeria monocytogenes, S. aureus* and *B. cereus.* They also concluded that the antibacterial effect was not affected by heat treatment and the ethanolic extract showed greater antimicrobial effect than the aqueous extract. The study further suggests

that both, ethanolic extract and protocatechuic acid, might be potent agents for use as food additives to prevent contamination from these bacteria.

A methanol-water extract of *H. sabdarifa* was effective against *E. coli* O157:H7 isolates from food, veterinary and clinical samples (Fullerton *et al*., 2011), with the highest concentration (10%) being the most effective. The crude extracts of *H. sabdarifa* seeds (200 mg/l) also showed antimicrobial effect against three types of Gram-negative bacteria. The extract exhibited higher activity against *Salmonella* followed by *Shigella* and *Enterobacter* (Nwaiwu *et al*., 2012).

Sekita *et al*. (2016) noticed that ethanol extract of *Houttuynia cordata* showed anti-bacterial effects against methicillin-resistant *S. aureus* (MRSA), and showed an anti-biofilm activity against MRSA.

Li *et al*. (2012) studied the antibacterial activity of the water extract of *H. cordata* against multi-drug resistant (MDR) *E. coli* isolates harboring the AcrA gene in order to determine its susceptibility for potential therapy. The water extract of *H. cordata* had antibacterial activity against MDR *E. coli* isolates tested with the highest and lowest zone diameters of inhibition of 29 and 13 mm at concentrations of 500 and 50 mg/ml respectively.

Liu *et al*. (2005) studied essential oil of *H. cordata* THUNB. for their antibacterial properties against *S. aureus* and *Sarcina ureae* and showed that essential oil of *H. cordata* possessed antibacterial effect. However, essential oil from different parts differed clearly in their antibacterial activities.

The essential oil of *L. camara* exposed a broad spectrum of antibacterial, antimicrobial, and antifungal activities (Siddiqui, 1995; Barre *et al*.,1997; Kumar *et al*., 2006).

Barreto *et al*. (2010) studied the antibacterial activity of the ethanolic extracts of *L. camara* leaves and roots against gram-positive and gram-negative strains standard

and multi-resistant bacteria isolated from clinical material are presented. The extracts demonstrated antibacterial activity against all tested bacteria viz., *S. aureus, Proteus vulgaris, P. aeruginosa, Víbrio cholareae*and *E. coli*.

Bhardwaj *et al*. (2015) studied the antimicrobial potentiality of *L. camara* as its leaf extracts exhibit good antimicrobial, fungicidal, insecticidal and nematicidal properties and the plant might be a novel source of antimicrobial drug. Four solvent phases viz, methanol, ethanol, acetone and aqueous were used for extraction of antimicrobial agent. The screening of antimicrobial property was done by well diffusion method. Leaves of *L. camara* showed excellent antibacterial activity in all the solvent phases used against both *E. coli* as well as *B. subtilis. L. camara* is effective against both the bacteria. The aqueous extract showed minimum ineffective antimicrobial activity against *E. coli*and *B. subtilis,* whereas acetone phase showed maximum activity against *B. subtilis* and ethanol against *E. coli* as shown in the terms of maximum zone of inhibition

P. attenuatum has antibacterial effects. Samy *et al*. (1998) reported that extracts of *P. attenuatum* has antibacterial activity against *E. coli*, *Proteus vulgaris*, *P. aeroginusa* and *Klebsiella aerogenes.*

Ohlyan *et al*. (2014) reported that methanol and ethanol extracts showed comparatively good antibacterial activity at higher doses (200 and 500 µg) against *S. aureus, E. coli* and *P. aeruginosa* but, ethyl acetate extract failed to show inhibition against any organism. The methanol extract gave hope for developing a good antibacterial compound by purifying the extract by chromatography and isolating the active constituents.

Tomczyk *et al*. (2007) reported that the aqueous extract of nine *Potentila species (P. rupestris, P. argentea, P. fruticoa, P. recta L, P. erecta, P. anserine, P. nepalensis* HOOK var Miss Willmott, *P. thuringiaca* BERNH ex LINK, *P. grandiflora* L.) show inhibitory effect on the various species of bacteria.

Radojevic *et al*. (2018) investigated the antimicrobial activities of methanol and ethyl acetate extracts from the whole plant of *Potentilla visianii* by microdilution method against *Bacillus cereus, Bacillus subtilis, S. aureus, P. aeruginosa, Proteus mirabilis, E. coli* and *Salmonella enterica*. Both the extract was shown to be antibacterial against the test bacteria.

Mahboubi *et al*. (2008) study the antimicrobial activities of methanol, aqueous, ethanol extracts of *P. vulgaris*. The best activity of aqueous extract was against *S. aureus, S. sobrinus and S. salivarius*. The best antimicrobial activity of ethanol extract was against *S. aureus, S. mutans, B. cereus* followed by *S. epidermidis, S. pneumoniae, S. pyogenes, S. sanguis, S. salivarius, P. aeruginosa, B. subtilis, C. albicans* and *S. marscenes*. The methanol extract of *P. vulgaris* exhibited the bactericidal activity against *St. mutans, S. aureus, S. epidermidis, S. sobrinus, S. sanguis, S. salivarius, S. dysenteriae, S. flexeneri, P. aeruginosa, E. coli, B. subtilis, B. cereus, C. albicans* and *C. glabrata*.

Psoyova *et al*. (2003) carried out screening of the antimicrobial activity of *P. vulgaris* and observed that *St. agalactiae, St. oralis, St. intermedius and St. gordonii* were inhibited by the organic extracts of *P. vulgaris*.

Kamal *et al*. (2014) investigates the antimicrobial activity of aqueous and ethanolic extracts of *P. vulgaris* against *E. coli* from urinary tract infection patients. Aqueous and ethanolic extract alone and in combination have positive effect against multi-drug resistant *E. coli* strain isolated from patients with urinary tract infection.

Rosmarinic acid or α-O-caffeoyl-3-4-dihydroxyphenyllactic acid is the multifunctional caffeic acid ester with antimicrobial activity against *Bacillus cereus, B. subtilis, B. polymyxa* and some Gram-negative bacteria but this antimicrobial activity is not related to phenolic compounds such as rosmarinic acid (Askun *et al*., 2009).

Xiu *et al*. (2017) compared the antibacterial activities of the extracts of *Sonchus species (S. oleraceus, S. arvensis, S. asper, S. uliginosus, S. brachyotus,* and *S.*

lingianus) in vitro by using the disc-diffusion method. According to their results, most of the *Sonchus* species extracts inhibited both Gram-negative and Gram-positive strains, which frequently cause food spoilage. The methanol extract of *S. oleraceus* showed the highest antibacterial activity of the tested *Sonchus* species extracts. The antibacterial activity of the methanol extract of *S. oleraceus* against *E. coli* and *S. enterica* at a dose of 0.5 mg of extract per disc was comparable to that of a positive control (ofloxacin, 20 mg per disc).

Another study led by Dao-Zong Xiaa *etal*. (2011) also confirmed the antibacterial effect of *S. oleraceus* against *S. aureus, S. typhi, Shigella dysenteriae,* andhaemolytic *Streptococcus. S. oleraceus* showed the most potent activity against *S. enterica* and *Vibrio parahaemolyticus*. It showed the strongest activity against *S. aureus* at a minimal inhibitory concentration of 0.02 mg mL−1. Furthermore, *S. aureus* was more susceptible than *E. coli* and *S. enterica* to *Sonchus* species extracts. This difference in antibacterial activities is probably related to the repulsion between the flavonoid (or phenolic) compounds and the surfaces of Gram-negative bacteria, which are covered with lipopolysaccharide. Therefore, the presence of flavonoids or phenolics in the *Sonchus* species extracts might play a role in the antibacterial activity. The flavonoid content of the tested *Sonchus* species extracts was similar. Thus, *Sonchus* species extracts could be considered good candidates as raw materials in antibacterial phyto-preparations.

Xiu *et al*. (2017) shows that ethanol extract from *S. brachyotus* DC. exhibited antimicrobial activity against *E. coli, Enterobacter cloacae, K. pneumoniae, Salmonella enterica, S. aureus,* and *Micrococcus luteus*; this is especially so in the case of *E. coli*. This study investigated the novel antibacterial mechanism of ethanol extract from *S. brachyotus* that shows an apoptosis-like response in *E. coli*.

Very limited information is available on the antibacterial activity of *S. palustris*. Osborn (1943) reported that *S. palustris* showed a slight antibacterial activity against *S. aureus* while *E. coli* are not susceptible to it.

Chapter 3

Sample Collection and Identification of selected Medicinal Plants

3.1 Introduction

Plant specimens were collected for the preparation of crude extract and voucher specimens. Research in any discipline of plant science demands proper collection and identification of the raw material i.e. species as its pre – requisite. This is particularly so, in case of research in medicinal plants, which are then used in the manufacture of life saving medicines. The material has to be identified initially and the improved taxonomic interpretation may follow subsequently. Wrong identification can lead to useless research and a disaster to one's health.

A collection of dried plant specimens is called a herbarium. Such collection are essential for future reference and systematic research. Herbarium specimens form the basis for most of our understanding of the patterns of variation in nature. They document the morphological variability of populations, species, and higher taxa, their geographic distributions, and their ecological characteristics, including blooming and fruiting times. In addition, small portions of a specimen can be removed (with permission) to study palynology, ultrastructure, micromorphology, anatomy, and (if the specimen is of sufficient quality) nucleotide sequences (Judd *et al.,* 2008).

Dried plant specimens also serve as scientific vouchers to document the presence of a species at a particular locality (in an environmental or floristic study) or the identity of a plant used in an experiment or from which a chromosome count, DNA sample, or chemical extract was obtained. Therefore, it is important to outline the steps involved in collecting, preserving, and identifying plants.

Plant collector must record locality, date of collection, and any other information concerning the plant that will not be evident in the pressed and dried specimen. The collector's name, along with the names of any individual accompanying the collector, should be included with the field data (Kutal *et al.,* 2021).

Plants may be identified after they have been collected, pressed, and dried, but it is easier to identify fresh or liquid – preserved material. Manual identification of medicinal plants is a time consuming process and need the help of experts for plants identification. Any vegetation survey should include, where possible, collection of at least one specimen of each species encountered. This is known as the voucher collection and should either be kept by the worker for future reference if needed, or stored in a local herbarium (Jane, 2004).

3.2 Study Site

Meghalaya, popularly known as the abode of clouds, is situated in the north eastern region of India as a narrow stretch of land, running between Bangladesh on the South and West and Assam on the North and East. The State of Meghalaya is located between 24˚58"N to 26˚07"N latitudes and 89˚48"E to 92˚51"E longitudes extending over an area of 22,429 sq km. Its recorded forest area is 17,217 sq km which accounts to about 76.76% of the state's geographical area (FSI, 2015). The state experiences a monsoonal type of climate and is endowed with rich vegetation which varies greatly according to the altitude of different areas as classified into agro-ecological regions. The richness and variety of vegetation ranging from sub–tropical to tropical is due to diverse topography and variation in rainfall, soil and temperature. Meghalaya is known for existence for the existence of large variety of plant species, many of which have medicinal properties (Haridasan and Rao, 1985 and 1987).

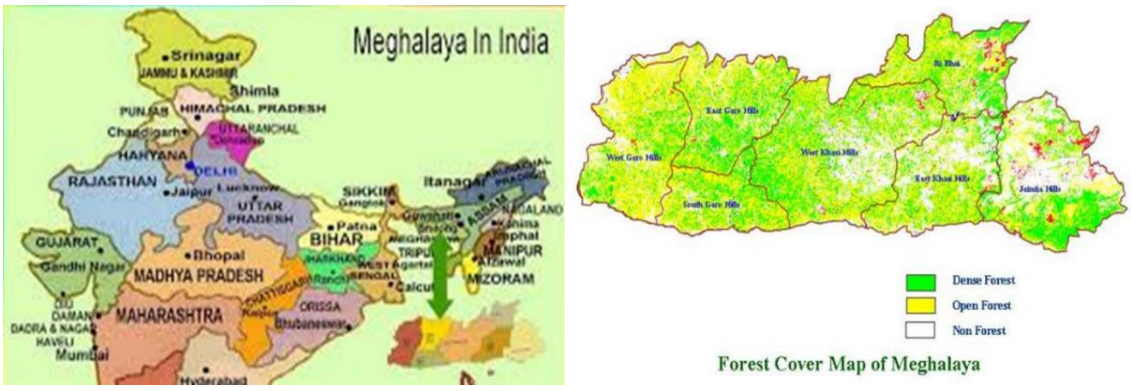


Figure 1: East Khasi Hills

The state is predominantly inhabited by tribal people who account for 89% of the total population of 23,06,069. The two main communities found in the state are: (i) the Hynniewtrep people consisting of the four ethnic groups Khasi, Jaintia, Bhoi and War, and inhabiting the eastern region of the states covering the six districts of East Khasi Hills, West Khasi Hills, South West Khasi Hills, RiBhoi, West Jaintia Hills, and East Jaintia Hills (ii) Garo community inhabits the five districts of West Garo Hills, East Garo Hills, South Garo Hills, North Garo Hills and South West Garo Hills.

East Khasi Hills district forms a central part of Meghalaya and covers a total geographical area of 2,748 km^2. It lies approximately between 25°07" & 25°41" N Lat. And 91°21" & 92°09" E Long. It has a population of 825,922 (as of 2011). As of 2011, it is the most populous district of Meghalaya's seven districts (Census of India, 2011).

The East Khasi Hills District is mostly hilly with deep gorges and ravines on the southern portion. The most important physiographic features of the district is Shillong Plateau interspersed with river valley, then fall sharply in the southern portion forming deep gorges and ravine in Mawsynram and Shella-Bholaganj bordering Bangladesh. The north of the district is bounded by the plain of Ri-Bhoi District gradually rising to the rolling grasslands of the Shillong Plateau interspersed with river valleys, then falls sharply in the Southern portion forming a deep gorges and ravines in Mawsynram and Shella-Bholaganj community and rural development block, bordering Bangladesh. The district is bounded by the Jaintia Hills District to the east and the West Khasi Hills District to the west.

The climate of the district ranges from temperate in the plateau region to the warmer tropical and sub-tropical pockets on the Northern and Southern regions. The whole of the district is influenced by the south-west monsoon which begins generally from May and continues till September. The weather is humid for the major portion of the year except for the relatively dry spell usually between December and March.

Table 1: Annual Climate Data of East Khasi Hills District, Meghalaya

Months	Average High (ºC)	Average low (ºC)	Average rainfall (mm)	Average rainy days
January	11	1	1	1
February	12	2	3	2
March	22	12	109	5
April	24	15	312	11
May	24	16	607	15
June	24	18	1111	18
July	24	19	1159	19
August	25	18	741	16
September	24	18	552	13
October	17	09	242	06
November	09	00	25	02
December	06	-03	15	01

3.3 Materials and Methods

3.3.1 Collection, identification and documentation of selected medicinal plants

Prior to the field work, all relevant literature of ethno-botanical researches were surveyed and studied, along with interview and consultation of the traditional medicine practitioners and ethnomedicine knowledgeable individuals, to identify a set of 15 plant species having ethnomedicinal value. For the selection of the plant species to study, randomized approach suggested by Albuquerque and Hanazaki (2006) was followed.

In this context, various localities of East Khasi Hills (EKH) were visited covering almost all the seasons of the year for the collection of plants, plant parts and informations regarding their ethnic and local uses. Extensive and intensive field work of different localities, especially the far off areas inhabited by backward people and rural areas were arranged in various phases so as to gather detail information on each and every species found useful in ethnobotanical studies.

For collecting information on ethnomedicinal aspects, the approaches and methodologies suggested by Jain (1989) was followed while for plant collection and herbarium preparation, routine methods suggested by Jain and Mudgal (1999) was followed.

Critical morphological studies were undertaken on the collected plant specimen using various literatures such as Forest Flora of Meghalaya, Vol I & II (Haridasan and Rao, 1985 and 1987), Flora of British India, Vol I – VII (Hooker 1872-1897), Flora of India (Sharma *et al.*, 1993), Check-list of Flora of Meghalaya (Mao *et al*., 2016).

All the selected potential ethno-medicinal plant were identified and authenticated at the Botanical Survey of India, Eastern Region, Shillong, Meghalaya and voucher specimens (herbarium) were deposited at the Assam Herbarium, BSI, Shillong, Meghalaya.

3.3 Results

The medicinal plants selected and documented during the present work are listed below in alphabetical order by botanical names, followed by family name, collection number, Assam Herbarium Accession Number, local/vernacular name, place of collection, brief description and use(s).

(i) ***Achyranthes aspera*** **L.** (Amaranthaceae)
Collection No.15
Date of collection: 5/12/2020
Assam Herbarium Accession No.096684
Local Name: Soh byrthit (Khasi)
Place of collection: Rynjah, EKH
Description: Annual herb that grows throughout India. (Anonymous CSIR, 2005) *A. aspera* L. is an erect or procumbent, annual or perennial herb of about 1- 2 meter in height, often with a woody base. Stems angular, ribbed, simple or branched from the base, often with tinged purple colour, branches terete or absolutely quadrangular, striate,

pubescent, leaves thick, ovate – elliptic or obovate – rounded, finely and softly pubescent on both sides, entire, petiolate, flowers greenish white, numerous in axillary or terminal spikes up to 75 cm long, seeds sub-cylindric, truncate at the apex, rounded at the base, reddish brown (Zafar *et al*., 2009).

Parts used: Leaves

Uses: Piles, Diuretic, Boils, Abscess, Painfull delivery, Antifertility, Rabies, Antidiabetic, Pneumonia, Menstrual disorders, Insect stings and Snakebite (Bidyasagar *et al*., 2017).

(ii) ***Acmella paniculata*** **(Wall.exDC.) R.K. Jansen** (Asteraceae)

Collection No.09

Date of collection: 15/4/2020

Assam Herbarium Accession No.096572

Local Name: Jasat (Khasi)

Place of collection: Mawlai, EKH

Description: Annual herbs, rooting at basal nodes, leaves opposite, ovate, heads numerous panicled, florets yellow.

Parts used: Flowers

Uses: Brushing teeth with inflorescence relieves toothache (Hynniewta, 2008); Crushed inflorescence is put into aching teeth to get relief from toothache (Meghalaya Biodiversity Board).

(iii) ***Ageratum conyzoides*** **(L) L.** (Asteraceae)

Collection No. 02

Date of collection: 14/4/2019

Assam Herbarium Accession No.096565

Local Name: Kynbat myngai (Khasi)

Place of collection: Mawlai, EKH

Description: An annual erect herb, leaves opposite, heads small in dense terminal corymbs, ray florets pale blue in colour.

Parts used: Whole plant

Uses: Leaf paste for cuts and injuries (Joseph and Kharkongor, 1981); Leaf paste is applied on cuts and wounds (antiseptic). Whole plant together with fruits of *Piper peepuloides* and rhizome of *Acorus calamus* L. are ground and the content is taken orally for treatment of cancer and ulcer. (Hynniewta, 2008)Paste of leaves and lime is applied to cuts acting as homeostatic (Kayang *et al*., 2005).

(iv) ***Bidens pilosa* L.** (Asteraceae)

Collection No. 03

Date of collection: 5/6/2019

Assam Herbarium Accession No.096566

Local Name: Soh byrthit (Khasi); Place of collection: Mawlai, EKH

Description: Branched herbs, leaves opposite, temate; leaflets ovate, heads in terminal panicles, ray florets white, disc florets yellowish, apical awn on achenes.

Parts used: Leaves

Uses: Leafs of *Bidens pilosa* along with leaf of *Drymaria diandra* are usedas antidote for snakebite (Kharkongor and Joseph, 1981). Leaves are grind and the juice is taken against gastric disorders (Hynniewta, 2010).

(v) ***Centella asiatica* (L.) Urb.** (Apiaceae)

Collection No. 04

Date of collection: 21/8/2019

Assam Herbarium Accession No.096567

Local Name: Khliang syiar (Khasi)

Place of collection: Mawkasiang, EKH

Description: Perennial herbs, stems creeping, rooting at nodes, leaves in rosettes, reniform, petiole long, imibels solitary or 2-5 together in axils of long bracts, flowers pink.

Parts used: Whole plant

Uses reported in literature: Leaves are boiled with small ginger and black pepper and the syrup taken for cough (Joseph and Kharkongor, 1981). Leaf is used for diarrhoea, headache, bodyache and wounds (Kumar *et al.*, 1987). Leaf is used for headache (Rao and Neogi, 1980). Juice of whole plant is used in eye infection, dysentery and stomachache (Baruah *et al*., 2006). Leaves are taken as raw to cure blood deficiency and helps in purification of blood. Whole plant is also taken for blood dysentery. Leaves are chewed and the paste is applied for wounds and injuries. Whole plant paste is taken for blood dysentery, diarrhoea, as brain tonic, heart tonic and applied locally on boils, mouth sores, and tumours (Hynniewta, 2008); Whole plant is ground and the juice is squeezed out of it and is used to get relieve from both diarrhea and dysentery (Laloo, 2011).

(vi) ***Garcinia pedunculata*** **Roxb. ex Buch.-Ham.** (Clusiaceae)

Collection No. 13

Date of collection: 7/12/2020

Assam Herbarium Accession No.096682

Local Name: U Sohdanei (Khasi)

Place of collection: Rongmesek, Ri– Bhoi

Description: They are evergreen polygamous trees with fluted trunk short spreading branches. They bear medium-sized, green coloured fruit that turns yellowish once ripe. Mature fruits range from 8-12 cm in diameter with a fleshy aril which encloses 4-8 seeds. About 35 species are reported to exist in India, many of which are endemic and economically important, with immense medicinal properties (Roberts, 1984).

Part Used: Fruit

Uses: The fruit is finely powdered after sun dried and used for dysentery (Laloo, 2011).

(vii) ***Gaultheria fragrantissima* Wall.** (Ericaceae)

Collection No. 11

Date of collection: 17/12/2018

Assam Herbarium Accession No.096574

Local Name: Jirhap/Lathynrait (Khasi)

Place of collection: Umtyngar, EKH

Description: Bushy shrubs, branches more or less trigonous, leaves lanceolate to ovate, shining, punctuate beneath, racemes axillary and terminal, flowers creamy white.

Parts used: Leaves and stem bark.

Uses: Paste made from leaves is applied to bone fractures and sprains (Kayang *et al*., 2005); leaf juice of *Gaultheria fragrantissima* Wall., *Clerodendron colebrookianum* Walp. and *Eucalyptus diarrhoea* Hook. is massaged over the body of persons suffering from rheumatism, arthritis and paralysis. In case of migraines and pneumonia the juice is applied over the forehead (Hynniewta, 2008); powdered leaf mixed with water is taken orally to treatdiarrhoae (Laloo, 2011).

(viii) ***Hibiscus sabdariffa* L.** (Malvaceae)

Collection No. 16

Date of collection: 21/11/2020

Assam Herbarium Accession No.096685

Local Name: Jajew (Khasi)

Place of collection: Madanrting/Nongrim, EKH

Description: Erect shrubs, stems red, leaves palmately lobed, flowers pale yellow with a bright purple centre, calyx blood red, enlarged and thick fleshy in fruits.

Uses: Leaf paste is applied for boils (Bidyasagar *et al*., 2017); Fleshy calyx and leaves cooked as vegetable (Hynniewta, 2010).

(ix) ***Houttuynia cordata*** **Thunb.** (Saururaceae)

Collection No. 05

Date of collection: 4/9/2018

Assam Herbarium Accession No.096568

Local Name: Jamyrdoh (Khasi)

Place of collection: Smit, EKH

Description: Perennial herbs, rootstock creeping, leaves cordate, spikes terminal with peduncle, presence of involucres, of 4-6 white petaloid bracts.

Parts used: Whole plant

Uses: Leaves eaten raw for blood purification and also applied to treat sores and boils (Rao, 1981a); Leaf juice is taken for cholera, dysentery, curing of blood deficiency and purification of blood (Hynniewta, 2008); Roots and leaves are eaten raw to treat amoebic dysentery (Laloo, 2011); Stomachache, Cholera, Dysentery and Diuretic (Bidyasagar *et al*., 2017).

(x) ***Lantana camara*** **L.** (Verbenaceae)

Collection No. 06.

Date of collection: 13/12/2018

Assam Herbarium Accession No.096569

Local Name: Sohpang khlieh. (Khasi)

Place of collection: Mawlai, EKH.

Description: Prickly shrubs, leaves ovate, spikes globose, flowers orange to pink, fruits shining, purplish blue when ripe.

Parts used: Flowers.

Uses: Leaf is used in skin diseases and to stop bleeding (Baruah *et al.*, 2006). Used as remedy for whooping cough, sprain, chronic inflammable skin and treatment for fever and jaundice (Meghalaya Biodiversity Board).

(xi) ***Piper attenuatum*Buch.-Ham. ex Miq.** (Piperaceae)

Collection No.12

Date of collection: 7/12/2020

Assam Herbarium Accession No.096681

Local Name: Sohmarit (Khasi)

Place of collection: Nartap, Ri-Bhoi

Description: *Piper attenuatum*, also known as oval leave pepper, is a plant in the Piperaceae family that inhabits the eastern tropical Himalayas, Assam, Khasi Hills, and the Nilgiris in India (Khare, 2008). It is a substitute for black pepper (*P. nigrum*) (Kokate *et al*., 2008). They are climbers. Stem is obviously ridged and furrowed. Petiole is shortest on leaves toward apex of stem, leaf blade is ovate-orbicular or ovate, membranous, glandular. Flowers are monoecious. Fruit is black, ovoid to globose. Flowering season is Oct-Dec (Flora of China, 1994).

Part Used: Fruit

Uses: Anti-Blennorliagic, Stomachic, Dyspepsia, Malaria, Haemorrhoids, Delirium, Tremors and Migraine (Bidyasagar *et al*., 2017).

(xii) ***Potentilla lineata* Trevir**. (Rosaceae)

Collection No. 10

Date of collection: 15/11/2018

Assam Herbarium Accession No.096573

Local Name: Lynniang (Khasi)

Place of collection: Umtyngar, EKH

Description: Perennial silky herbs, rootstock very stout, leaflets clothed with silvery hairs, flowers panicled or corymbose.

Parts used: Roots

Uses: The root stocks are believed to strengthen the gums and teeth and also reported to be used in diarrhea. Slices of the rootstock are chewed with betelnut, lime and betel leaf

locally. It has been reported to be antidiabetic (Kayang *et al.*, 2005); roots are edible and effective against high blood pressure (Hynniewta, 2008).

(xiii) ***Prunella vulgaris* L.** (Lamiaceae)
Collection No. 07
Date of collection: 2/10/2018
Assam Herbarium Accession No.096570
Local Name: Jahynwet (Khasi)
Place of collection: Rynjah, EKH
Description: Perennial herbs, stem quadrangular, erect, leaves petioled ovate, whorls in dense heads, flowers lipped, purple.
Part Used: Leaves
Uses: Tender leaf paste is applied on cuts and wounds for quick healing (Hynniewta, 2008).

(xiv) ***Sonchus oleraceus* (L.) L.** (Asteraceae)
Collection No.08
Date of collection: 23/11/2018
Assam Herbarium Accession No.096571
Local Name: Jhurkthang. (Khasi)
Place of collection: Rynjah, EKH
Description: Erect laticiferous herbs with hollow stems, sagittate leaves and yellow heads.
Part Used: Leaves
Uses: Jaundice, Diuretic, Diaphoretic, Antiseptic, Coughs, Phthisis, Bronchitis, Asthma, Pertusis and Demulcent (Bidyasagar *et al.*, 2017). Leaves are eaten raw as salad (Hynniewta, 2010).

(xv) ***Sonchus palustris* L.** (Asteraceae)

Collection No.14

Date of collection: 17/10/2020

Assam Herbarium Accession No.096683

Local Name: Jalynniar (Khasi)

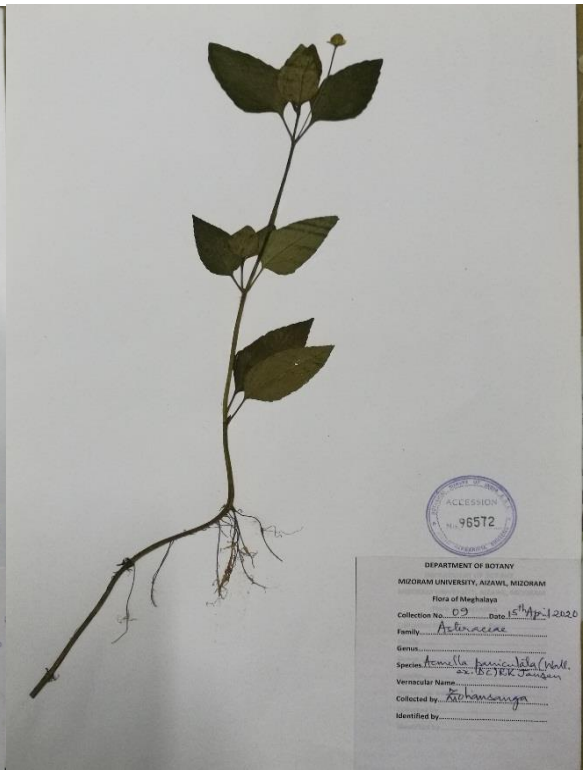
Place of collection: Pokseh, EKH

Description: Herbs, perennial, rhizomatous, leaves radical, cauline, glabrous, entire or spinous toothed, rounded or acute at apex, heads cylindric, involucral bracts many seriate, ligules yellow achenes dark yellowish.

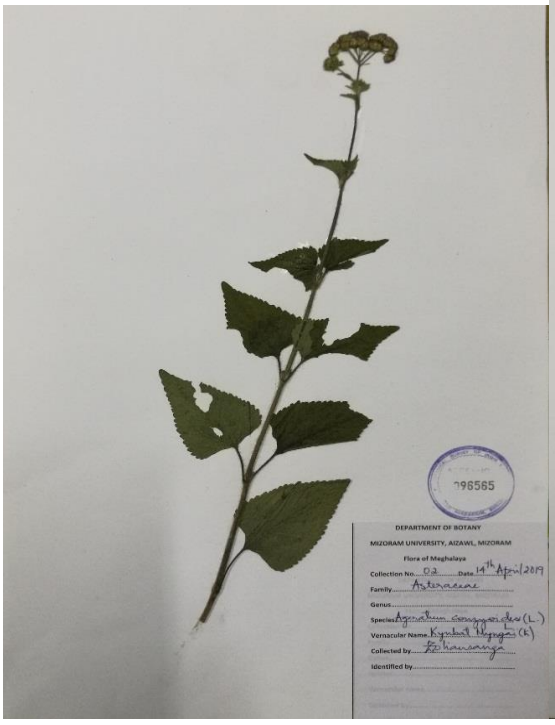
Parts used: Whole plant

Uses: Mainly eaten as vegetable leaves but are claim to be useful for treatment of high blood pressure and diabetis.Young leaves taken raw to stop white discharge in women. Whole plant taken raw to cure anaemia (Hynniewta, 2010).

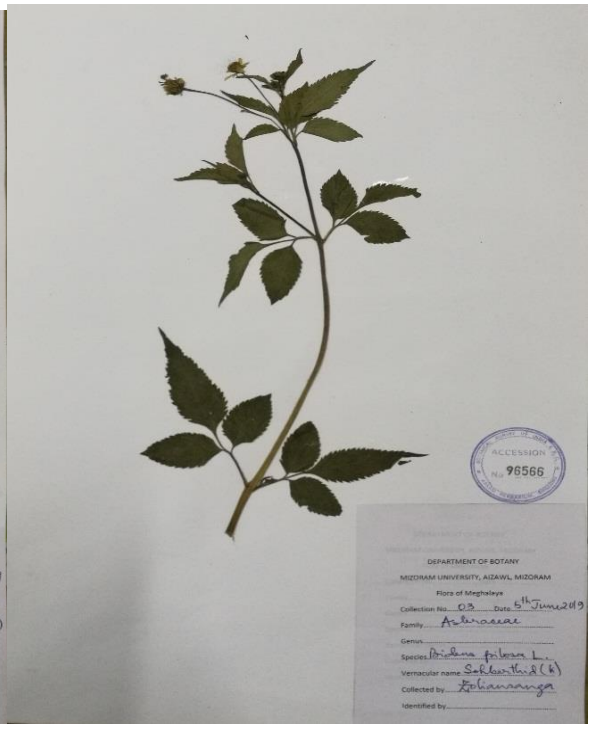
(i) Achyranthes aspera L.



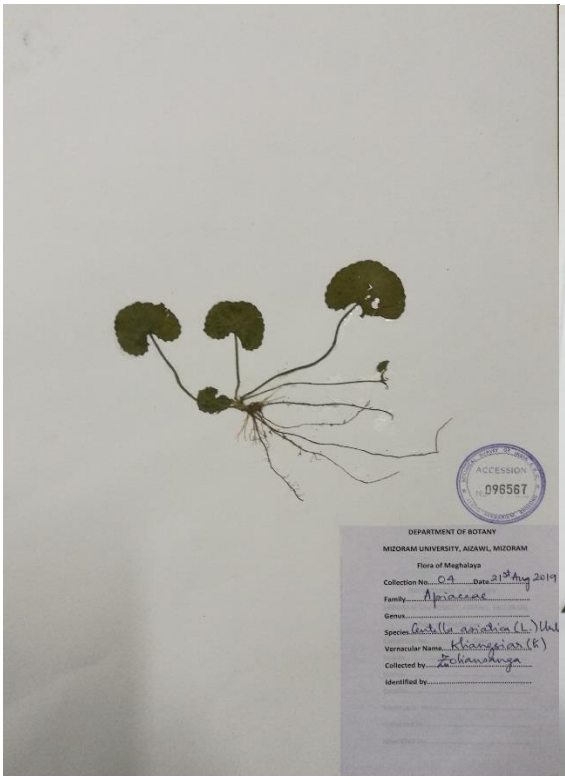
(ii) Acmella paniculata (Wall ex. DC) R.K. Jansen



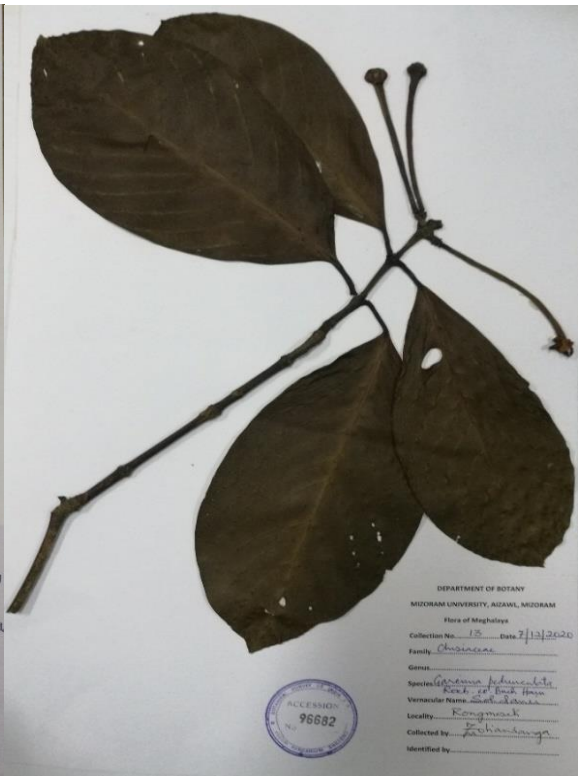
(iii) Ageratum conyzoides (L.) L.



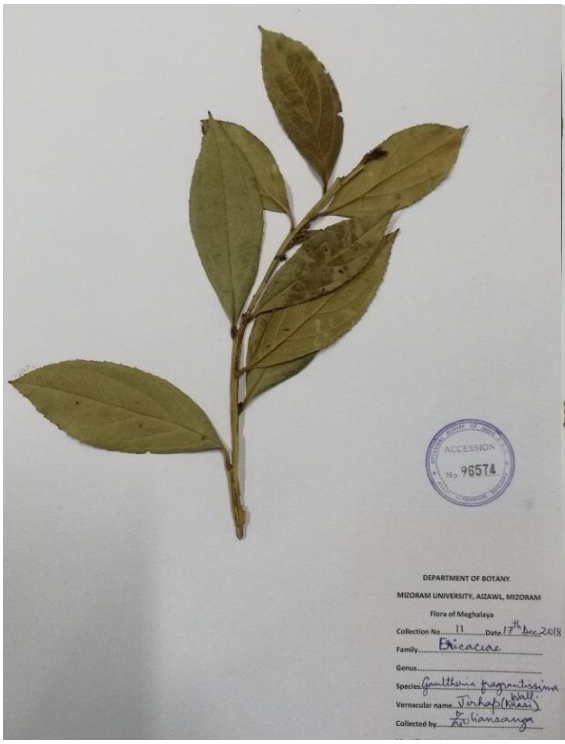
(iv) Bidens pilosa L.



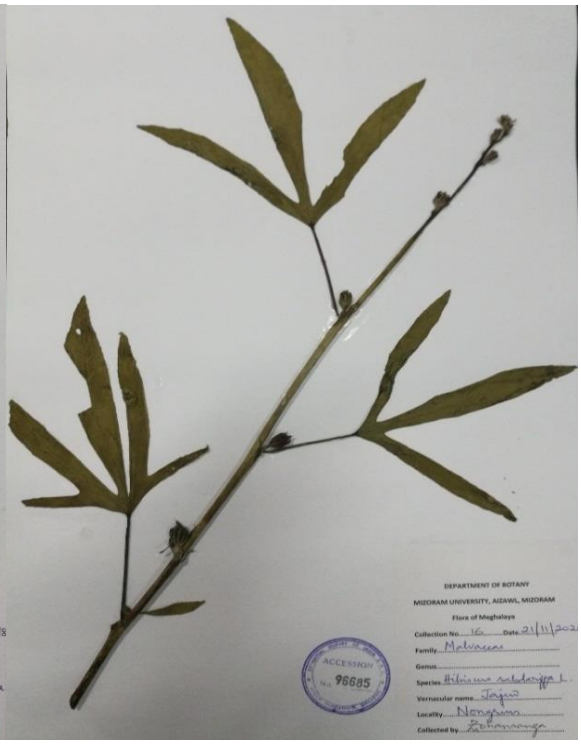
(v) Centella asiatica (L.) Urb.



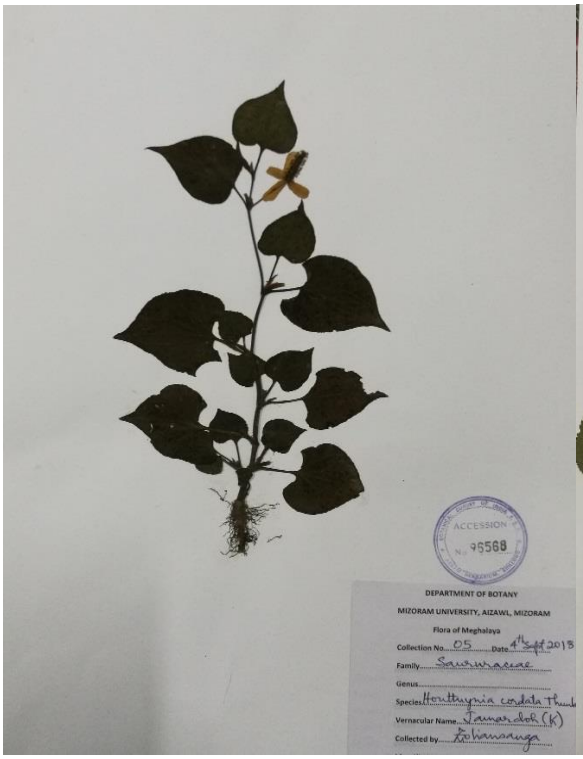
(vi) Garcinia pedunculata Roxb. Ex. Buch.-Ham



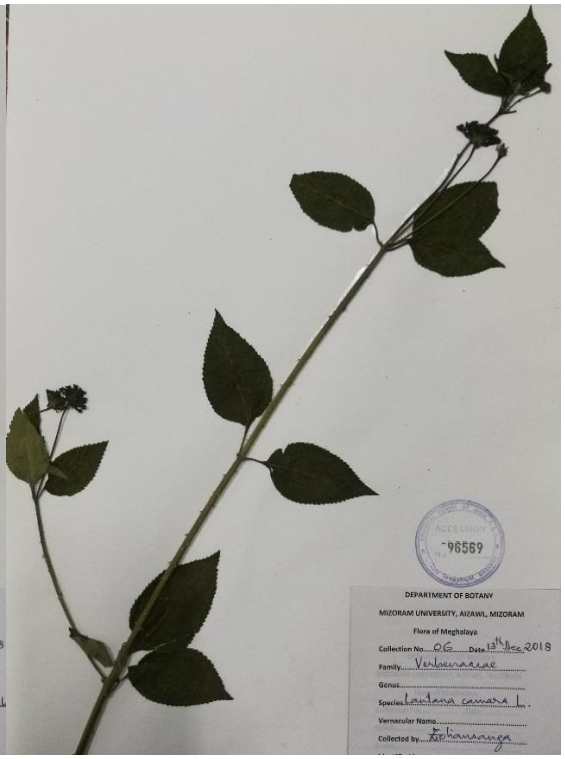
(vii) Gaultheria fragrantissima Wall.



(viii) Hibiscus sabdarifa L.

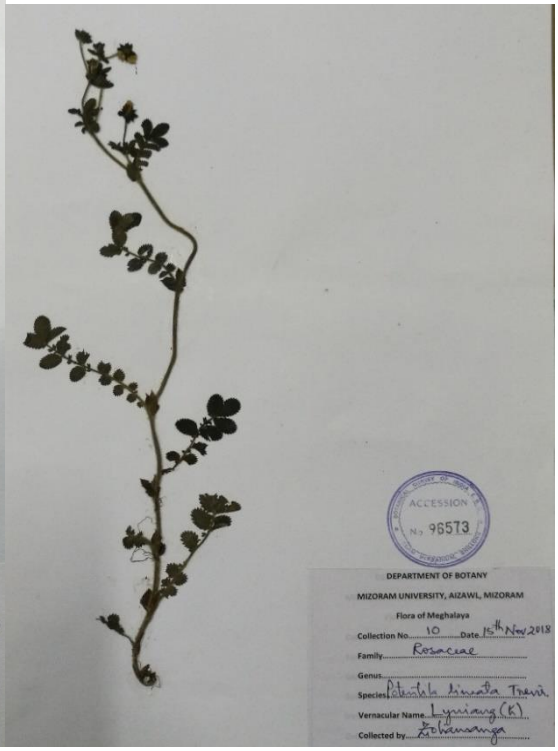


(ix) Houttuynia cordata Thunb.

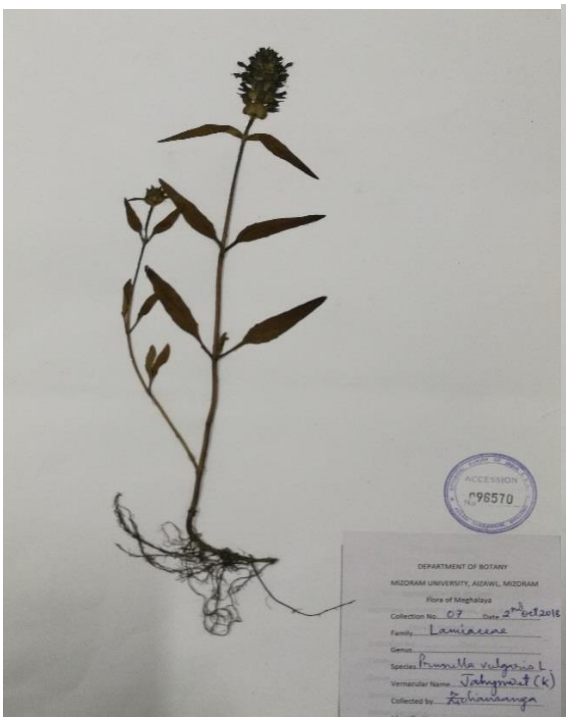


*(x) Lantana camara*L.

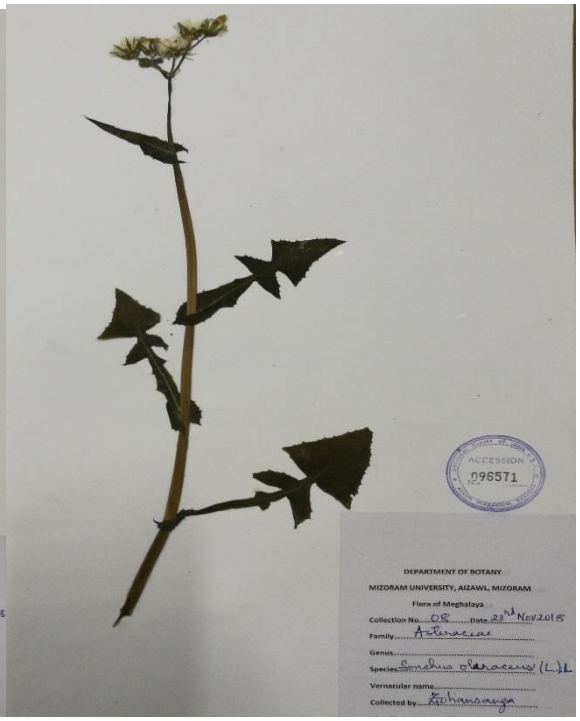
*(xi) Piper attenuatum*Buch.-Ham.ex Miq.



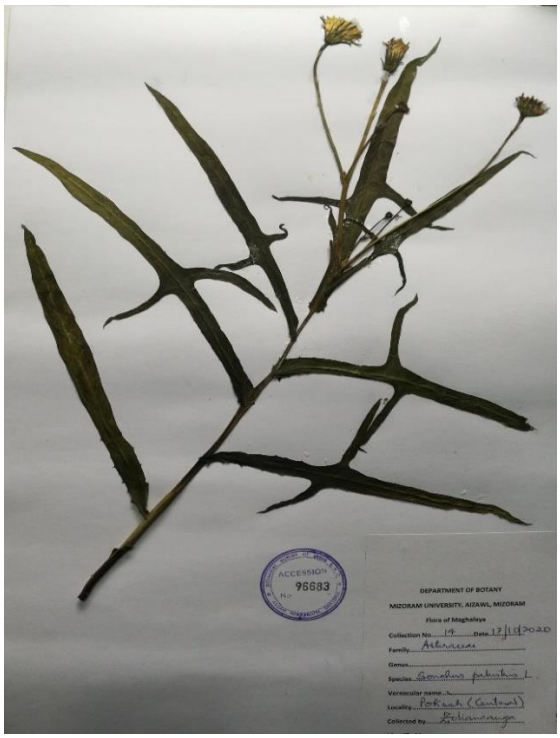
(xii) Potentilla lineata Trevir.



(xiii) Prunella vulgaris L.



(xiv) Sonchus oleraceus (L.) L.



(xv) Sonchus palustris L.

Plates 1(i-xv): Herbarium of slected medicinal plants

3.5 Discussion

As per the report of the State Level Planning Committee, the state of Meghalaya has 850 species of medicinal plants, 377 of which are used by 70-88% of the state's population for primary health care needs. Hynniewta (2010) recorded 172 species of medicinal plants from the three districts of Meghalaya i.e. East Khasi Hills, West Khasi Hills and Ri-Bhoi.

In the present study, 15 medicinal plants were selected for antibacterial activity and phytochemical analysis. Out of the 15 plants selected, 5 plants belong to the family of Asteraceae, one plant each belong to the family of Amaranthaceae, Apiaceae, Clusiaceae, Ericaceae, Lamiaceae, Malvaceae, Piperaceae, Rosaceae, Sauraceae and Verbenaceae.

In the investigation of medicinal plants, a relevant moment, which can set the course of the work and its impact on all points of view, was the criterion used for the selection of the plant species to study. In the present study, random approach was followed in the selection of 15 medicinal plants. The randomized investigations involves random selection and collection of plant species for study, according to the plant availability. When carried out in regions with high diversity and endemism, the probability of finding novel substances, bioactive or not, is certainly higher in this type of selection (Maciel *et al*., 2002, Oliveira *et al*., 2011). It is an indispensable approach, once it can demonstrate the potential of different plant species that had never been investigated. According to Souza Brito (1996), this type of selection provides an endless source of new structures, since nature is a vast chemical laboratory.

Several important authors recognize the randomized approach as an approach without criteria. Calderon (2000) and others who worked with forest plots, do not identify this form of selecting plants for research, as random. However, there are many

mistaken views and criticisms about this approach due to its randomness, which does not mean the absence of criteria (Albuquerque and Hanazaki, 2006).

The specimens collected were as complete as possible and healthy. They are collected from its typical habitat. Flowers and fruit were included, as well as vegetative parts wherever possible. Clearly, in most cases, this is impossible since ripe fruit and flowers do not usually occur at the same time.

Chapter 4

Screening of Antibacterial Activity of Selected Medicinal Plants

4.1 Introduction

The use of medicinal plants for the treatment, prevention and cure of diseases accompanies man since the earliest civilizations (Firmo*et al*., 2011). The presence of significant secondary metabolites and the identification of biologically active compounds (Duarte *et al.*,2004; Calixio,2005), makes the herbal alternative often effective for developing new therapeutic strategies for the treatment of infectious diseases (Ming,1998; Vargas *et al.*,2004; Calixto,2005). Between 1981 and 2002, of the 877 new molecules introduced into the pharmaceutical market, 49% were substances isolated from natural products (Newman *et al.*, 2003). In 2010, herbal medicines accounted for approximately 15% of the capital of the world pharmaceutical industry (Niero,2010).

Herbal drugs made from medicinal plants have been used from ancient times to treat various diseases and their antimicrobial properties make them a rich source of many potent drugs (Srivastava *et al*., 2005). The use of herbal medicinal plants has always played a positive role in the control or prevention of diseases such as diabetes, heart disorders and various cancers (Mohanta, 2003). Some medicinal plants have been used in the production of various drugs singly or in combination and even as principal raw material for the production of other conventional medicines (Tahir *et al.*,2012).

The study of contemporary medicine has yielded promising and commendable results on the antibacterial activity of medicinal plant extracts as potential drugs that can be added along with the contemporary drugs (Coopoosamy*et al*.,2007).

Plant extracts have been developed and proposed for the elimination of pathogenic microorganisms, because of the resistance that microorganisms have built against antibiotics (Saraswati,2011). In the last few decades, many bacterial organisms have continued to show increasing resistance against current antimicrobial agents

(Nascimento *et al.*,2000). The long-term use of antimicrobials has led to the selection of mutant pathogenic microorganisms that are resistant to medications (Vargas *et al*.,2004.), generating a serious public health problem (Duarte *et al*., 2004).

Antibiotics have saved the lives of millions of people and have contributed to the major gains in life expectancy over the last century. However, the clinical efficacy of many existing antibiotics is being threatened by the emergence of multi-drug resistant (MDR) pathogens (Bandow *et al.*, 2003). There is recent appearance of strains with reduced susceptibility as well as, undesirable side effects of certain antibiotics (Cunha,2001). Infectious diseases caused by resistant microorganisms are associated with prolonged hospitalizations, increased cost, and greater risk for morbidity and mortality. Resistance is an especially vexing problem for people with impaired immune systems, such as AIDS, cancer patients and recipients of organ transplants. The promiscuous use of antibiotics accounts for a major part of the community burden of antibiotic use and contributes dramatically to the rising prevalence of resistance among major human pathogens. Vancomycin-resistant *Enterococci* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), MDR *Mycobacterium tuberculosis* and MDR Gram-negative bacteria are recognised as the most difficult healthcare-associated infections to control and treat. The development of extended-spectrum β-lactamases (ESBLs) and carbapenemases that target Gram-negative bacteria has resulted in infections that can be extremely difficult to treat leading to substantial increased illnesses and death rate. The effect is pronounced in third world as the costly replacement drugs for treating the highly resistant infectious diseases are unaffordable.

The resistance problem demands that a renewed effort be made to screen various medicinal plants for their potential antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins, phenolic compounds, steroids, resins, fatty acids and gums which are capable of producing definite physiological action on body. Another driving factor that encouraged scientists to search for new antimicrobial substances from various sources including medicinal plants has

been the rapid rate of plant species extinction. Medicinal plants are relied upon by 80% of the world's population (Irum*et al*.,2010) and in India there is a rich tradition of using herbal medicine for the treatment of various infectious diseases, inflammations, injuries and other diseases. Many of the plant materials used in traditional medicine are generally proved more effective and relatively cheaper than modern medicine (Mann *et al.*, 2008) against certain ailments while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu*et al.*, 1999).

Despite abundant literature on the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been exploited for clinical use as antibiotics. A significant part of the chemical diversity produced by plants is thought to protect plants against microbial pathogen. Hence, they have been proven to have antimicrobial importance both in vivo and in vitro (Gibbons,2004). This fact opens the way for research aimed at identifying new antimicrobial agents (Antunes *et al.*, 2006; Guimaraes *et al.*,2010).

4.2 Materials and Methods

4.2.1 Preparation of plant extract

The randomly collected plants were brought to the laboratory and thoroughly washed in running water to remove debris and dust particles and then rinsed using distilled water and finally air dried at room temperature before grinding them to powdered form using pestle and mortar / mechanized grinder.

(i) Cold Extraction

(a) Aqueous extraction – Aqueous extracts of plant materials were prepared by taking 10 gm of air dried powdered plant material in 25 ml of sterilized distilled water (30 – 40˚C) for 48 hours. It was filtered through Whatman filter paper No. 1 and centrifuge at 10,000 g for 15 min and the supernatants were used for phytochemical screening. Aqueous extracts were used directly after adjusting their pH to 7.0 (Gurinder and Daljit, 2009).

(b) Organic extraction - Organic extracts of plant materials were prepared using three different solvents with increasing polarity – chloroform, ethanol and methanol. The dried plant materials of 10 gm each were extracted by maceration in different solvents (25 ml) for 48 hours at room temperature in a dark place with intermittent shaking. Each extracted material were filtered through Whatman filter paper No.1 and centrifuged at X10000g for 15min and the supernatant were used for phytochemical and antibacterial activity screening (Gurinder and Daljit, 2009).

(ii) Hot extraction

Organic extraction - Methanol and ethanol extracts of *P.lineata* and *G.pedunculata* were prepared using Soxhlet hot extraction apparatus by continuous hot percolation method. The dried powdered plant materials of 10 gm each were extracted in two different solvents (25 ml) until the solvents become clear. Each extracted material were filtered through Whatman filter paper No.1 and centrifuged at X10,000g for 15min and the supernatant were used for LC-ESI-MS and comparative study of antibacterial activity of *P.lineata*and *G.pedunculata* (Karthika*et al*.,2014).

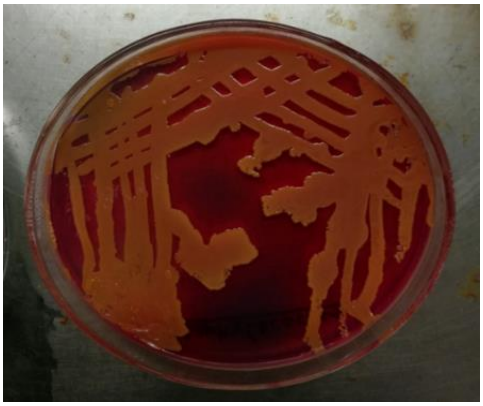
The extracts were evaporated to dryness in a rotary shaker (150 rpm; 50° C) and were stored at 4° C till further use.

4.2.2 Collection and maintenance of bacterial test cultures

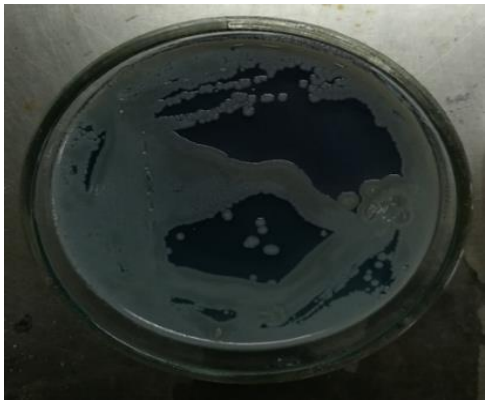
The test microorganisms used in the study were clinical isolates of *Escherichia coli* AF06,*Staphylococcus aureus*AM12, *Enterococcus faecalis* AF24, *Enterobacter cloacae* AM03, *Pseudomonas aeruginosa* CM07 and *Klebsiella pneumoniae* CF09. The microorganisms were isolated from urine samples collected from patients. The collected samples were streak on selective media and characteristic colony morphological study was conducted. The isolated colonies were sub – cultured to obtain pure culture and then the pure culture were then subjected to a biochemical test for identification using Vitek Automated Machine in the laboratory of Department of Microbiology, Woodland Hospital, Shillong.

The pure cultures of the clinical isolates of the test microorganisms were maintained on different agar slants - *Staphylococcus aureus* AM12 (MSA), *Enterococcus faecalis* AF24 (CLED), *Enterobacter cloacae*AM03(McConkey), *Escherichia coli* AF06(EMB), *Pseudomonasaeruginosa* CM07 (Cetrimide), *Klebsiella pneumoniae* CF09 (McConkey).

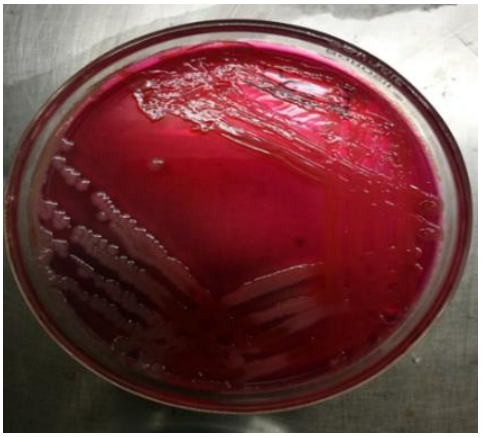
The solution of each media were pour into Mc Cartney bottles at equal amount of 10 millilitres. The bottles were then closed and placed in an autoclave and sterilized for about 15 minutes at 121°C. The autoclave was left to cool before the bottles containing the media are removed. The bottle was then placed in a laminar flow slanting at around 45°Cand left to cool forming agar slants. The bacterial microorganisms were streaked on agar slant. The streaked agar slants were placed in an incubator at 37°C overnight. The test microorganisms were subsequently sub - cultured after every 48 hours to maintain their viability.



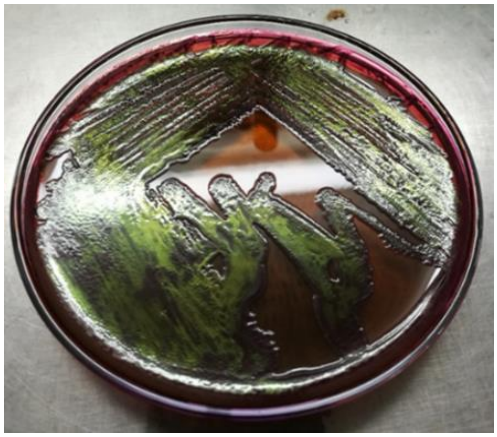
i) Staphylococcusaureus AM12



ii) Enterococcus faecalis AF24

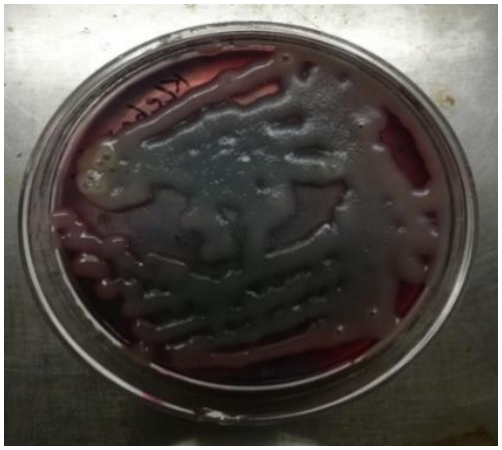


iii) Enterobacter cloacae AM03



iv) Escherichia coli AF05

v) Pseudomonas aeruginosa CM07



vi) Klebsiella pneumoniae CF09

Plates 2 (i-vi): UTI clinical bacterial isolates

4.2.3Antibacterial Susceptibility Testing (AST) of bacterial clinical isolates against standard antibiotics by Disc Diffusion Method

The susceptibility of the six(6) reference strain of test bacteria to twelve (12) commonly employed different antibiotics viz. Ceftazidime CAZ 30mcg/disc, Gentamicin GEN 10mcg/disc, Piperacillin PI 100mcg/disc, Amikacin AK 30mcg/disc, Cefepime CPM 30mcg/disc, Aztreonam AT 30mcg/disc, Cefoperazone CPZ 75mcg/disc, Ciproflaxacin CIP 5mcg/disc, Levoflaxacin LE 5mcg/disc, Imipenem IPM 10mcg/disc, Meropenem MRP 10mcg/disc and Piperacillin/Tazobactam PIT 100/10mcg/disc was assessed by disc diffusion method using HiMedia'sDodeca Disc for easy and relevant comparision and to ascertain the effectiveness of the 15 medicinal plant extracts.

4.2.4 Screening of antibacterial activity of selected medicinal plants

4.2.4.1 Preparation of standardized inoculum

The test bacteria were grown on Mueller-Hinton Agar (MHA, HiMedia) plate for 16–18 hr. at 37 ± 2°C. Well-isolated colonies were suspended in sterile Mueller-Hinton Broth (MHB, HiMedia) and the turbidity was adjusted against 0.5 McFarland standard to comprise approximately 1.5 x 10^8 CFU/ml.

4.2.4.2 AST of bacterial clinical isolates against selected medicinal plants by Disc Diffusion Method

Antibacterial activity of the methanol extract of the 15 medicinal plants towards different clinical isolates were measured in terms of zone of inhibition using disc diffusion method as stated by Kirby - Bauer *et al*. (1966). It is the recommended method of European Committee on Antimicrobial Susceptibility Testing (EUCAST) and National Committee for Clinical Laboratory Standard (NCCLS),USA.

The isolates to be tested were, firstly, inoculated/spread on the surface of MHA using sterile spreader. Sterile 6-mm diameter blank discs (HiMedia) impregnated with 250 µl of different plant extracts stock solution (200 mg/ml) were place on the inoculated MHA plate as test disc. Blank disc impregnated with 250 µl methanol were

used as negative control for methanol plant extract. Disc of ampicillin (10 mcg) and methicillin (5mcg) antibiotics (HiMedia) were used as reference antibiotics. All test plates were incubated at 37 ± 2°C for 16 to 24 hours and the diameter of zones of inhibition produced by the plant extracts were measured. For each combination of extract and the microbe, the experiments were repeated thrice.

Antibiotic sensitivity test of the test bacteria against standard antibiotics were also determined by disc diffusion method.

4.2.4.3AST of bacterial clinical isolates against selected medicinal plants by Well Diffusion Method

The susceptibility of different bacterial clinical isolates to the methanol extract of the selected 15 ethnomedicinalplantswere measured in terms of zone of inhibition using agar well diffusion assay. The plates containing MHA were spread with the inoculum. Wells (8mm diameter) were cut out from agar plates using a sterilized stainless steel well borer and filled with 250 µl of the plant extracts (200 mg/ml). Wells filled with 250 µl methanol were used as negative control for methanol plant extract. Disc of ampicillin (10 mcg) and methicillin (5 mcg) antibiotics (HiMedia) were used as reference antibiotics. The plates inoculated with different bacteria were incubated at 37° C for 16 to 24 hours and diameter of any resultant zone of inhibition were measured. For each combination of extract and the bacterial strain, the experiment was repeated thrice.

4.2.4.4 Comparative study of the Antimicrobial Activity of *P. lineata* and *G. pedunculata* against the UTI clinical bacterial isolates by Disc Diffusion and Well Diffusion Assay

The antibacterial activity of the methanol extract and ethanol extract of the selected 2 ethnomedicinal plants viz. *P. lineata* and *G. pedunculata*were determined following the same procedure as was mention in screening of antibacterial activity by Disc Diffusion (Bauer – Kirby,1966) and Well Diffusion Method.

Both the methanol extract and ethanol extract were subjected to antibacterial activity assay against the six clinical isolates. All the bacterial pathogens

(*Staphylococcus aureus* AM12,*Enterococcus faecalis* AF24, *Enterobacter cloacae* AM03, *Escherichia coli*AF05, *Pseudomonas aeruginosa* CM07 *and Klebsiella pneumoniae* CF09)were exposed to the plant extract in triplicates.

4.2.4.5MIC Determination by Broth Microdilution Method supplemented with Resazurin

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the plant extracts were determined by using broth microdilution method, as described previously by Wiegand *et al.*(2008) supplemented with Resazurin dye (Elsinkh,2016) based on Clinical Laboratory Standard Institute. Two-fold serial dilution of plant extract with starting concentration of 200 mg/ml was prepared using Mueller-Hinton broth or MHB (HiMedia) as diluent. Each set-up was carried out in triplicate in sterile 2.0 ml Eppendorf tubes. Negative control (inoculum and MHB only) was also maintained. Solvent blanks and positive controls were also included.

The bacterial inoculum was prepared as described above for the disc-diffusion method. Fifty (50) µl of the adjusted inoculum were added into each Eppendorf tube containing 400 µl of MHB and 500 µl of each dilution of plant extract in the dilution series, and mixed. The capped microdilution vials were incubated at 37 ± 2°C for 16 to 20 hours in an ambient air incubator. Tests were performed in triplicate.

After incubation for 24 h at 37°C, resazurin (0.015 %) was added to all the Eppendorf tubes (150 µl per vial), and further incubated for 2–4 h for the observation of color change. On completion of the incubation, the Eppendorf tubes with the lowest concentration of the extract that shows no color change (blue resazurin color remained unchanged) was scored as the MIC value.

4.2.4.6 Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by plating directly the content of Eppendorf tubes with concentrations higher than the MIC value.

The lowest concentration of the extract that did not permit any growth was taken as the MBC.

4.2.4.7 Time-kill assay/Bactericidal activity

Time-kill assays were performed by the broth macro-dilution method in accordance with the CLSI guidelines. All the susceptible exponentially growing susceptible test bacteria in mid-logarithmic growth phase were adjusted to the 0.5 McFarland standard. Then, each of the standardized bacterial suspension of all the susceptible test bacteria (50 µl) were inoculated into several Eppendorf tubes (0,1,2,3,4,5,6,7 and 8 h) of MHB containing 450 µl of MHB and 500 µl of the extracts with final concentrations corresponding to 1X MIC (i.e.25 mg/ml of MEGP for *Enterobacter cloacae* AM03, *E.coli* AF05and*Klebsiella pneumoniae* CF09*;* and 12.5 mg/ml of MEGP for *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24and *Pseudomonas aeruginosa* CM07*;* 50 mg/ml of MEPL for*Staphylococcus aureus* AM12and*Enterococcus faecalis* AF24) . The final density of bacteria was approximately 4-5x10^5 CFU/mL. The capped microdilution vials were incubated at 37 ± 2°C for 16 to 20 hours in an ambient air incubator. Then, each particular tube were removed at specific time intervals of incubation i.e. 0,1,2,3,4,5,6,7 and 8 h. The numbers of viable cells were determined by the plate count technique which involved plating 25 µl on a MHA plate.The number of bacteria remaining in each sample was plotted over time to determine the rate of killing. A growth control comprising the bacterial strain without the test extract was included in each trial. Tests are performed in triplicate.

4.3. Results

4.3.1 AST of bacterial clinical isolates against standard antibiotics by Disc Diffusion Method

For easy and relevant comparision as well as to ascertain the effectiveness of the extract of *G.pedunculata* and *P.lineata,* AST of bacterial clinical isolates against standard antibiotics were conducted by Disc Diffusion Method using HiMedia'sDodeca Disc. The result of the antibacterial susceptibility test is given in Table–2 and Fig.2. The different cultures of the clinical bacterial isolates responded to standard antibiotics in a

variable manner resulting in various size of zones of inhibition. The size of zone of inhibition range from 10.3±0.5 (Piperacillin/Tazobactam PIT 100/10mcg against *K. pneumoniae* CF09) to 59.7±0.5 (Levoflaxacin LE 5mcg against*S. aureus* AM12).

Table 2: AST of bacterial clinical isolates against standard antibiotics

SN	**Standard antibiotics**	***S.aureus* AM12 (mm)**	***E.faecalis* AF24 (mm)**	***E.cloacae* AM03 (mm)**	***E.coli* AF05 (mm)**	***P.aeruginosa* CM07 (mm)**	***Klebsiella pneumoniae* CF09 (mm)**
1	Ceftazidime CAZ 30mcg	16.0±0.9	19.0±0.9	0	25.7±0.5	25.3±1.0	10.3±0.5
2	Gentamicin GEN 10mcg	30.3±0.5	23.0±0.9	18.7±0.5	19.7±0.5	26. 7±0.5	23.0±0.9
3	Piperacillin PI 100mcg	45.0±0.9	25.7±0.5	11.3±0.5	27.0±0.9	15. 7±0.5	10.3±0.5
4	Amikacin AK 30mcg	42.3±0.5	23.0±0.9	18.3±0.5	18.7±0.5	41.0±0.9	25.3±1.0
5	Cefepime CPM 30mcg	42.3±0.5	23.0±0.9	0	36.7±0.5	30.3±0.5	23.7±0.5
6	Aztreonam AT 30mcg	20.7±0.5	14.3±0.5	0	28.7±0.5	25.7±0.5	13.3±0.5
7	Cefoperazone CPZ 75mcg	41.0±0.9	29.3±0.5	11.3±0.5	34.7±0.5	27.7±1.0	13.7±0.5
8	Ciproflaxacin CIP 5mcg	43.7±0.5	29.3±0.5	39.7±0.5	40.0±0.9	51.3±1.0	31.7±0.5
9	Levoflaxacin LE 5mcg	59.7±0.5	40.0±0.9	26.7±0.5	38.3±1.0	52.0±0.9	39.7±0.5
10	Imipenem IPM 10mcg	35.0±0.9	36.3±0.5	22.3±0.5	33.7±0.5	31.3±1.0	24.3±1.0
11	Meropenem MRP 10mcg	30.0±0.9	31.0±0.9	22.3±0.5	33.7±0.5	29.7±0.5	23.3±0.5
12	Piperacillin/ Tazobactam PIT 100/10mcg	23.3±0.5	32. 7±0.5	15. 67±0.5	28.0±0.9	19.7±0.5	10.3±0.5

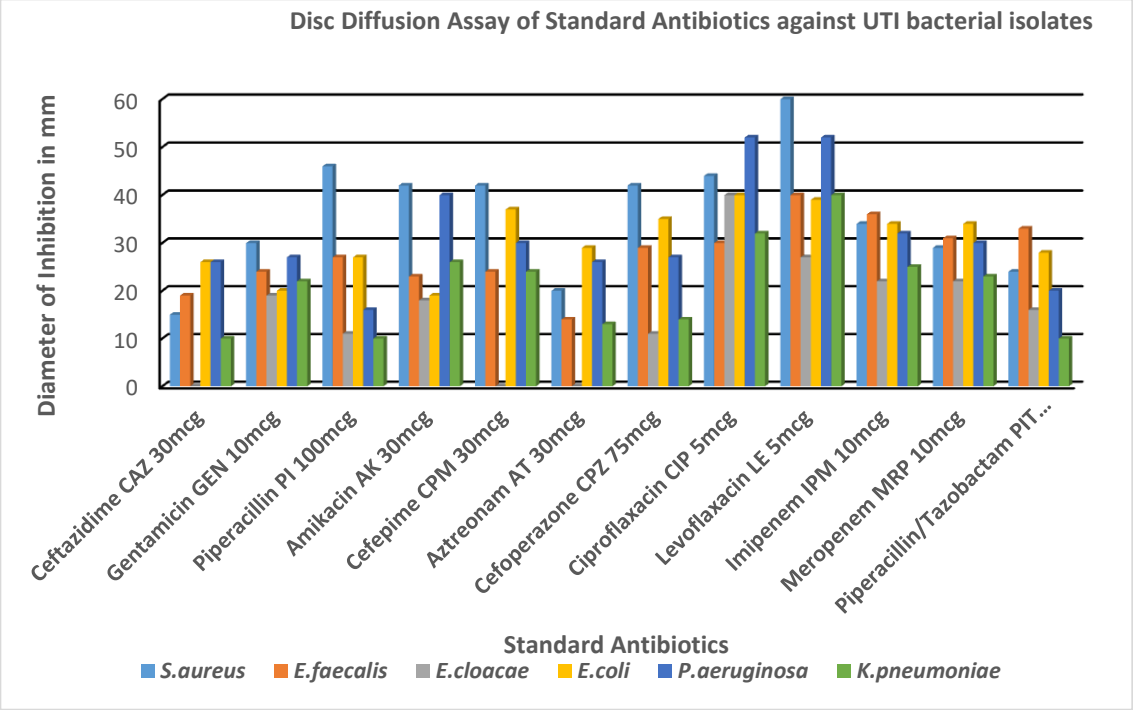


Figure 2: Disc diffusion assay of standard antibiotics against test bacteria

4.3.2AST of bacterial clinical isolates against selected medicinal plants by Disc Diffusion Method and Well Diffusion Method

All the bacterial pathogens (*Staphylococcus aureus* AM12,*Enterococcus faecalis* AF24, *Enterobacter cloacae* AM03, *Escherichia coli*AF05, *Pseudomonas aeruginosa* CM07 *and Klebsiella pneumoniae* CF09*)* were exposed to the extract of 15 selected medicinal plants in triplicates and they showed varied antimicrobial activity (Table 3 and Plates 3-32). All the methanol negative control discs did not produce any zone of inhibition against any of the tested clinical isolates.

All the tested bacteria were susceptible to *Garcinia pedunculata* while none of the extract of *A.aspera, A.paniculata, A.conzyoides, C.asiatica, H.sabdarifa, H.cordata, P.attenuatum, P.vulgaris, S.oleraceus and S.palustris* were active against any of the tested bacterial species.

Staphylococcusaureus AM12 was susceptible to *B.pilosa, G.pedunculata, G.fragrantissima* and *P.lineata* while both *S.aureus*AM12 and *E.faecalis*AF24 were

susceptible to *G.pedunculata*, *G.fragrantissima*and *P.lineata*. *E. cloacae* AM03, *E.coli* AF05and *K. pneumoniae* CF09 were inhibited by only *G.pedunculata* while *P.aeruginosa* CM07 was susceptible to only *G.pedunculata* and *L.camara*.

It is noteworthy that *G.pedunculata* shows antibacterial activity against *E.cloacae* AM03, *P.aeruginosa* CM07 and *K. pneumoniae* CF09which are not susceptible to the standard antibiotics namely Ampicillin and methicillin.

Table 3: Antibacterial activity of selected 15 plant extracts against bacterial pathogens.

SN	**Medicinal plants**	***S. aureus* AM12 (mm)**	***E. faecalis* AF24 (mm)**	***E. cloacae* AM03 (mm)**	***E.coli* AF05 (mm)**	***P. aeruginosa* CM07 (mm)**	***K. pneumoniae* CF09 (mm)**
1	*Achyranthes aspera*	-ve	-ve	-ve	-ve	-ve	-ve
2	*Acmellapaniculata*	-ve	-ve	-ve	-ve	-ve	-ve
3	*Ageratum conzyoides*	-ve	-ve	-ve	-ve	-ve	-ve
4	*Bidenspilosa*	+ve	+ve	-ve	-ve	-ve	-ve
5	*Centellaasiatica*	-ve	-ve	-ve	-ve	-ve	-ve
6	*Garcinia pedunculata*	+ve	+ve	+ve	+ve	+ve	+ve
7	*Gaultheria fragrantissima*	+ve	+ve	-ve	-ve	-ve	-ve
8	*Hibiscus sabdarifa*	-ve	-ve	-ve	-ve	-ve	-ve
9	*Houttuynia cordata*	-ve	-ve	-ve	-ve	-ve	-ve
10	*Lantana camara*	-ve	-ve	-ve	-ve	+ve	-ve
11	*Piper attenuatum*	-ve	-ve	-ve	-ve	-ve	-ve
12	*Potentilalineata*	+ve	+ve	-ve	-ve	-ve	-ve
13	*Prunella vulgaris*	-ve	-ve	-ve	-ve	-ve	-ve
14	*Sonchusoleraceas*	-ve	-ve	-ve	-ve	-ve	-ve
15	*Sonchus palustris*	-ve	-ve	-ve	-ve	-ve	-ve

Achyranthes aspera

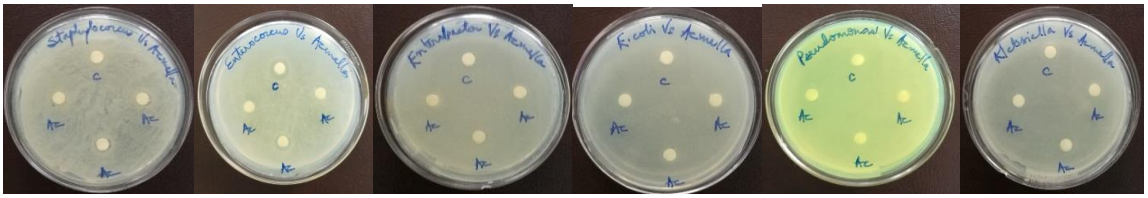
a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 3 (a-f):Disc diffusion assay showing antibacterial activity of *Achyranthes aspera* methanol extract on different test organisms.

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

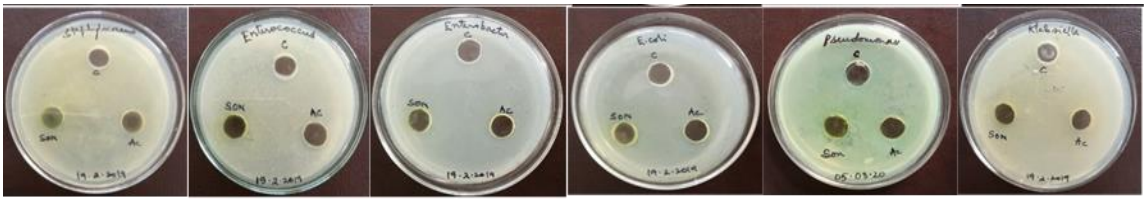
Plate 4 (a-f): Well diffusion assay showing antibacterial activity of *Achyranthes aspera* methanol extract on the different test organisms.

Acmella paniculata



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

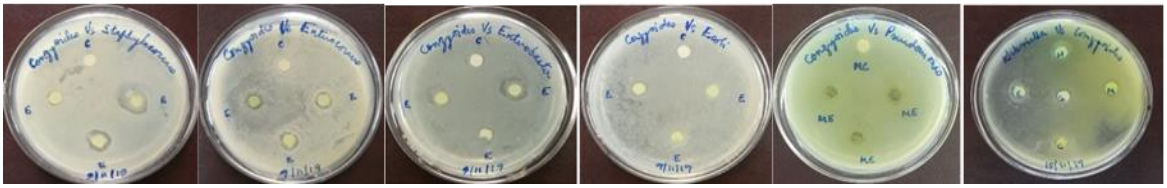
Plate 5 (a-f):Disc diffusion assay showing antibacterial activity of *Acmella paniculata*methanol extract on different test organisms.



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

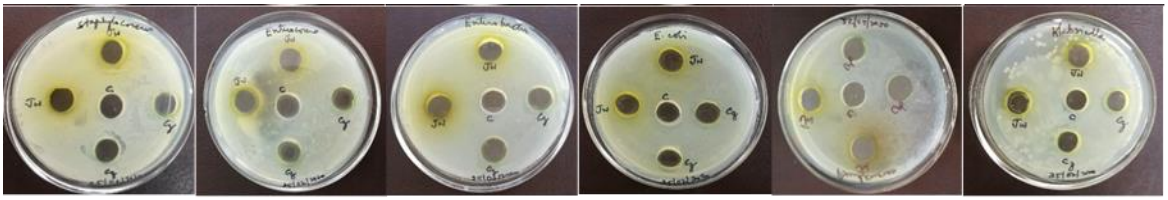
Plate 6 (a-f): Well diffusion assay showing antibacterial activity of *Acmellapaniculata* methanol extract on the different test organisms.

Ageratum conzyoides



a) *S. aureus* b) *E. faecalis* c) *E. cloacae*d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

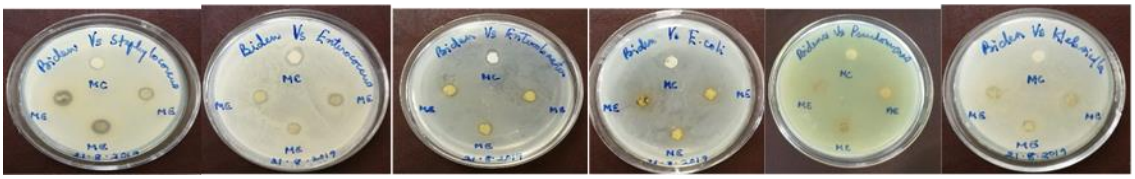
Plate 7 (a-f):Disc diffusion assay showing antibacterial activity of *Ageratum conzyoides* methanol extract on different test organisms.



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 8 (a-f): Well diffusion assay showing antibacterial activity of *Ageratum conzyoides* methanol extract on the different test organisms.

Bidens pilosa



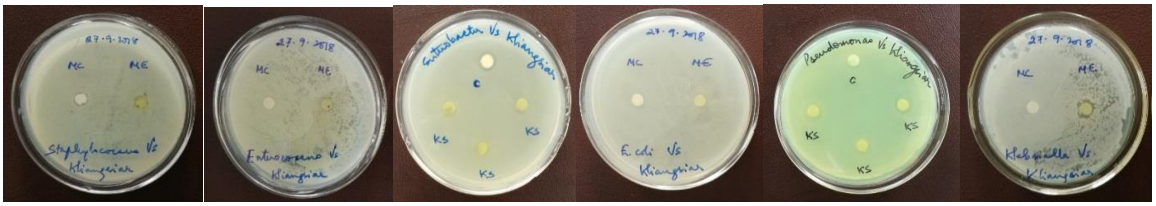
a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 9 (a-f):Plates of Disc diffusion assay showing antibacterial activity of *Bidenspilosa* methanol extract on different test organisms.

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

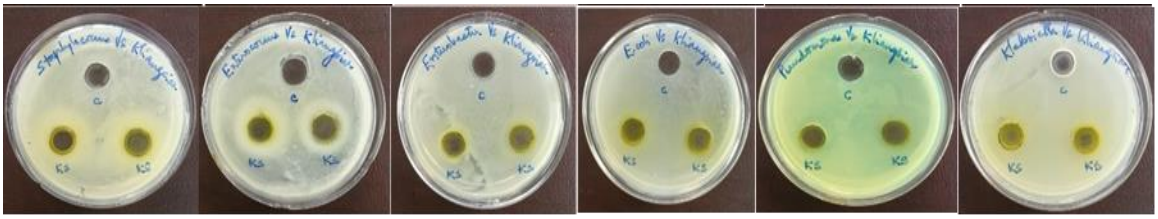
Plate 10 (a-f): Plates of disc diffusion assay showing antibacterial activity of *Bidenspilosa* methanol extract on the different test organisms.

Centella asiatica



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

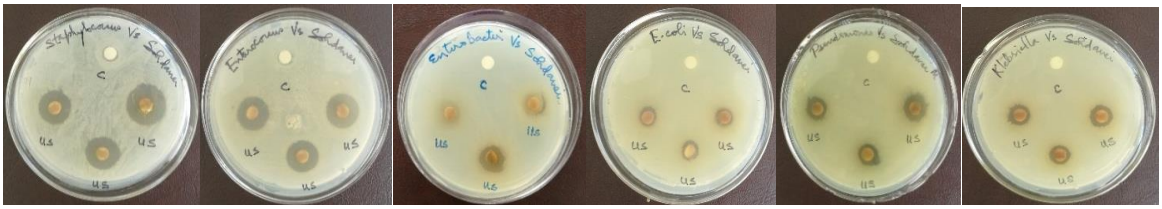
Plate 11 (a-f):Disc diffusion assay showing antibacterial activity of *Centellaasiatica*methanol extract on different test organisms.



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 12 (a-f): Well diffusion assay showing antibacterial activity of *Centellaasiatica* methanol extract on the different test organisms.

Garcinia pedunculata



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 13 (a-f):Disc diffusion assay showing antibacterial activity of *Garcinia pedunculata* methanol extract on different test organisms.

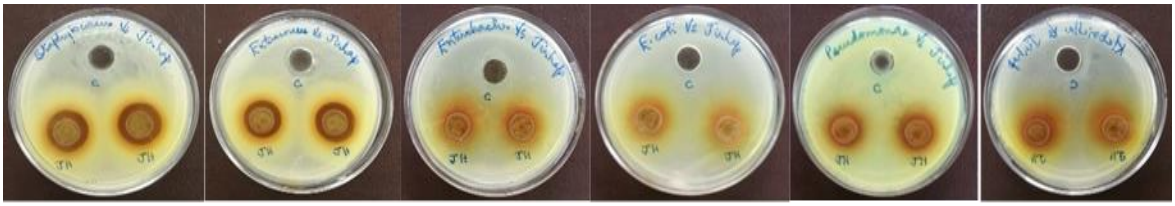
a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 14 (a-f): Well diffusion assay showing antibacterial activity of *Garcinia pedunculata* methanol extract on the different test organisms.

Gaultheria fragrantissima

a) *S. aureus* b) *E. faecalis* c) *E. cloacae*d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

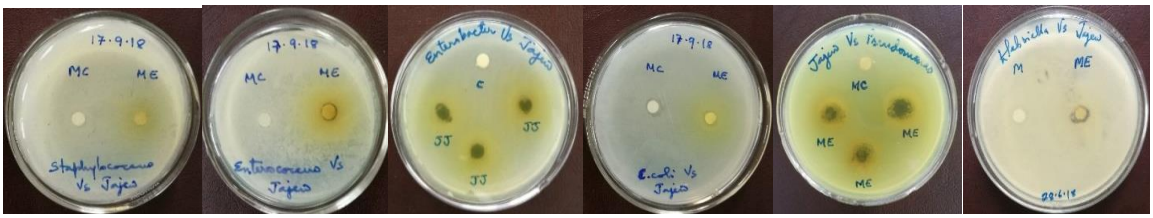
Plate 15 (a-f):Disc diffusion assay showing antibacterial activity of *Gaultheria fragrantissima* methanol extract on different test organisms.



a) *S. aureus* b) *E. faecalis* c) *E. cloacae*d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 16 (a-f): Well diffusion assay showing antibacterial activity of *Gaultheria fragrantissima* methanol extract on the different test organisms.

Hibiscus sabdarifa



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 17 (a-f):Disc diffusion assay showing antibacterial activity of *Hibiscus sabdarifa* methanol extract on different test organisms.

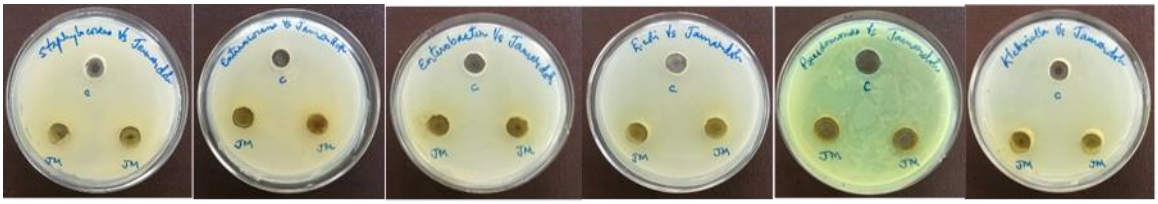
a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 18 (a-f): Well diffusion assay showing antibacterial activity of *Hibiscus sabdarifa* methanol extract on the different test organisms.

Houttuynia cordata

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

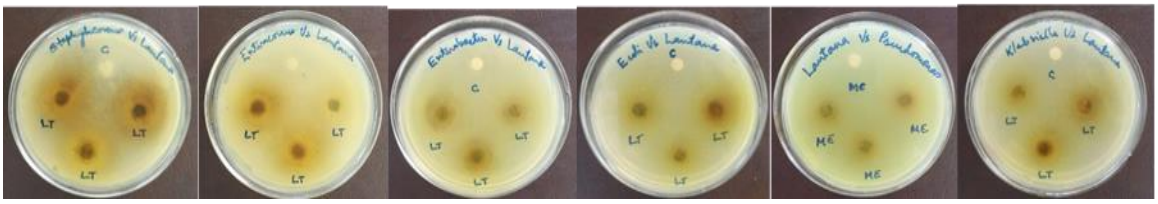
Plate 19 (a-f):Disc diffusion assay showing antibacterial activity of *Houttuynia cordata* methanol extract on different test organisms.



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

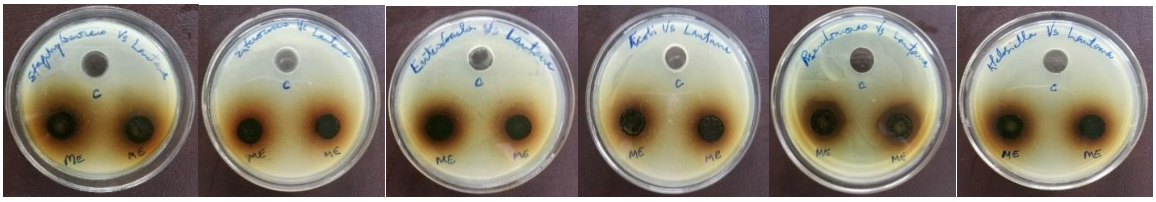
Plate 20 (a-f): Well diffusion assay showing antibacterial activity of *Houttuynia cordata* methanol extract on the different test organisms.

Lantana camara



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 21 (a-f):Disc diffusion assay showing antibacterial activity of *Lantana camara* methanol extract on different test organisms.



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 22 (a-f): Well diffusion assay showing antibacterial activity of *Lantana camara* methanol extract on the different test organisms.

Piper attenuatum

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 23 (a-f):Disc diffusion assay showing antibacterial activity of *Piper attenuatum*methanol extract on different test organisms.

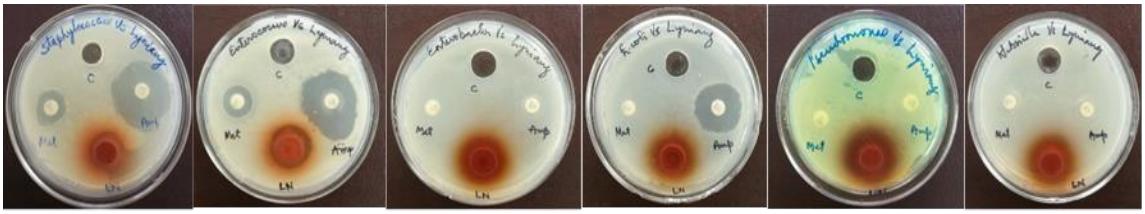
a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 24 (a-f):Disc diffusion assay showing antibacterial activity of *Piper attenuatum*methanol extract on different test organisms.

Potentila lineata

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

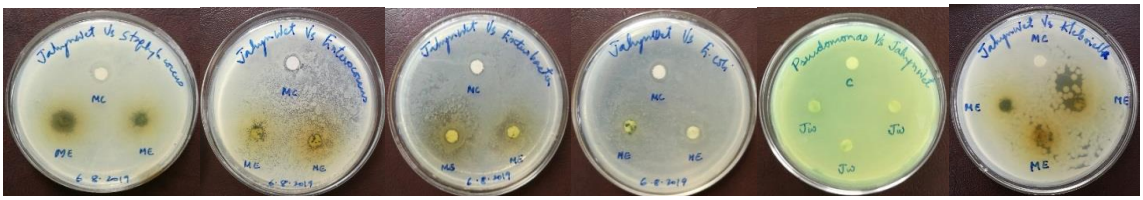
Plate 25 (a-f):Disc diffusion assay showing antibacterial activity of *Potentilalineata*methanol extract on different test organisms.



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

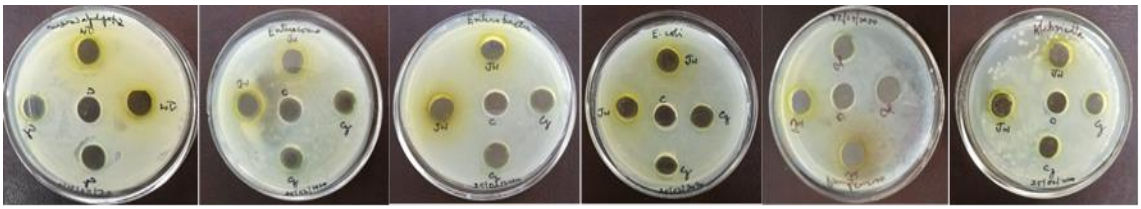
Plate 26 (a-f):Well diffusion assay showing antibacterial activity of *Potentilalineata*methanol extract on different test organisms.

Prunella vulgaris



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 27 (a-f):Disc diffusion assay showing antibacterial activity of *Prunella vulgaris*methanol extract on different test organisms.



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 28 (a-f):Well diffusion assay showing antibacterial activity of *Prunella vulgaris* methanol extract on different test organisms.

Sonchus oleraceus

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 29 (a-f):Disc diffusion assay showing antibacterial activity of *Sonchus oleraceus* methanol extract on different test organisms.

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 30 (a-f): Well diffusion assay showing antibacterial activity of *Sonchus oleraceus* methanol extract on the different test organisms.

Sonchus palustris

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 31 (a-f):Disc diffusion assay showing antibacterial activity of *Sonchus palustris*methanol extract on different test organisms.

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

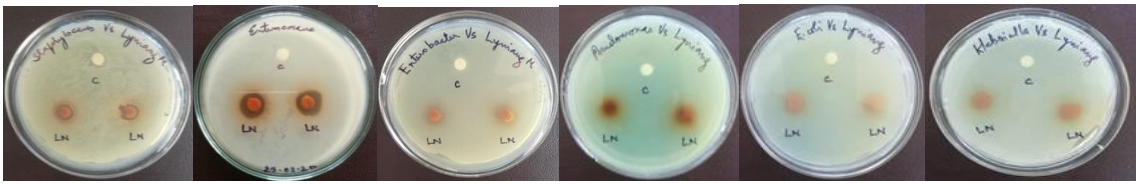
Plate 32 (a-f): Well diffusion assay showing antibacterial activity of *Sonchus palustris* methanol extract on the different test organisms.

4.3.3 Comparative study of the Antibacterial Activity of *P.lineata* and *G.pedunculata* against the bacterial clinical isolates - Disc Diffusion Assay and Well Diffusion Assay

The antibacterial activity of the methanol extract and ethanol extract of the selected 2 ethnomedicinal plants viz. *P.lineata* and *G.pedunculata* were determined by the disc and well diffusion method. It was observe that both the organic solvent extract of *P.lineata* produced very effective and notable antimicrobial activities against the two tested Gram positive bacteria (*Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24but Gram negative bacteria (*Enterobacter cloacae* AM03, *E.coli* AF05, *Pseudomonas aeruginosa* CM07, *Klebsiella pneumoniae* CF09) were not susceptible to the extract.

On the other hand, the organic solvent extract of *G.pedunculata* were inhibitory to all the six bacterial isolates and are more potent than that of the extract of *P.lineata*. All the methanol and ethanol negative control discs did not produce any zone of

inhibition against any of the tested clinical isolates. The results were compared with those of ampicillin and methicillin as standard antibiotics. The solvent extract (50 mg/ml) of both *P.lineata* and *G.pedunculata* were not as potent as ampicillin (10 mcg) but are quite effective as methicillin (5 mcg) antibiotics or even better (Plates 33-40;Fig. 3& 4; Table 4 & 5)



a) *S. aureus* b) *E. faecalis*c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 33: Disc diffusion assay showing antibacterial activity of *P.lineata* methanol extract on the different test organisms.

a) *S.aureus* b) *E.faecalis* c) *E.cloacae* d) *E.coli* e) *P.aeruginosa* f) *K. pneumoniae*

Plate 34: Disc diffusion assay showing antibacterial activity of *P. lineata* ethanol extract on the different test organisms

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 35: Disc diffusion assay showing antibacterial activity of *G.pedunculata* methanol extract on different test organisms.

a) *S.aureus*b) *E.faecalis*c) *E.cloacae*d) *E.coli* e) *P.aeruginosa* f) *K. pneumoniae*

Plate 36: Disc diffusion assay showing antibacterial activity of *G.pedunculata* ethanol extract on different test organisms

Table 4:Antibacterial activity of *P.lineata* and *G.pedunculata* extracts and some of the standard antibiotics against UTI bacterial isolates - Disc Diffusion Assay

Test Bacteria	Diameter of Zone of Inhibition (mm) are given as mean ± SD; (n = 3)					
	MEGPDD	MEPLDD	EEGPDD	EEPLDD	AMP	MET
S.aureus AM12	16.7±0.5	13±0.9	14±0	11.6±0.5	24.3±0.5	11.6±0.5
E.faecalis AF24	19±0	15.3±0.5	16±0.9	14±0.9	26.3±0.5	13.3±0.5
E.cloacae AM03	10.7±0.5	0	11±0	0	0	0
E.coli AF05	10±0	0	10±0	0	17.7±0.5	0
P.aeruginosa CM07	10.3±0.5	0	10.3±0.5	0	0	0
K. pneumoniae CF09	10.3±0.5	0	10.3±0.5	0	0	0

Note: MEGPDD = Methanol Extract of *G.pedunculata* (Disc Diffusion); MEPLDD = Methanol Extract of *P.lineata* (Disc Diffusion); EEGPDD = Ethanol Extract of *G.pedunculata* (Disc Diffusion); EEPLDD = Ethanol Extract of *P.lineata* (Disc Diffusion); AMP = Ampicillin 10 mcg; MET = Methicillin 5 mcg

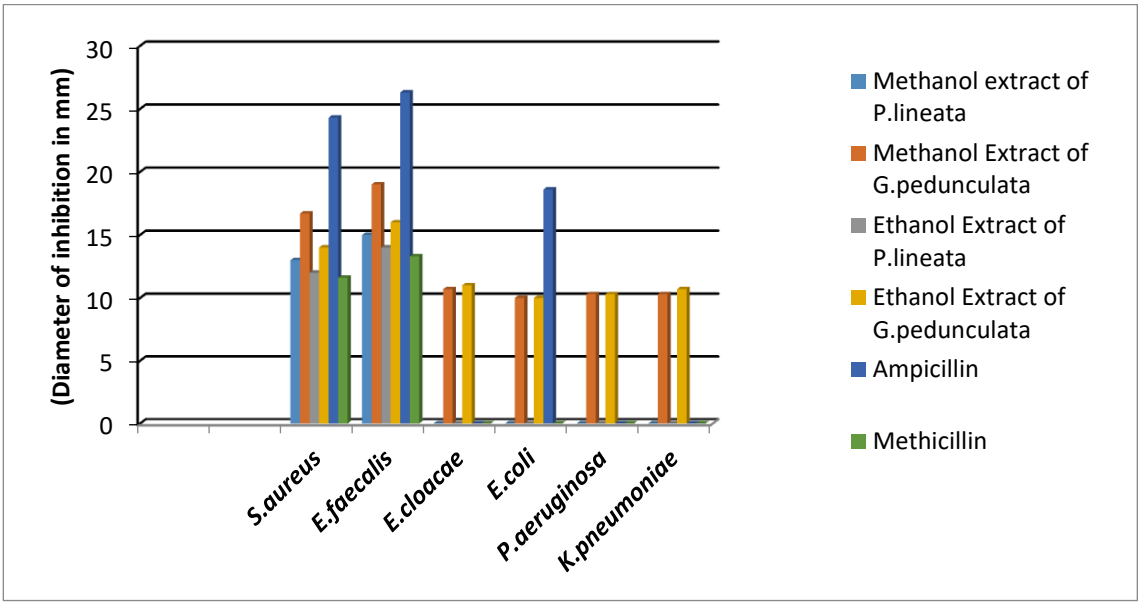
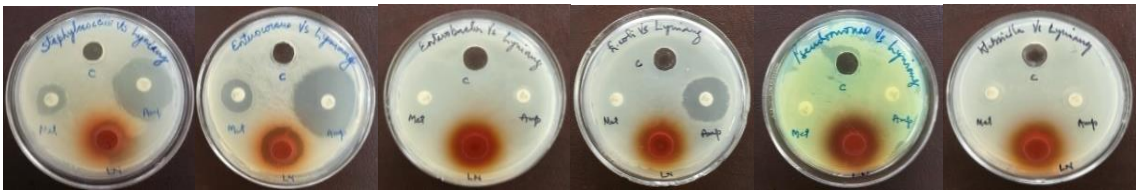


Figure 3: Disc diffusion assay of organic solvent extract of *P.lineata* and *G.paniculata*



a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumonia*

Plate 37: Well diffusion assay showing antibacterial activity of *P.lineata* methanol extract on the different test organisms.

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumonia*

Plate 38: Well diffusion assay showing antibacterial activity of *P.lineata* ethanol extract on the different test organisms.

a) *S. aureus* b) *E. faecalis* c) *E. cloacae* d) *E. coli* e) *P. aeruginosa* f) *K. pneumoniae*

Plate 39: Well diffusion assay showing antibacterial activity of *G.pedunculata* methanol extract on the different test organisms.

a)*S. aureus*b) *E. faecalis*c) *E. cloacae*d) *E. coli*e) *P. aeruginosa*f) *K. pneumoniae*

Plate 40: Well diffusion assay showing antibacterial activity of *G.pedunculata* ethanol extract on different test organisms.

Table 5:Antibacterial activity of *P.lineata* and *G.pedunculata* extracts and some of the standard antibiotics against UTI bacterial isolates - Well Diffusion Assay

Test Bacteria	Diameter of Zone of Inhibition (mm) are given as mean ± SD; (n = 3)					
	MEGPWD	**MEPLWD**	**EEGPWD**	**EEPLWD**	**AMP**	**MET**
S.aureus AM12	30±0.9	15±0.9	16.7±0.5	14±0.9	24.3±0.5	11.6±0.5
E.faecalis AF24	34±0.9	18±0.9	23.7±0.5	17±0.9	26.3±0.5	13.3±0.6
E.cloacae AM03	25.7±0.5	0	17±0.9	0	0	0
E.coli AF05	26.7±0.5	0	22±0.9	0	17.7±0.5	0
P.aeruginosa CM07	29.3±1.0	0	20.7±0.5	0	0	0
K. pneumoniae CF09	23.3±0.5	0	14±0.9	0	0	0

Note: MEGPWD = Methanol Extract of *G.pedunculata* (Well Diffusion); MEPLWD = Methanol Extract of *P.lineata* (Well Diffusion); EEGPWD = Ethanol Extract of *G.pedunculata* (Well Diffusion); EEPLWD = Methanol Extract of *P.lineata* (Well Diffusion); AMP = Ampicillin 10 mcg; MET = Methicillin 5 mcg

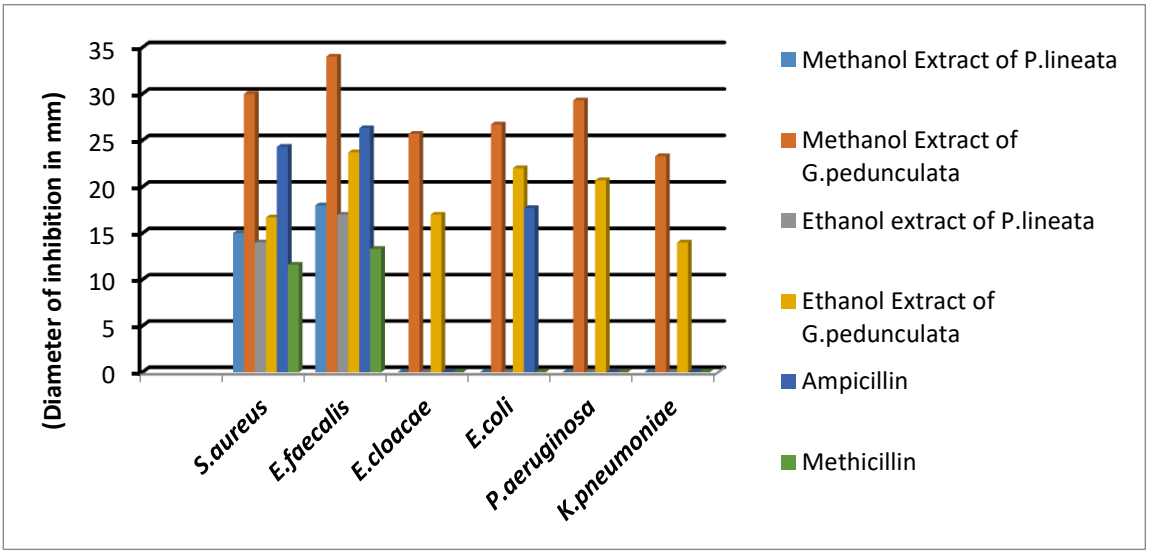


Figure 4: Well diffusion assay of organic solvent extract of *P.lineata* and*G. pedunculata*

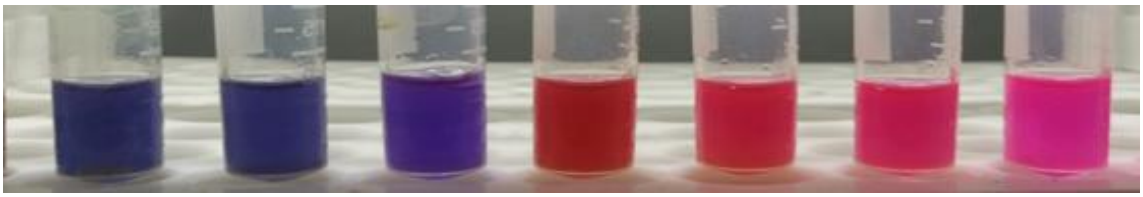
4.3.4 Minimum Inhibitory Concentration (MIC) of *P.lineata*

The MICs of only methanol extract of *P. lineata* for the susceptible bacterial isolates was determined, though both the methanol and ethanol extracts showed antimicrobial activities against Gram-positive bacteria, *Staphylococcus aureus AM12*and *Enterococcus faecalis AF24*. The extract is approximately equally potent against *Enterococcus faecalis AF24*(fig.5b)and *Staphylococcus aureus AM12* (fig.5a) with MICs being 25 mg/ml.

50 25 12.5 6.25 3.13 1.56 0.78

(mg/ml)

Figure 5(a): MIC of MEPL against *Staphylococcus aureus* AM12. The Eppendorf tubes with the lowest concentration of the extract i.e. 25 mg/ml that shows no color change (blue resazurin color remained unchanged) is scored as the MIC value



50 25 12.5 6.25 3.13 1.56 0.78

(mg / ml)

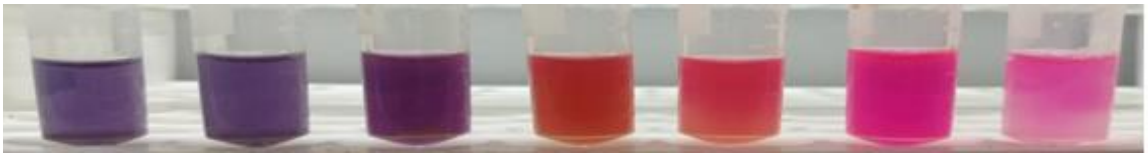
Figure 5(b): MIC of MEPL against *Enterococcus faecalis* AF24.The Eppendorf tubes with the lowest concentration of the extract i.e. 25 mg/ml that shows no color change (blue resazurin color remained unchanged) is scored as the MIC value

4.3.5 Minimum Bactericidal Concentration (MBC) of *P.lineata*

The methanol and ethanol extracts of *P.lineata* were not bactericidal for all the tested Gram negative bacteria but were effective towards both the tested Gram positive bacteria. The MBC values of methanol extract is 100 mg/ml for both *Staphylococcus aureus* AM12 and *Enterococcus faecalis* AF24.

4.3.6 Minimum Inhibitory Concentration (MIC) of *G. pedunculata*

The MICs of the methanol extract of *G.pedunculata* for the susceptible bacterial isolates were determined. The methanolic extracts showed antimicrobial activities against both Gram - positive bacteria and Gram - negative bacteria. The extract is approximately equally potent against *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 *and Pseudomonas aeruginosa* CM07, with MIC being 12.5 mg/ml (Figure 17a, 17b & 17e). Against *Enterobacter cloacae* AM03, *E.coli* AF05and*Klebsiella pneumoniae* CF09, the MIC of the extract was observed to be approximately 25 mg/ml (Figure 6c, 6d & 6f).



50 25 12.5 6.25 3.12 1.56 0.78

(mg / ml)

Figure 6(a) : MIC of MEGP against *Staphylococcus aureus* AM12. The Eppendorf tubes with the lowest concentration of the extract i.e. 12.5 mg/ml that shows no color change (blue resazurin color remained unchanged) is scored as the MIC value.

50 25 12.5 6.25 3.12 1.56 0.78

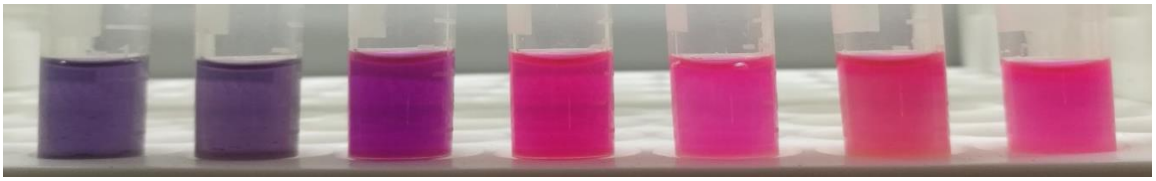
(mg / ml)

Figure 6(b): MIC of MEGP against *Enterococcus faecalis* AF24. The Eppendorf tubes with the lowest concentration of the extract i.e. 12.5 mg/ml that shows no color change (blue resazurin color remained unchanged) is scored as the MIC value

50 25 12.5 6.25 3.13 1.56 0.78

(mg / ml)

Figure 6(c): MIC of MEGP against *Enterobacter cloacae* AM03. The Eppendorf tubes with the lowest concentration of the extract i.e. 25 mg/ml that shows no color change (blue resazurin color remained unchanged) is scored as the MIC value.



50 25 12.5 6.25 3.13 1.56 0.78

(mg / ml)

Figure 6(d): MIC of MEGP against *E.coli* AF05. The Eppendorf tubes with the lowest concentration of the extract i.e. 25mg/ml that shows no color change (blue resazurin color remained unchanged) is scored as the MIC value

50 25 12.5 6.25 3.13 1.56 0.78

(mg / ml)

Figure 6(e): MIC of MEGP against *P.aeruginosa* CM07. The Eppendorf tubes with the lowest concentration of the extract i.e. 12.5 mg/ml that shows no color change (blue resazurin color remained unchanged) is scored as the MIC value

0.78 1.56 3.13 6.25 12.5 25 50

(mg / ml)

Figure 6(f): MIC of MEGP against *K. pneumoniae* CF09. The Eppendorf tubes with the lowest concentration of the extract i.e. 25 mg/ml that shows no color change (blue resazurin color remained unchanged) is scored as the MIC value.

4.3.7 Minimum Bactericidal Concentration (MBC) of *G.pedunculata*

The methanol extracts of *G.pedunculata* showed antibactericidal activity for all the tested Gram negative bacteria as well as Gram positive bacteria. The MBC values was found to be approximately 25 mg/ml against *Staphylococcus aureus* AM12,*Enterococcus faecalis* AF24and *Pseudomonas aeruginosa* CM07while 50 mg/ml was the observed MBC against *Enterobacter cloacae* AM03, *E.coli* AF05and *K. pneumoniae* CF09.

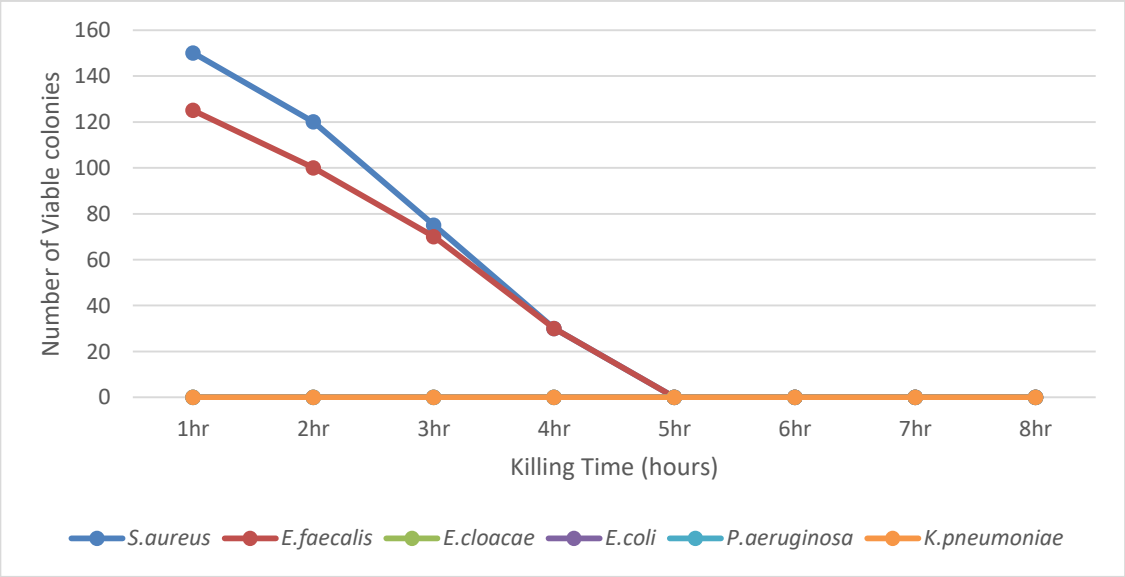
4.3.8 Time-kill Assay

Time-kill assays have been widely used for in vitro investigations of new antimicrobial agents as these provide descriptive (qualitative) information on the pharmacodynamics of antimicrobial agents(Lewis,2007).

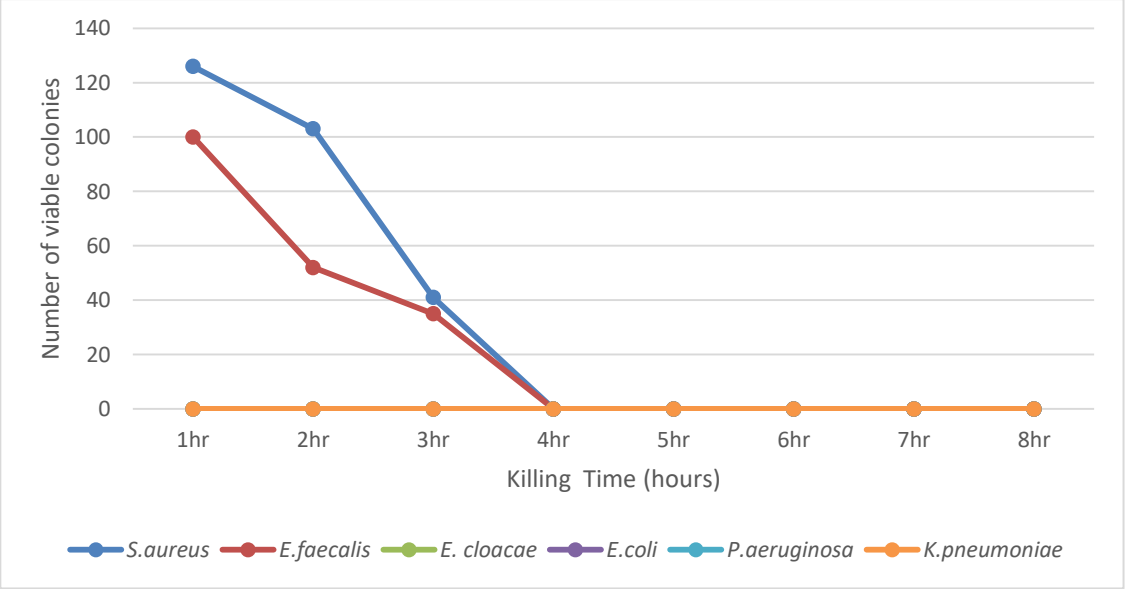
With regards to MEPL, only MEPL with activity towards susceptible bacterial strains was selected for time-kill studies. The kinetic interaction between susceptible bacteria and MEPL was examined at the concentration of 1X MIC (25 mg/ml) and 2X MIC (50 mg/ml). The kill kinetic profiles of MEPL displayed rapid killing rate towards both the susceptible Gram positive bacteria, *S. aureus*AM12 and *E.faecalis*AF24, showing complete destruction after 5 h exposure at 1X MIC (Fig.7a) and after 4 h exposure at 2X (Fig.7b).

In the present study, the kinetic interaction between susceptible bacteria and MEGP was also examined at the concentration of 1X MIC (12.5mg/ml for *S.aureus*AM12, *E. faecalis* AF24&*P.aeruginosa* CM07 and 25 mg/ml for *E. cloacae*AM03, *E.coli* AF05&*K. pneumonia* CF09), and 2X MIC. The kill kinetic profiles of MEGP displayed rapid bactericidal activity towards all susceptible strains, showing complete destruction after 7 h exposure at 1X MIC (Fig.8a) and after 5 h exposure at 2X MIC(Fig.8b). As expected from the determined MBC/MIC ratios, the time-kill assays for MEGP towards *S.aureus* AM12, *E. faecalis*AF24, *E. cloacae*AM03, *E.coli* AF05, *P.aeruginosa* CM07 and *K. pneumoniae* CF09 were consistent with bactericidal characteristic. The kill kinetic profiles of MEGP exhibited varying degrees of bactericidal activities depending on the tested strains. The killing rate of MEGP at 1XMIC was slower against *E. cloacae*AM03 and *K. pneumonia* CF09(complete killing were only seen after 7 h interaction) than against *E.coli* AF05 and *P.aeruginosa* CM07 (complete killing were only seen after 6 h interaction) but the killing rate of MEGP at 2X MIC was found to be similar for all tested Gram negative bacteria - *E. cloacae*AM03, *E.coli* AF05, *P.aeruginosa* CM07 and *K. pneumonia* CF09(complete killing were seen after 5h).MEGP exhibited a faster killing rate against *S.aureus*AM12 and *E.faecalis* AF24, showing bactericidal activity only after 4 h at

both 1X MIC and 2X MIC indicating that increase concentration does not have any significance.

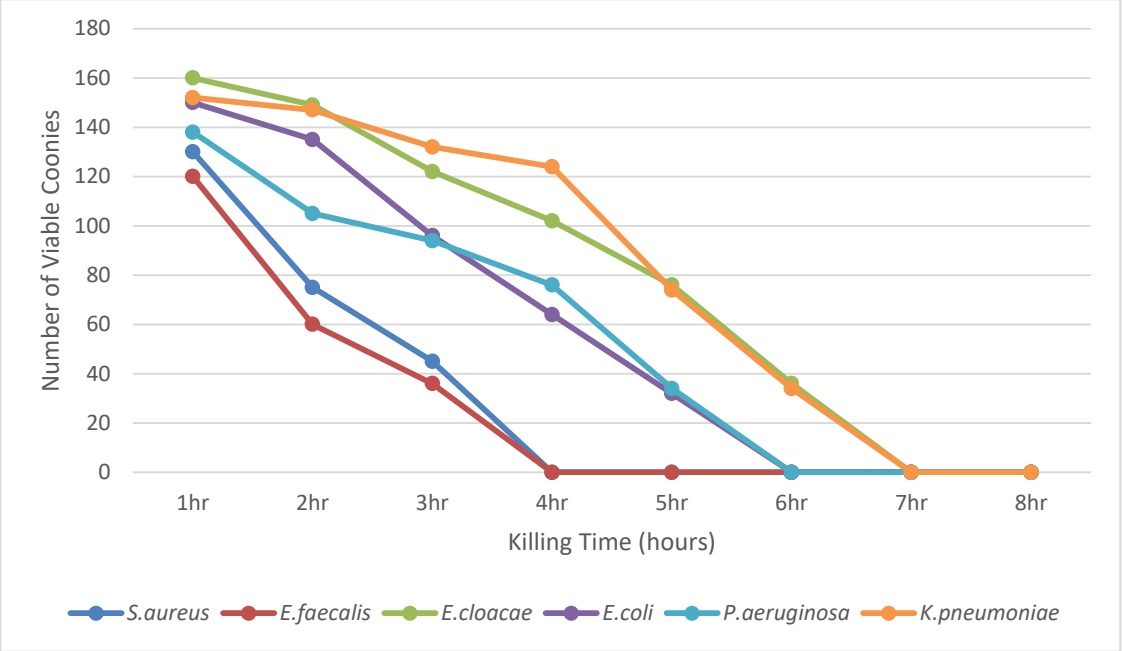


(a) : Time-kill studies of MEPL 1XMIC

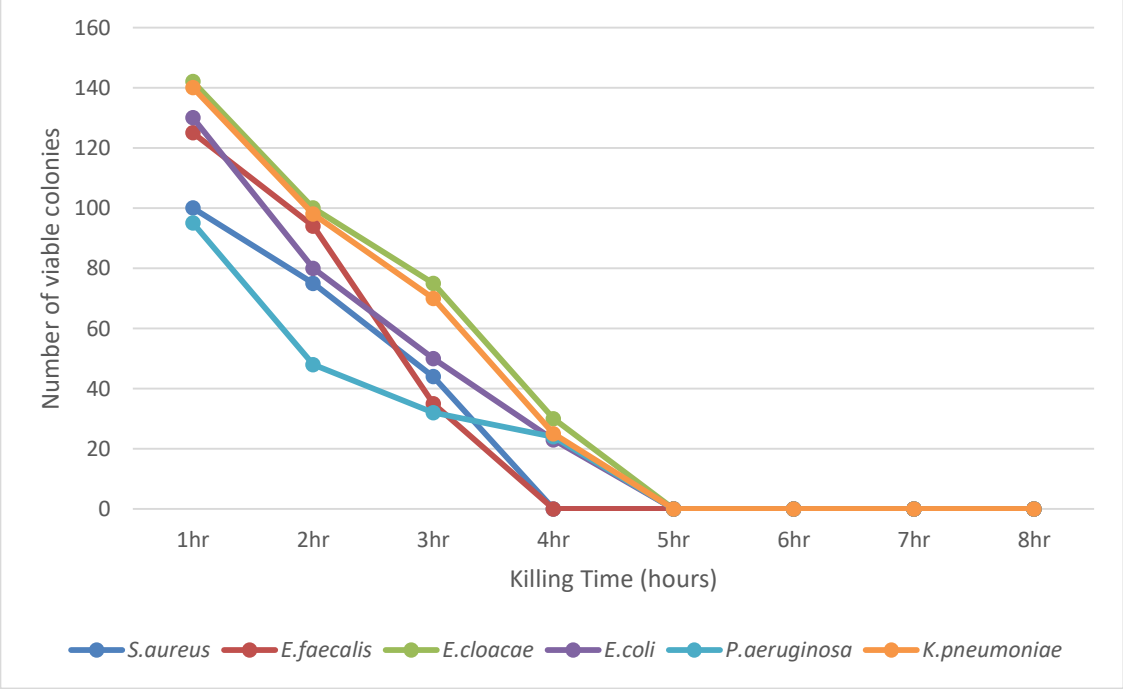


(b) : Time-kill studies of MEPL 2XMIC

Figure 7 (a & b) : Time-kill studies of MEPL



(a) : Time-kill studies of MEGP 1XMIC



(b) :Time-kill studies of MEGP 2XMIC

Figure 8 (a & b): Time-kill studies of MEGP

4.4 Discussion

According to the 31st edition of CLSI performance standard for antimicrobial susceptibility testing,*E.coli* AF05was susceptible to all the 12 standard antibiotics tested;*E. cloacae* AM03was resistant to CAZ,PI,CPM,AT,CPZ and PIT but susceptible to GEN,AK,CIP and LE;*K. pneumoniae* CF09was susceptible to GEN,AK,CIP,LE,IPM and MRP but resistant to CAZ,PI,CPM,AT,CPZ and PIT;*P. aeruginosa* CM07was susceptible to CAZ,GEN,AK,CPM,AT,CIP, LE and IPM; *S. aureus* AM12was susceptible to GEN,CIP and LE;*E. faecalis* AF24was susceptible to CIP and LE.All the five(5) test bacteria viz. *S. aureus* AM12, *E. faecalis* AF24, *E. coli* AF05, *P. aeruginosa* CM07and *K. pneumoniae* CF09, were susceptible to the twelve (12) different antibiotics except *E. cloacae* AM03 which showed resistance towards Ceftazidime, Cefepime and Aztreonam.

There are many factors affecting the performance of antibacterial sensitivity test (AST). The quality of the medium plays major role in antimicrobial susceptibility system. Free electrolyte present in the medium can affect sensitivity of few antimicrobial agents. Hence, the culture medium (Mueller Hinton Agar) used in AST should be monitored to make sure that it contains acceptable concentrations of divalent cations such as Ca^{++} and Mg^{++}, as the increased concentration of Ca^{++} and Mg^{++} results in decreased activity of, for instance, Aminoglycosides and Tetracyclines whereas the decreased concentration of Ca^{++} and Mg^{++} will have the opposite effect (Richard *et al.*, 1979). If MHA contains excessive amounts of thymine or thymidine content, they will reversibly inhibit the action of certain antimicrobial agents such as Sulfonamides, Trimethoprim, Trimethoprim-Sulphomethoxazole etc. which gives smaller or less distinct or even no zones and will be misinterpreted as resistant antibiotics. If zone of inhibition of Sulfonamides, Trimethoprim, Trimethoprim-Sulphomethoxazole will be $\leq$ 20 mm for *E. faecalis* ATCC 29212 can interpret as medium contains high thymine or thymidine content. MHA is low in thymine and thymidine content and it can be used successfully to study the susceptibility of antibiotics(HiMedia Biosciences, 2021).

The pH of the prepared medium is another important parameter of disc diffusion method. The pH of prepared medium should fall within acceptable range which is 7.2 to 7.4 as per CLSI. If the pH is outside the stated parameters, the zone sizes will be altered, with the degree of alteration determined by the antibiotic group. For e.g. an increase in pH will results in a decrease in Penicillin and Tetracycline zones and increase in quinolones, macrolids and aminoglycosides zone; conversely, decrease in pH will results in increase in Penicillin and Tetracycline zones and decrease in quinolones, macrolids and aminoglycosides zone. pH test should perform as a part of the routine quality control tests for each batch of medium prepared (Kincses*et al.,* 2021).

Depth of agar medium in petri plates also affect antimicrobial activity test. Variation in depth of medium will affect the zone of inhibition which can give false positive or negative results. If the depth of agar is too thin, larger zones will appear as the antimicrobial compound will diffuse further than it should. If the depth of agar is too thick, smaller zones will appear as the diffusion of antimicrobial agent will be restricted.Hence, it is advisable to pour the plates on uniform bench to achieve depth of 4 to 5 mm.

The inoculum level is also one of the most important, and variable factors in susceptibility testing, and will have a profound effect on the zone size obtained. The zone edge is determined when the critical mass is achieved. If the inoculum is heavy, then the critical mass will be achieved in a shorter time and the zone will be smaller. If the inoculum is light, the critical mass takes longer to develop, the antibiotic diffuses further and a larger zone is achieved. It is advisable to use a technique which yields a uniform suspension of correct number of organisms i.e. 10^5-10^6 cells/ml. Even if the density of culture is adjusted due to improper inoculation false results could be obtained. Inoculation method includes correct and even swabbing of plates and correct placement of discs (Linton, 1958).

Incubation for correct time and correct temperature is also very important parameter. For example, incubation at temperature above 35°C may fail to detect

Methicillin Resistant *Staphylococci*. At temperatures lower than 35°C, the rate of growth of most bacteria is prolonged. Diffusion of antimicrobial agents also tends to be slow. Increased CO_2 atmosphere results in decreased pH, which affects the activity of some antimicrobial agents. The plates should therefore be incubated in ambient air atmosphere unless CO_2 is necessary for the growth of the organisms. Hence, calibration of instruments is a must at all stages.

The antibacterial activity of the selected 15 ethnomedicinal plantswere evaluated by disc and well diffusion method measuring the diameter of zone of inhibition. *Potentilalineata* and *Garcinia pedunculata*extract were observed to exhibit largest zone of inhibition and therefore, were chosen for comparative study.It was observe that both the organic solvent extract of *P.lineata* produced very effective and notable antimicrobial activities against the two tested Gram positive bacteria (*S. aureus* AM12 and*E. faecalis* AF24)but Gram negative bacteria (*E. cloacae* AM03, *E.coli* AF05, *P. aeruginosa* CM07and*K. pneumoniae* CF09) were not susceptible to the extract.Tomczyk*et al*. (2007) reported that the aqueous extract ofnine *Potentila* species (*P.rupestris,P. argentea,P.fruticoa,P. recta* L,*P.erecta,P. anserine,P.nepalensis*HOOK var Miss Willmott,*P. thuringiaca*BERNH ex LINK,*P. grandiflora* L.) show inhibitory effect on the various species of bacteria. In the present study, one more species of *Potentilla*, named *P.lineata* antibacterial activity and bioactive component was studied. The methanol and ethanol extract of *P.lineata* show moderate antibacterial activity against Gram positive bacteria(*S. aureus*and *E. faecalis*) but no activity was observed against Gram negative bacteria (*E. cloacae, E.coli,P. aeruginosa,K. pneumoniae*). This observation is in good agreement with the findings of Tomczyk*et al.*(2007) since the plant under investigation also belongs to the same genus.

The antibacterial activity can be attributed to its phytochemical constituents detected in LC-ESI-MS study viz. catechin and epicatechin since these flavonoid compound are observed to have inhibitory effect on many species of bacteria (Taylor *et al.*, 2005). The present study also reveals that Gram positive bacteria are more susceptible than Gram negative bacteria against the methanol and ethanol extract of

P.lineata. The relative complexity of the cell wall of Gram negative bacteria than that of Gram positive bacteria may be a factor that require further investigation.

The investigation also revealed the potency of *G.pedunculata* extract as an effective antibacterial agent against both Gram positive bacteria (GPB) and Gram negative bacteria (GNB). *G.pedunculata* are evaluated to be bactericidal against the tested bacteria. The antibacterial activity may be due to an individual compound, mangostin or synergistic effect of more than one compound present in the medicinal plant extract detected in LC-ESI-MS study(Priscilla *et al*., 2007).

The antibacterial activity may be due to an individual compound or synergistic effect of more than one compound present in the medicinal plant extract. Heymsfield*et al.* (1998), Kumar *et al.*(2013) and Aravindakshanpillai*etal.*(2016) reported xanthones, biflavonoids, benzophenones, benzoquinones, and triterpenes are bioactive chemicals found in *Garcinia* species that exhibit antibacterial, antifungal, antioxidant, and cytotoxic properties(Heymsfield*et al*., 1998; Kumar *et al*., 2013; Aravindakshanpillai*et al*., 2016).

The antibacterial activity of MEGP was quantitatively evaluated by determining its minimum inhibitory concentration (MIC) values and minimum bactericidal concentration (MBC). As the bigger zone of inhibition was observed with methanol extract of *G.pedunculata* (MEGP) than that of ethanol extract of *G.pedunculata*(EEGP), MEGP was preferred to EEGP for determination of MIC and MBC.The MEGP is considered to be bactericidal rather than bacteriostatic since its MBC/MIC ratio is $\leq$ 4.An antimicrobial agent is considered bactericidal if the MBC is not more than fourfold higher than the MIC (Levison,2004).

The antimicrobial activity of different species of *Garcinia* had been investigated by various researchers with an encouraging result till date. Negi *et al.* (2008) reported thatcertain foodborne pathogens and spoilage bacteria, including *B.cereus, B.coagulans, B.subtilis, S.aureus,* and *E.coli* are sensitive to crude hexane and chloroform extracts of *Garciniacowa* and *Garciniapedunculata* fruit rinds.Torrungruang*et al.* (2007) also

observed that *G.mangostana*pericarp extract exert antibacterial activity against cariogenic *Streptococcus mutans.*

Priya *et al.* (2010) study revealed the antibacterial activity of pericarp extract of *G.mangostana* against *S. aureus, Staphylococcus albus* and *Micrococcus luteus*.Mangosteen pericarp extract has long been known for its broad-spectrum antibacterial activity against a number of GPB and GNB, particularly those linked to skin infections, diarrhoea, TB, and acne. α-mangostin, one of the xanthone derivatives derived from mangosteen extract, has been shown to have the strongest antibacterial action (Iinuma*et al*., 1996; Sundaram*et al*., 1983; Mahabusarakum*et al*., 1983; Mahabusarakam*et al*., 1986; Sakagami *et al*., 2005; Suksamrarn*et al*., 2003).

The present investigation revealed the potency of both methanol and ethanol extracts of *G.pedunculata* as an antibacterial agent. *G.pedunculata* is effective as an inhibitory agent against both GPB as well as GNB. The antibacterial activity can be attributed to its phytochemical constituents (Priscilla *et al*., 2007).

It can be concluded that MEGP possess potent and differential activity against Gram-positive and Gram-negative bacteria pathogens while MEPL shows antibacterial activity towards only Gram-positive bacteria. As the antibacterial activities and bacterial killing rates of MEPL and MEPL were different from each other, it is likely that different mechanisms are involved. Further investigation is needed to determine the mechanism(s) of action of these extracts in order to strengthen their potential as therapeutic antibiotics. MEGP, in particular, with its potent and specific antibacterial profile, deserve further investigation.

Time-kill studies have provided valuable information on the rate, concentration and potential action of MEGP and MEPL in vitro. Time-kill assays have been widely used for in vitro investigations of new antimicrobial agents as these provide descriptive (qualitative) information on the pharmacodynamics of antimicrobial agents (Lewis, 2007). Time-kill curves that monitor bacterial growth and death over a wide range of

antimicrobial concentrations have been frequently used to evaluate the effect of antimicrobials over time. Information about the effects of antimicrobials covering a wide range of antimicrobial concentrations below and above the MIC is particularly valuable for pathogens where data about PK/PD effects are limited.

In summary and consistent with the MBC/MIC ratios, at its MIC value, MEGP were found to be bactericidal towards all the tested bacteria after 7 hours exposure, whereas MEPL was observed to be bactericidal towards only tested Gram-positive bacteria after 5 hours exposure. MEGP possess potent and differential activity against Gram-positive and Gram-negative bacteria pathogens while MEPL shows antibacterial activity towards only Gram-positive bacteria.

Chapter 5

Qualitative and Quantitative Phytochemical Screening of Selected Medicinal Plants

Introduction

Phytochemicals (from the Greek word "phyto", meaning plant) are biologically active, naturally occurring chemical compounds found in plants (Hasler *et al.,* 1999). They protect plants from disease and damage and contribute to the plant's color, aroma and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called as phytochemicals (Gibson *et al.*, 1998).

Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds (Costa *et al.*, 1999). Many phytochemicals, particularly the pigment molecules, are often concentrated in the outer layers of the various plant tissues. The quantitative amount vary from plant to plant depending upon the variety, processing, cooking and growing conditions (King *et al.*, 1999). These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. There are more than thousand known and many unknown phytochemicals. It is well-known that plants produce these chemicals to protect themselves, but recent researches demonstrate that many phytochemicals can also protect human against diseases (Narasinga, 2003).

Phytochemicals may be either primary or secondary metabolites, which are naturally occurring in the leaves, vegetables or roots that have defense mechanism and protect from various diseases. Primary metabolites are proteins, carbohydrates,

chlorophyll, lipids and common sugars, which are synthesized during photosynthesis, and these organic compounds are essential for plant life and growth and development (Wadood *et al.*, 2013). Secondary metabolites are tannins, flavonoids, phenolics, saponins and alkaloids, which are synthesized by the plant during development and are time, tissue and organ specific (Linga *et al*., 2012).

Secondary plant metabolites (Phytochemicals) have been extensively investigated as a source of medicinal agents (Krishnaraju, 2005). Plants can synthesize and accumulate a great variety of phytochemicals in their cells including saponins, tannins, flavonoids, cyanogenic, phenolic compounds, lignins, lignans, alkaloids and glycosides (Okwu, 2004). Plants also have a great potency of antimicrobial activity due to the presence of phenolic compounds and essential oils (Aboaba and Efuwape, 2001). Medicinal plants have been known to produce an array of phytochemicals with recognized antibacterial activity belonging to chemical structural classes: phenolic, terpenoids, alkaloids, lectins, polypeptides, and polyacetylenes but the most bioactive constituents are alkaloids, tannins, flavonoids, and phenolic compounds (Hill, 1952). The screening of plant extractsand plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes (Afolayan, 2003). Numerous studies have identified compounds within herbal plants that are effective antibiotics (Afolayan, 2003). Some of the commonly used traditional remedies have already produced compounds that are effective against antibiotic-resistant strains of bacteria (Kone *et al*., 2004).

Alkaloids are a group of naturally occurring compounds that contain nitrogen and can be neutral or have weak acidic properties (Mc Naught, 1997). They may also sometimes contain oxygen, Sulphur, more rarely other elements such as chlorine, bromine, and phosphorus (Schardl *et al*., 2007). They are mainly secondary metabolites of plants but can also be produced by a variety of organisms including bacteria, fungi, and animals (Kittakoop *et al*., 2014). They dissolve in water poorly but readily dissolve in organic solvents (Shi *et al*., 2104). They are divided into five major groups namely:

true alkaloids (contain nitrogen in heterocyclic and originate from amino acids), proto alkaloids, polyamine alkaloids, peptide and cyclopeptides alkaloids and pseudoalkaloids (Faulkner *et al*., 2006). In the recent past, based on the analogy that one containing a *non-heterocyclic nucleus* and the other having the *heterocyclic nucleus,* alkaloids have been divided into *two* major categories- (*a*) *Non-heterocyclic Alkaloids* (*b*) *Heterocyclic Alkaloids*

Flavonoids or bioflavonoids are polyphenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain having the carbon skeleton C6 - C3 - C6. Flavonoids or bioflavonoids are secondary metabolites of plants (Mc Naught, 1997). Flavonoids or bioflavonoids are named from the Latin word *flavus*, meaning yellow, and are ubiquitous in plants; these compounds are the most abundant polyphenolic compounds (Prasad *et al*., 2010; Castellarin *et al.,* 2007). There are over 5000 naturally occuring flavonoids that have been characterized from various plants according to their chemical structure (Ververidis *et al*., 2007).

Flavonoids can be divided into multiple subgroups according to the substitution patterns of the ring C, and flavonoids within the same class can be differentiated by the substitution of A and B. There are six major subgroups of flavonoids, including flavonols (including quercetin, kaempferol, and myricetin), flavanones (including eriodictyol, hesperetin, and naringenin), isoflavones (including daidzein, genistein, and glycitein), flavones (including apigenin and luteolin), flavans-3-ol (including catechin), and anthocyanins (including cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin) (Havsteen, 2002; Prasain *et al.*, 2010; Middleton, 1998).

Saponins are naturally occurring surface-active glycosides. The word 'saponin' is derived from the Latin letters 'sapo' means soap and traditionally saponin-containing plants have been utilized for washing. They are mainly produced by plants (Alfalfa, Soyabean, Lucerne, Berseem, Yucca, Mahua, Guar etc.), but also by lower marine animals (sea cucumbers, starfish etc.) and some rhizo bacteria. They derive their name

from their ability to form stable, soap-like foams in aqueous solutions (Yoshiki *et al*., 1998).

Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone (sapogenin) which may may be triterpenoid or steroid in nature. The aglycone may contain one or more unsaturated C–C bonds. The oligosaccharide chain is normally attached at the C3 position (monodesmosidic), but many saponins have an additional sugar moiety at the C26 or C28 position (bidesmosidic). The great complexity of saponin structure arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these moieties on the aglycone.They are a class of chemical compounds found in various plant species and they are amphipathic glycoside grouped structurally by having one or more hydrophilic glycosides moieties combined with lipophilic triterpene (Hostettmann and Martson, 1995). In plants, saponins are known to provide protection against microbes and fungi (Riguera, 1997).

According to the nature of the aglycone, saponins are classified into steroidal saponin and triterpenoidal saponin. They are also sometimes classified as monodesmoside (one sugar chain) or bidesmoside (two sugar chain) on the basis of number of sugar moiety.

Tannin is astringent vegetable product found in a wide range of plants parts ranging from the barks, roots, seeds, fruits, leaves, galls and roots. The name ‘tannin’ is derived from the French ‘tanin’ (tanning substance) and is used for a range of natural polyphenols (Rompp, 1997). They occur naturally in plants and are water soluble phenolic compounds of the higher molecular weight of about 500 - 3000 containing phenolic hydroxyl groups that make them to effectively cross-link with proteins and other macromolecules (Ramakrishnan, 2006). The tannins are broadly classified into two groups: (i) *Hydrolysable tannins* (ii) *Condensed tannins*

Phytosteroids constitute a diverse group of natural products. Biosynthetically, they are derived from *S*-squalene-2,3-epoxide via acetate-mevalonate pathway. Among the plant steroids, phytosterols are ubiquitous in the plant kingdom (Kamal, 2014). Phytosteroids are plant steroids that may ormay not act as weak hormones in the body. They share a common basic ring structure, cyclopentanoperhydrophenanthrene, withanimal steroids though they are not equivalentbecause of varying chemical groups attachedto the main ring in different positions.

When a hydroxyl group is attached to the C-3 atom of the A-ring and an aliphatic side chain of 8-10 carbon is attached to the C-17 atom of the D-ring of steroid nucleus, it is called sterol. Phytosterols have a double bond, typically between C-5 and C-6 of the sterol moiety, whereas this bond is saturated in phytostanols. It occurs in animals, plants, fungi and bacteria. Sterols which are obtain from the plant kingdom called phytosterol. Eg. Stigmasterol, Sitosterol (Ngoci *et al*., 2011).

Cardiac glycosides, which are highly toxic and found in a number of plants, are usually phytochemicals consisting of an aglycone (structurally related to steroid hormones) linked to one or more sugar molecules. The general structure of a cardiac glycoside consists of a steroid nucleus to which a sugar (glycoside) is attached at C-3 and an unsaturated conjugated lactone ring/an R group is attached at C17 position.The steroid nucleus consists of four fused rings to which other functional groups such as methyl, hydroxyl, and aldehyde groups can be attached to influence the overall molecule's biological activity. Common monosaccharides found in cardiac glycosides are glucose, rhamnose and 6-deoxy monosaccharide (Harborne, 1973).

According to the type of aglycone (unsaturated lactone ring) attached to the C17 position of the cardiac glycoside, the cardiac glycoside can be classified into two types: the cardenolide (with a five-membered unsaturated lactone ring) and the bufadienolides (with a six-membered unsaturated lactone ring) (Afolabi *et al*., 2014).

5.2 Materials and Methods

5.2.1 Qualitative phytochemical analysis

Preliminary qualitative phytochemical analysis of the crude extract of the 15 plants collected was determined as as per standard methods described by Brain and Turner (1975) and Evans (1996).

5.2.1.1 Alkaloid

Alkaloids (Dragendorff Test) – About 2 ml methanol extract of plant material powder was mix with 1% HCl and then keep in a boiling water bath for 2 minutes. Appearance of brownish-red precipitate/orange precipitate on addition of few drops of Dragendorff reagent respectively indicated the presence of alkaloids.

5.2.1.2 Flavonoid

Flavonoids (Shinoda Test) – To the ethanol extract (2ml) of plant material, conc. HCl was added and then followed by addition of magnesium ribbon. Formation of pink-red color indicated the presence of flavonoids.

5.2.1.3 Saponin

Saponin (Foam Test) - Appearance of froth and its persistence on addition of 5 ml distilled water to 0.5 ml of water extract indicated presence of saponin.

5.2.1.4 Tannin

Tannins (Ferric Chloride Test) - Formation of blue-black precipitate/brownish green on addition of 2 ml of 5% $FeCl_3$ to 2 ml of water plant extract indicated the presence of Tannins.

5.2.1.5 Steroid

Steroids (Liebermann-Burchard reaction) - Appearance of blue-green ring on addition of 2 ml acetic anhydride and conc. H_2SO_4 to 2ml chloroform extract of plant material indicated the presence of terpenoids.

5.2.1.6 Cardiac glycoside

Cardiac glycosides (Keller-Kiliani test) - Add 1 ml glacial acetic acid to 2 ml of methanol plant extract, followed by the addition of $FeCl_3$ and conc. H_2SO_4. Appearance of green-blue color indicated the presence of cardiac glycosides.

5.2.2 Quantitative phytochemical analysis

The alkaloid content was determined gravimetrically by following Harborne (1973) Method. The flavonoids were estimated spectrophotometrically by the method of Zhishen *et al*. (2010) whereas saponins and tannins were estimated by the method of Hiai *et al.* (1976) and Van-Burden and Robinson (1981) respectively.

5.2.2.1 Estimation of Alkaloid Content

Quantitative determination of alkaloid was persued gravimetrically according to the methodology by Harborne (1975). Five (5) g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed. The results were expressed in terms of μg of alkaloid / mg of crude extract sample.

5.2.2.2 Estimation of Flavonoid Content

Preparation of standard solution*:* Ten (10) mg quercetin was weighed and made up to 10ml with Methanol in a 10ml volumetric flask. From the above solution (1mg/ml), 1ml was pippeted out and made up to 10ml with Methanol to get 100mcg/ml quercetin standard solution (stock solution). From the stock solution, solutions of concentration 25, 50, 75, 100, 125 and 150 mcg/ml were prepared. To each of these 4ml water was added followed by 0.3ml of 5% sodium nitrite. After 5min 0.3ml of 10% $AlCl_3$ (aluminium chloride) solution and at the 6th minute 2ml of 1M NaOH/Sodium hydroxide was added. The total volume was made up to 10ml with distilled water. A blank was prepared without addition of aluminium chloride solution. The solutions were mixed well and the absorbance was measured against the blank at 510nm using UV-Visible spectrophotometer. A standard graph was plotted using various concentrations of quercetin and their corresponding absorbance.

Preparation of sample solution: The total flavanoids content of each plant extract was estimated by method described by Zhishen *et al*. (2010). Based on this method, 5 mg of each sample extract were mixed with 4 ml of distilled water and subsequently with 0.30ml of $NaNO_2$ solution (10%). After 5min, 0.30ml $AlCl_3$ solution (10%) was added followed by 2.0ml of NaOH solution (1%) to the mixture. Immediately, the mixture was thoroughly mixed and absorbance was then determined at 510 nm versus blank. Standard curve of quercetin was prepared (0-12 mg/ml) and the results were expressed in terms of µg of quercetin equivalents / ml of crude extract sample.

5.2.2.3 Estimation of Saponin content

Saponin contents in different plant materials were estimated by prescribed colorimetric methods (Hiai *et al.*, 1976). Five (5) mg of plant extract were dissolve in 5 ml 80% aqueous methanol and 50 µl of solution was taken in different test tubes to which 0.25 ml of vanillin reagent (8%, w/vin 99.9% ethanol) was added. Test tubes were placed in ice-cold water bath and 2.5 ml of 72% (v/v) H_2SO_4 was added slowly on

the inner side of the wall. After mixing the content in each tube, these were left as such for 3 min. Warm the tubes to 60^0C for 10 min using a water bath and then cooled them in ice-cold water bath. Absorbance was measured at 544 nm using spectrophotometer against the reagent blank and prepared the standards curve. The content of total saponin was expressed in terms of µg of saponin / ml of crude extract sample.

5.2.2.4 Estimation of Tannin content

Tannin content was determined using the method described by Van-Burden and Robinson (1981). Five (5) mg of the sample extract was weighed into 100ml of plastic bottle 50ml of distilled water was added and shaken for 1 hour in a mechanical shaker. This was filtered into 50ml volumetric flask and made up to the mark. Then 5ml of the filtrate was pipetted out into a test tube and 3ml of 0.1 M $FeCl_3$ in 0.IN HCl and 0.008M potassium ferrocyanide was added and mixed. The absorbance of the resultant solution was measured in a spectrophotometer at 120nm wavelength within 10mins. A blank sample was prepared and the color also developed and read at the same wavelength. A standard was prepared using tannic acid and measured. The results was expressed in terms of µg of tannic acid equivalents / ml of crude extract sample.

5.2.3 Thin layer chromatography

Phytochemical analysis by TLC were carried out by following the method of Harborne (Harborne, 1998). TLC is a quick, sensitive, and inexpensive technique, which separates the number of components present in any non-volatile complex mixture or plant sample using a suitable solvent for separation of different components.

For the separation of different phytochemical compounds in the methanol extract of 15 ethnomedicinal plants, the extract was spotted manually using a capillary tube on pre-coated silica gel 60 F254 TLC plates (15X5 cm with 3 mm thickness). The spotted plates were developed in the appropriate solvent system. Different compositions of the mobile phase were tried in order to separate and obtain better resolution of the different secondary metabolites and to detect the suitable mobile phase as per the method of

Wagner *et al*. (Wagner, 1984, 1996). The various combinations of mobile phase allowed the separation of different components of the plant extracts that had distinct Rf values and develop TLC fingerprint profile. For each extract, six different solvent system were used as developing solvents. These were Chloroform : Methanol (4 : 2), Ethyl Acetate : Methanol : Water : Glacial Acetic Acid (1.35 : 0.5 : 0.5 : 0.5), Chloroform : Methanol (6 : 1), Methanol : Water (6 : 4), Hexane : Ethyl Acetate (4 :1) and Ethyl Acetate : Methanol : Water (8.1 : 1.1 : 0.8). After the separation of phytochemical constituents, the spraying reagents namely, Dragendroff reagent (for solvent system I), 5% Ferric Chloride solution (for solvent system II & IV), Vanillin Sulphuric Acid (for solvent system III &VI), Vanillin Phosphoric Acid (for solvent system V) were used to detect the bioactive compounds. After derivatization with proper spraying reagent, the bands/spots developed were noted and Rf values were calculated by using the following formula:

Retention time (Rf) = Distance travelled by the solute/Distance travelled by the solvent

The Rf value is a measure of the distance a compound travels. In each case the TLC spots were visualized under day light or UV light (254 nm or 356 nm).

5.3 Results

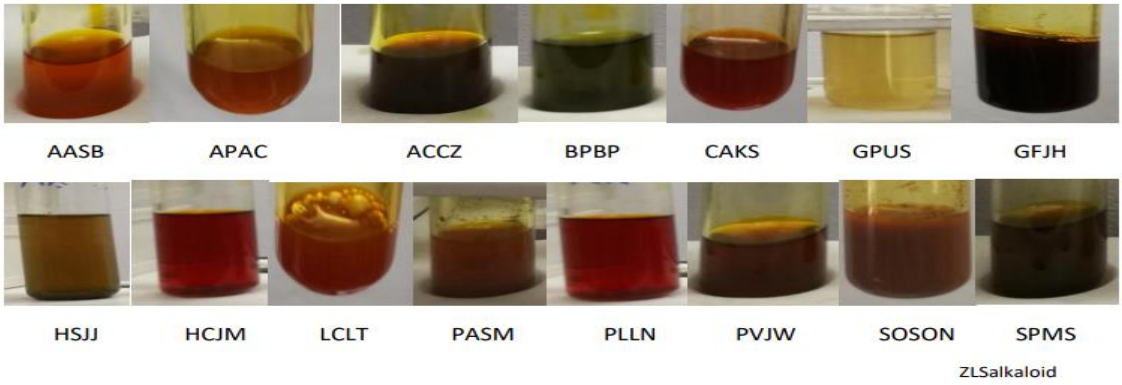
5.3.1 Qualitative Phytochemical Test

The result of the phytochemical group tests for extract of 15 (fifteen) medicinal plants is given in Table 6 and Fig. 9. The result of the phytochemical group test revealed that there was great diversity in the phytochemical content of various species of plants.

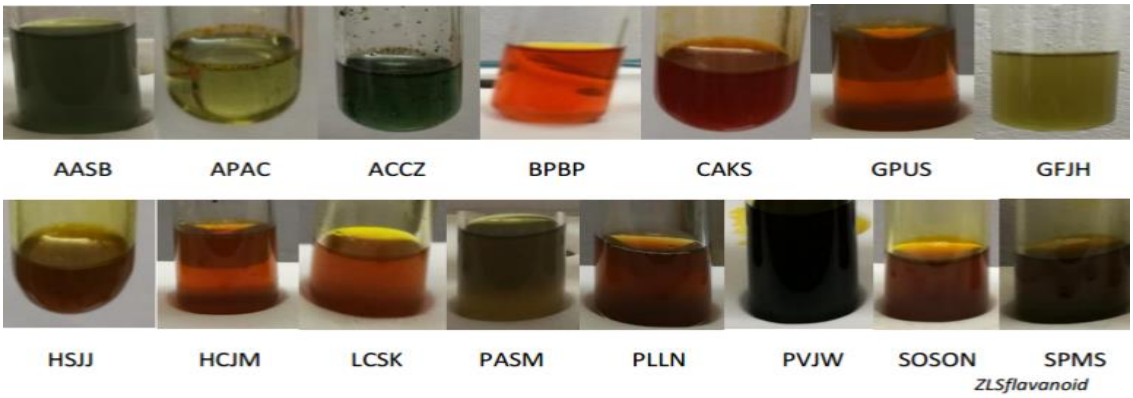
Table 6: Preliminary phytochemical group tests of the crude extract of the fifteen (15) medicinal plants.

SN	Medicinal plants	Alkaloid	Flavonoid	Saponin	Tannin	Steroid	Cardiac glycoside
1	*Achyranthes aspera*	+	-	+	+	+	+
2	*Acmella paniculata*	+	-	-	-	+	-
3	*Ageratum conzyoides*	+	-	-	-	+	+
4	*Bidens pilosa*	-	+	+	-	+	-
5	*Centella asiatica*	+	+	-	+	+	+
6	*Garcinia pedunculata*	-	+	-	-	-	-
7	*Gaultheria fragrantissima*	-	-	-	+	+	-
8	*Hibiscus sabdarifa*	-	+	-	-	+	+
9	*Houttuynia cordata*	+	+	-	-	+	-
10	*Lantana camara*	+	+	+	+	+	+
11	*Piper attenuatum*	+	-	-	-	+	-
12	*Potentilla lineata*	+	+	-	+	+	-
13	*Prunella vulgaris*	+	+	+	+	+	-
14	*Sonchus oleraceus*	+	+	+	+	+	-
15	*Sonchus palustris*	+	+	-	+	+	-

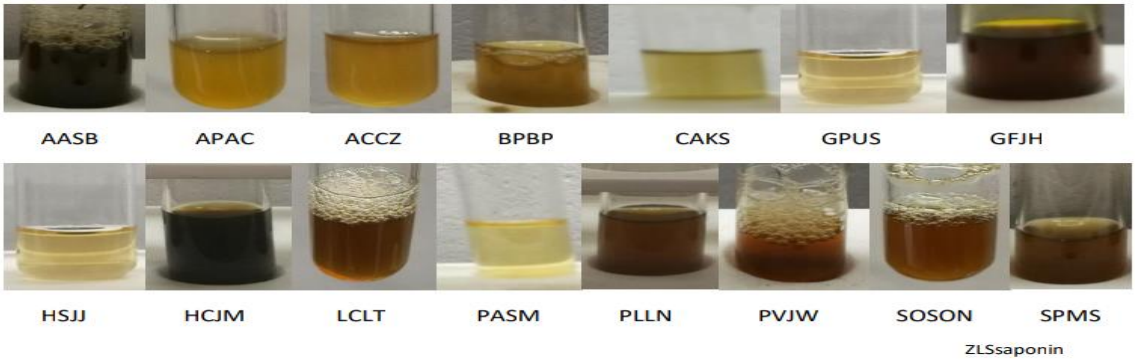
Note: + (Positive) = present - (Negative) = absent.



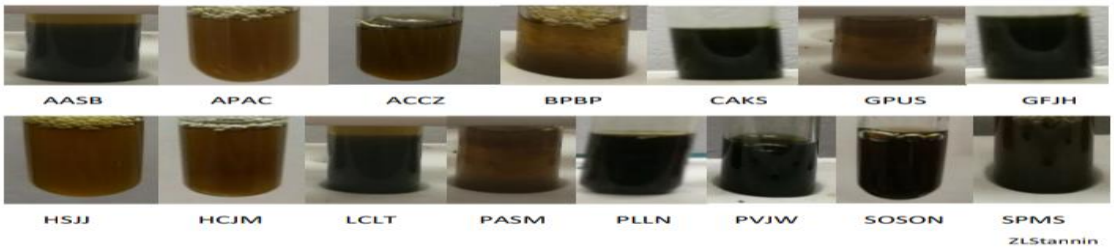
i) Dragendorff Test: Appearance of brownish-red precipitate/orange precipitate on addition of few drops of Dragendorff reagent respectively indicated the presence of alkaloids.



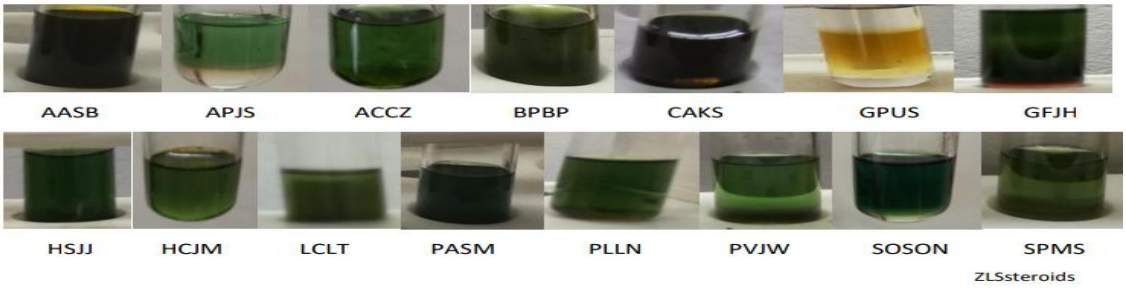
ii) Shinoda Test: Formation of pink-red color indicated the presence of flavonoids



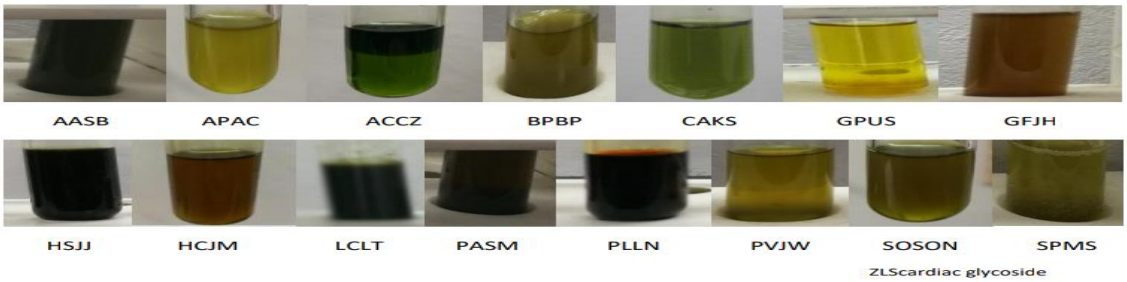
iii) Foam Test: Appearance and persistence of froth indicate presence of saponin



iv) Ferric chloride Test: Formation of blue-black precipitate/brownish green indicate presence of tannin



v) Libermann Test: Appearance of blue-green ring indicate presence of steroids



vi) Keller Kiliani Test : Appearance of green-blue color indicated the presence of cardiac glycosides)

Figure 9: Qualitative Phytochemical Test

Note: PLLN-*Potentilla lineata*; HSJJ-*Hibiscus sabdarifa*; LCLT-*Lantana camara*; HCJM-*Houttuynia cordata*; GPUS-*Garcinia pedunculata*; CAKS-*Centella asiatica*; SPMS-*Sonchus palustris*; GFJH-*Gaultheria fragrantissima*; PASM-*Piper attenuatum*; AASB-*Achyranthes aspera*; PVJW-*Prunella vulgaris*; ACCZ-*Ageratum conzyoides*; BPBP-*Bidens pilosa*; APAC-*Acmella paniculata*; SOSON-*Sonchus oleraceus;* CFCF-*Caffeine;* QCT-*Quercetin;* SPN-*Saponin;* TATA-*Tannic acid;* SSSS- *Stigmasterol;* DGDG-*Digoxin*

5.3.2 Quantitative Phytochemical Test

5.3.2.1 Estimation of Alkaloid content

Alkaloid content in the extract of 15 (fifteen) ethnomedicinal plants were shown in the Table 7. *S. palustris* contain highest total alkaloid content viz.13.2 µg/ml while the least alkaloid content (2.6 µg/ml) was estimated for *A. aspera.*

5.3.2.2 Estimation of Flavonoid content

Flavonoid content in the extract of each selected ethnomedicinal plants were shown in the Table 7. *S. oleraceus* contain highest total flavonoid content viz.155.97 µg/ml while the least flavanoid content (50.135 µg/ml) was estimated for *S. palustris.*

5.3.2.3 Estimation of Saponin content

Saponin content in the extract of the selected ethnomedicinal plants were shown in the Table 7. *B. pilosa* contain highest total saponin content viz.74.73 µg/ml while the least saponin content (47.36 µg/ml) was estimated for *P. vulgaris*.

5.3.2.4 Estimation of Tannin content

Tannin content in the extract of the selected ethnomedicinal plants were shown in the Table 7. *C. asiatica* contain highest total tannin content viz.71.77 µg/ml while the least tannin content (15.04 µg/ml) was estimated for *S. oleraceus*.

5.3.3. Thin Layer Chromatography

The present study was oriented towards the phytochemical screening of the 15 ethnomedicinal plants and development of TLC fingerprints using TLC technique.

The results of Thin Layer Chromatography are presented in Table 11. Clear separated resolved bands/spots were observed in three solvent systems i.e., Chloroform: Methanol (6:1), Hexane : Ethyl acetate (4:1) and Ethyl acetate : Methanol:Water:Glacial acetic Acid (1.35:0.5:0.5:0.5) after being sprayed with reagent. Therefore, they are recommendable as a solvent system for further analysis.

The evaluations of various plants extract showed presence of different bioactive compounds as indicated by varying number of spots on a TLC plate and different Rf values (Table 8). The TLC profiles are depicted in Plate 41 (a-f).

Table 7: Quantitative phytochemical test:

SN	Medicinal plants with part used	Alkaloid (µg/mg)	Flavonoid (µg/ml)	Saponin (µg/ml)	Tannin (µg/ml)
1	*Achyranthes aspera* (leaves)	2.6±0.6	ND	63.54±2.25	69.05±3.29
2	*Acmella paniculata* (flower)	7.0±1.5	ND	ND	ND
3	*Ageratum conzyoides* (leaves)	9.4±3.5	ND	ND	ND
4	*Bidens pilosa* (leaves)	ND	79.29±0.01	74.73±1.29	ND
5	*Centella asiatica* (leaves)	12.2±4.5	60.89±4.87	ND	71.77±1.55
6	*Garcinia pedunculata* (fruit)	ND	101.89±6.82	ND	ND
7	*Gaultheria fragrantissima* (leaves)	ND	ND	ND	40.46±2.71
8	*Hibiscus sabdarifa* (fruit)	ND	94.06±0.47	ND	ND
9	*Houttuynia cordata* (leaves)	13±2.5	127.43±4.88	ND	ND
10	*Lantana camara* (leaves)	6.4±2.5	120.34±0.31	63.37±2.89	53.43±0.15
11	*Piper attenuatum* (fruit)	4.2±3.0	ND	ND	ND
12	*Potentilla lineata* (root)	7.4±2.5	72.39±18.50	ND	57.92±0.07
13	*Prunella vulgaris* (leaves)	8.6±2.5	82.86±9.74	47.36±1.0	62.17±11.02
14	*Sonchus oleraceus* (leaves)	7.0±3.0	155.97±3.9	60.61±1.93	15.04±0.37
15	*Sonchus palustris* (leaves)	13.2±1.5	50.135±6.8	ND	61.99±5.81

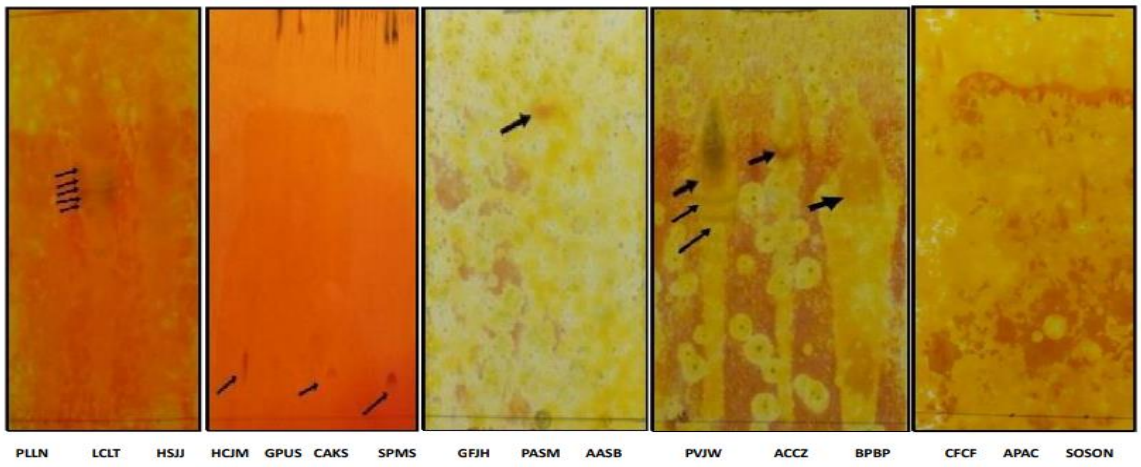
Note: i) Quantitative value are given as mean ± SD; (n = 3) ii) ND = Not Determined

Table 8: Thin Layer Chromatographic study of methanol extract of the fifteen (15) medicinal plants

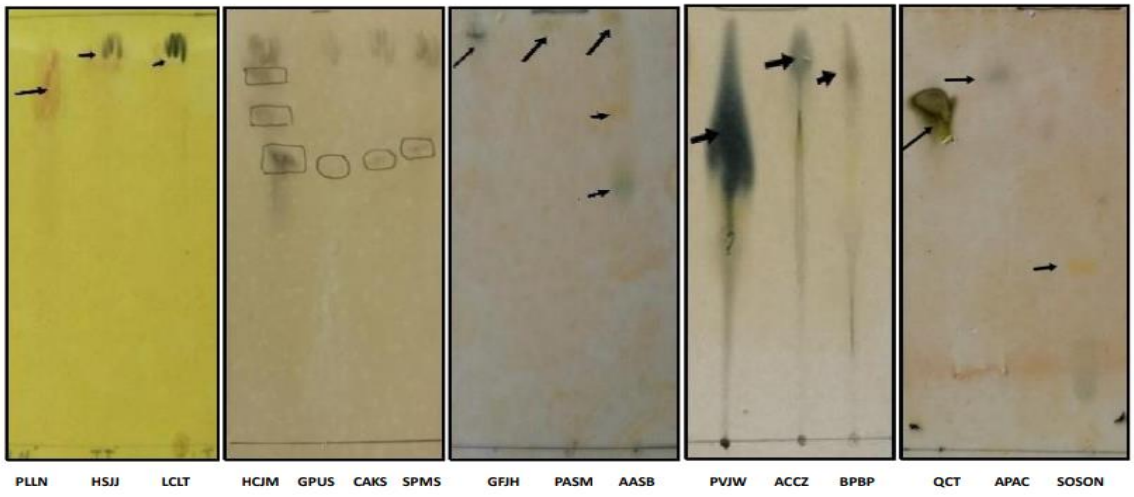
SN	Medicinal Plants	Rf value in different Developing Solvent System					
		$CHCl_3$: MeOH 3:2 Alkaloid	**EA : MeOH : H2O : GAA 1.35:0.5:0.5:0.5 Flavonoid**	**$CHCl_3$: MeOH 6:1 Saponin**	**MeOH: H2O 6:4 Tannin**	**Hx:EA 4:1 Steroid**	**EA : MeOH : Hx 8.1:1.1:0.8 Cardiac glycoside**
1	*Achyranthes aspera* (leaves)	No spot	0.99,0.76 0.60	0.88	0.84	0.95,0.84 0.09	0.87,0.83
2	*Acmella paniculata* (flowers)	No spot	0.85	0.62,0.54	No spot	No spot	No spot
3	*Ageratum conzyoides* (leaves)	0.64	0.75	0.63,0.54 0.50,0.44	0.92	0.96,0.64	0.93
4	*Bidens pilosa* (leaves)	0.56	0.72	0.63	0.92	0.87,0.71	0.93
5	*Centella asiatica* (leaves)	0.11	0.65	0.84,0.67 0.57,0.46 0.41,0.15	0.83	0.95,0.48	No spot
6	*Garcinia pedunculata* (fruit)	No spot	0.64	0.18,0.20	0.51	0.95,0.90 0.48	No spot
7	*Gaultheria fragrantissima* (leaves)	No spot	0.95	0.82,0.67	0.84	0.96,0.17	0.60
8	*Hibiscus sabdarifa* (leaves)	0.67,0.52 0.49	0.91	0.60,0.52	0.68,0.64	0.98,0.94 0.75,0.64 0.59,0.53 0.48,0.35	0.90 0.77
9	*Houttuynia cordata* (leaves)	0.14	0.65,0.75 0.85	0.80,0.41 0.14	0.86,0.74 0.51	No spot	0.87,0.35 0.51
10	*Lantana camara* (leaves)	0.61,0.57 0.55,0.53 0.51	0.91	0.59,0.52 0.47,0.35 0.29,0.27 0.22	No spot	0.98,0.94 0.88,0.75 0.60,0.50 0.43,0.35 0.28,0.19	No spot

						0.10	
11	*Piper attenuatum* (fruit)	0.75	0.96	0.92	No spot	0.96,0.88 0.77,0.56 0.44,0.32 0.09	0.77,0.54
12	*Potentilla lineata* (root)	No spot	0.82	0.62	No spot	0.98,0.94 0.50,0.34 0.28,0.13	No spot
13	*Prunella vulgaris* (leaves)	0.57,0.54 0.50	0.61	0.66,0.51 0.50,0.43	0.90	0.93,0.13	0.93,0.49 0.20
14	*Sonchus oleraceus* (leaves)	No spot	0.41	0.70,0.66 0.62,0.57	No spot	No spot	No spot
15	*Sonchus palustris* (leaves)	0.08	0.68	0.87,0.73 0.66,0.50 0.14	0.86	0.98,0.94 0.65,0.55 0.48,0.33 0.31,0.25	0.89,0.81 0.72,0.61

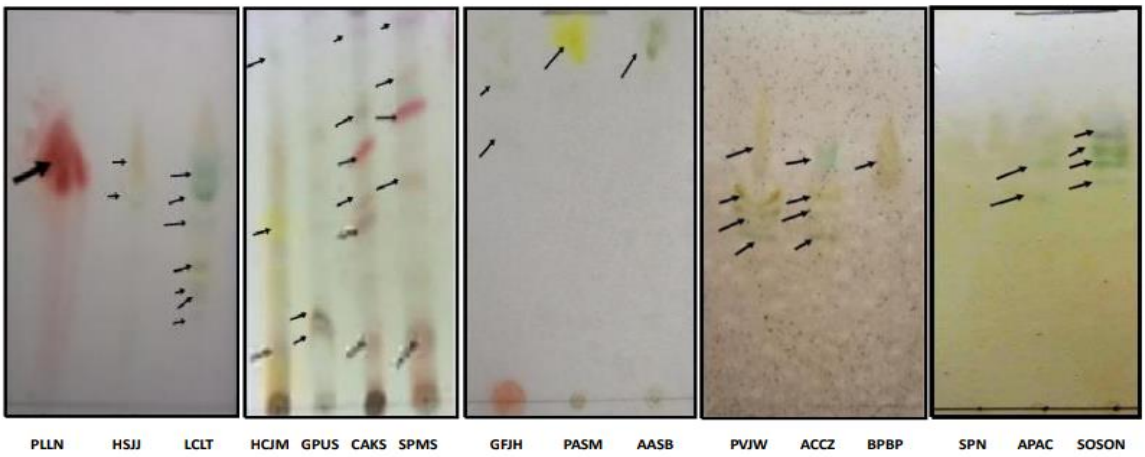
Note: Hexane = Hx, ethyl acetate = EtOAc, chloroform = CHCl3, methanol = MeOH, water = H2O, and glacial acetic acid = GAA.



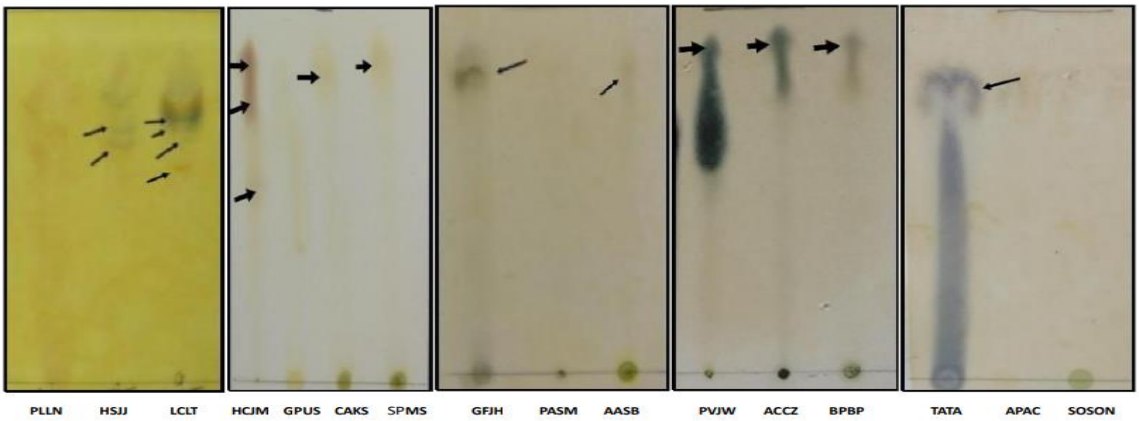
(a): TLC plates developed in Solvent System I



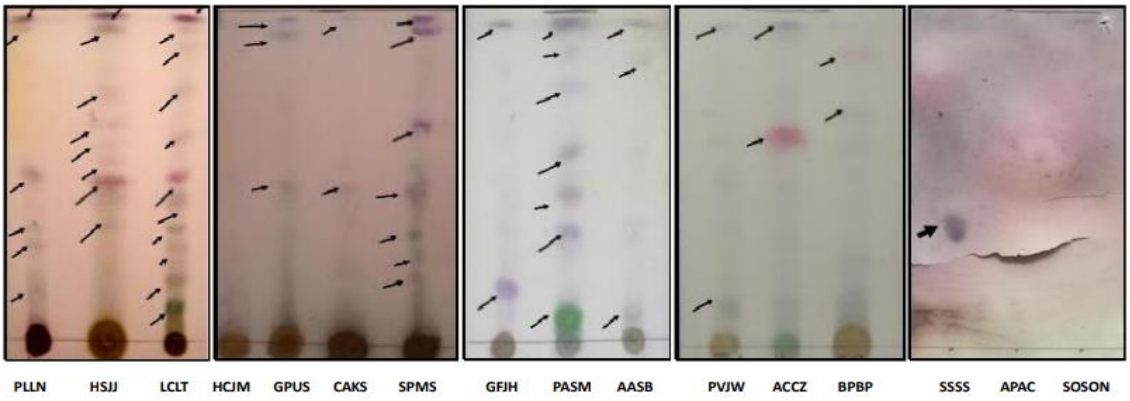
(b): TLC plates developed in Solvent System II



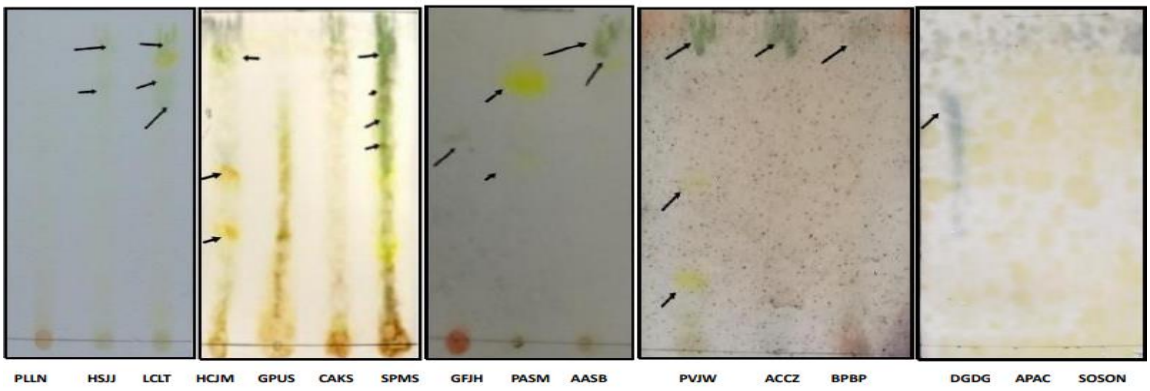
(c): TLC plates developed in Solvent System III



(d): TLC plates developed in Solvent System IV



(e): TLC plates developed in Solvent System V



(f): TLC plates developed in Solvent System VI

Plate 41 (a–f): TLC fingerprint / profile of methanol extract of 15 ethnomedicinal plants in different Solvent Systems (Arrow indicates band).

Note: PLLN-*Potentilla lineata*; HSJJ-*Hibiscus sabdarifa*; LCLT-*Lantana camara*; HCJM-*Houttuynia cordata*; GPUS-*Garcinia pedunculata*; CAKS-*Centella asiatica*; SPMS-*Sonchus palustris*; GFJH-*Gaultheria fragrantissima*; PASM-*Piper attenuatum*; AASB-*Achyranthes aspera*; PVJW-*Prunella vulgaris*; ACCZ-*Ageratum conzyoides*; BPBP-*Bidens pilosa*; APAC-*Acmella paniculata*; SOSON-*Sonchus oleraceus;* CFCF-*Caffeine;* QCT-*Quercetin;* SPN-*Saponin;* TATA-*Tannic acid;* SSSS- *Stigmasterol;* DGDG-*Digoxin*

5.4 Discussion

Phytochemical screening revealed variations and diversity in the content of phytochemical compounds, qualitatively and quantitatively, for the 15 selected medicinal plants investigated. The variations and diversity was observed not only between the family and genus, but also between the species.

Alkaloid were found to be present in eleven (11) of the medicinal plants Viz. *Achyranthes aspera, Acmella paniculata, Ageratum conzyoides, Centella asiatica, Houttuynia cordata, Lantana camara, Piper attenuatum, Potentilla lineata, Prunella*

vulgaris, Sonchus oleraceus and *Sonchus palustris*. *S. palustris* contain highest total alkaloid content viz.13.2 µg/ml while the least alkaloid content (2.6 µg/ml) was estimated for *A. aspera*. Alkaloid have a wide range of pharmacological activities such as antiasthma, antimalarial, anticancer, cholinomimetic, vasodilatory, antiamyhyrithic, analgesic, antibacterial and antihyperglycemic activities (Cushnie and Lamb, 2014). Some alkaloids have been known to possess psychotropic and stimulant activities and have been used as recreational drugs and entheogenic rituals (Blankenship *et al*., 2005). Alkaloids have great antimicrobial activity against bacterial pathogens such as *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus* and *Pseudomonas aureginosa* (Maatalah *et al*., 2012).

Some of the alkaloids such as morphine and cordine have been found to be active not only against bacterial and fungal pathogens but also trypanosomes and plasmodia (Freiburghaus *et al*., 1996; Omulokoli *et al*., 1997). Some of the Alkaloids found in dietary food materials have also been found to contain microbiocidal and antidiarrheal effect in the small intestines where they show the ability to intercalate with the microbial genetic material (Ghoshal *et al*., 1996; Phillipson and Niell, 1997). Other studies carried out on alkaloids extracted from a variety of medicinal plants in Nigeria showed a great antimicrobial activity against both Gram-negative and Gram-negative bacteria and also showed great antifungal activity (Garba and Okeniyi, 2012).

Flavonoid were detected in ten (10) of the medicinal plants Viz. *Bidens pilosa, C. asiatica, Garcinia pedunculata, Hibiscus sabdarifa, H. cordata, L. camara, P. lineata, P. vulgaris* and *S. oleraceus*. *S. oleraceus* contain highest total flavonoid content viz.155.97 µg/ml while the least flavanoid content (50.135 µg/ml) was estimated for *S. palustris*. In plants, flavonoid are responsible for floral pigmentation, ultraviolet ray's filtration in higher plants and symbiotic nitrogen fixation. They are also known to have inhibitory activities against organisms that cause plant diseases for example *Fusarium oxysporum* (Galoetti *et al*., 2008). Flavonoids have been known to possess antimicrobial activity against bacterial, fungal and viral microorganisms (Cowan, 1999). They are usually known for their antimicrobial activity of inhibiting the synthesis of the nucleic

acids, tampering with the integrity of the cytoplasmic membrane function and the energy metabolism process. Flavonoids from some medicinal plants have been found to inhibit the synthesis of the nucleic acids, cause permeability of the inner bacterial membrane and a dissipation of the membrane potential of Gram negative and Gram positive bacteria (Cushnie and Lamb, 2005). Some of the bioactive components that have been isolated from flavonoids have been found to contain antifungal, antibacterial and insecticidal activities (Abdel *et al.,* 2013). Previous studies carried out have shown that when mixed with antibiotics they have synergistic activity and suppress many pathogenic microorganisms in numerous *in vitro* and *in vivo* studies (Cushnie and Lamb, 2011; Manner *et al*., 2013). Additional *in vivo* studies have shown that flavonoids can be used as pharmaceutical drugs for bacterial infections or through the dietary intake to offer protection against infection (Zamora *et al*., 2012).

In this research, appreciable quantities of saponin was present in five (5) of the medicinal plants Viz. *A. aspera, B. pilosa, L. camara, P. vulgaris* and *S. oleraceus*. *B. pilosa* contain highest total saponin content viz. 74.73 µg/ml while the least saponin content (47.36 µg/ml) was estimated for *P. vulgaris*. Saponins are also considered as one of the natural antimicrobial products that make up the defense system of the plants and some can be beneficial rather than harmful to animals (Rupasighe *et al*., 2003; Hubert *et al*., 2005). There has been evidence of the presence of saponins in traditional medicine preparations where the administration is through oral means that is expected to lead to the hydrolysis of glycosides from terpenoids (Asl *et al*., 2008). Studies carried out have shown medicinal plant extracts fractions rich in saponins are effective against microorganisms such as *Escherichia coli, Salmonella typhi, Aeromonas hydrophilia* and other fungal pathogens such as *Candida albicans* (Deshpande *et al*., 2013). Saponins antimicrobial activity is attributed mainly to its capability of lysing microorganism's membranes rather than the surface tension of the extracellular medium (Asl *et al*., 2008). Apart from antimicrobial activity, saponins have shown other biological properties with its cytotoxic activity on cancer or tumor cells being considered the most important one (Yokosuka and Mimaki, 2009). Other plants are known to produce

steroidal saponins for example cholestane glycosides which are known to have a broad spectrum of biological activity such as cytotoxic activity, antifungal, antibacterial and in vivo antitumor activities (Li *et al*., 2012).

Tannin was detected in eight (8) of the medicinal plants viz. *A. aspera, C. asiatica, Gaultheria fragrantissima, L. camara, P. lineata, P. vulgaris, S. oleraceus* and *S. palustris*. *C. asiatica* contain highest total tannin content viz.71.77 µg/ml while the least tannin content (15.04 µg/ml) was estimated for *S. oleraceus*. Thus, from the result of this research, the leaves of *A. aspera, C. asiatica, G. fragrantissima, L. camara, P. vulgaris, S. oleraceus* and *S. palustris* and root of *P. lineata* may be an ideal sources for tannin extraction. Tannins are generally found in plants and they are thought to function as chemical defenses against pathogens and herbivores (Gedir *et al*., 2005). They have been commercially used primarily in the preservation of leather, making glue stains and mordant (Kanth *et al*., 2009). It has also been used in the vegetable industry in different concentration in pickling process to provide protection against bacteria, mold, and yeasts (Andrade *et al*., 2005).

Steroid was found to be present in all the selected medicinal plants except *G. pedunculata* while Cardiac glycoside were detected in five (5) of the medicinal plants viz. *A. aspera, A. conzyoides, C. asiatica, Hibiscus sabdarifa* and *L. camara*. Cardiac glycosides (also called cardenoloids) are used in treatment of congestive heartfailure, whereby they inhibit Na+/K+-ATPasepump that causes positive ionotropic effectsand electrophysiological changes. This strengthen heart muscle and the power ofsystolic concentration against congestive heartfailure (Ogunwenmo *et al*., 2007; Ngoci *et al*., 2011). They are also used in treatment of atrial fibrillation, flatter, and they act as emeticsand as diuretics (Harborne, 1973; Awoyinka *et al*., 2007; Ngoci *et al*., 2011).

The non-detection of certain phytochemicals in the medicinal plants does not absolutely rule out their presence in the plants. The phytochemicals in the plant material could be below the limit of detection, or they are insoluble in the solvent used for the extraction. Another important factor to be consider is the part of the plant used for the extraction of the phytochemicals since secondary may not be equally distributed in the

plant organs. The solvent of plant extraction has an important effect on biologically active content but there are contradicting findings. For instance, while Cheung *et al*. (2003) reported that methanol can extract the higher amount of phenolic compounds than aqueous extract, Mohaddese *et al*. (2015) showed that the highest amount of phenolic is present in aqueous extract but the total flavonoid content is higher in the methanol extract. Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure (Tiwari, 2011). This, therefore, underscores the need to try as much solvents as possible in screening plant parts for phytochemicals. Diversity and difference in the amount of phytochemicals content can be attributed to many factors like growth conditions (solar radiation, temperature, precipitation and relative humidity), genetic inbuilt etc.

Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds (Costa *et al.*, 1999). Many phytochemicals, particularly the pigment molecules, are often concentrated in the outer layers of the various plant tissues. The quantitative amount vary from plant to plant depending upon the variety, processing, cooking and growing conditions (King *et al.*, 1999).

TLC profiling of all 15 extracts gives an impressive result that direct towards the presence of number of phytochemicals. Various phytochemicals give different Rf values in different solvent system. Compound showing high Rf value in less polar solvent system have low polarity and with less Rf value have high polarity. This variation in Rf values of the phytochemicals provides a very important clue in understanding of their polarity and also helps in selection of appropriate solvent system for separation of pure compounds by Column Chromatography (Bennett and Heftmann, 1962). Mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract. The selection of appropriate solvent system for a particular plant extract can only be achieved by analyzing the Rf values of compounds in different solvent system (Sherma and Fried, 1996).

Chapter 6

Phytochemical Analysis of Selected Medicinal Plants Using LC-ESI-MS

5.1 Introduction

Garcinia pedunculata and *Potentila lineata* were selected for LC-ESI-MS analysis since they exhibit impressive antibacterial activity. *Garcinia* contains a high amount of Vitamin C and is used as a heart tonic. *Garcini*a is the source for a natural diet ingredient (-) hydroxycitric acid. HCA, (1,2-dihydroxypropane-1,2,3-tricarboxylic acid) which is an anti-obesity compound present in the fruit rind and leaves of *Garcinia* and is known to inhibit lipid and fatty acid synthesis in living systems (Lewis and Neelakandan, 1965). HCA is also hypocholesterolamic agent (Lowenstein, 1971; Sullivan *et al*., 1972; Sullivan and Triscari, 1977). On a dry weight basis, HCA constitutes about 20-30% of the fruit (Cheek, 2004).

Mukherjee *et al*. (1995) reported that *Garcinia* contain bioactive compounds such as xanthones, biflavonoids, benzophenones, benzoquinones, and triterpenes which have antibacterial, antifungal, antioxidant, and cytotoxic effects. *P. lineata* syn *P. fulgens* is rich in polyphenolic and triterpene constituents (Laloo *et al*., 2017; Tomczyk and Latte, 2009; Jaitak *et al*., 2010; Kumar *et al*., 2013). A novel bioflavonoid potifulgene (Epiafzelchin-6-o-8´´ epiafzelchin) along with epicatechin have been isolated from the root parts of *P. fulgens*. Two new ursane type triterpenoids, Fulgic acid A (Bhattari, 1993) and Fulgic acid B were identified and characterized (Farooqui, 1998).

Choudhary *et al.* (2017) had undertaken extensive studies to evaluate the anticariogenic effects of the plant and search for potent anticariogenic phenolic molecules. Polyphenolic compounds viz. Afzelechin, Epiafzelechin, Epigallocetechin, Epigallocetechin gallate, Epicatechin, Catechin, Afzelechin(4β→8) epicatechin, Epiafzelechin (4β→8) epicatechin, Catechin (4α→8) epicatechin, Afzelechin(4α→8)

catechin and Afzelechin (4α→8) epiafzelechin (Choudhary *et al*., 2017) were isolated from the roots of *P. fulgens* using semi-preparative HPLC. Ursolic acid and epicatechin were the major compounds in the *Potentilla* root extract.

In another study, Choudhary *et al*. (2013) isolated 2α,3α,20β-trihydroxyrus-13-en-28-oic acid, 2α,3β,20β-trihydroxyurs-13en-28-oic, p-hydroxy benzaldehyde and gallic acid from ethyl acetate extract *of P. fulgens* and they exhibited good antioxidant activity. Phenolic compounds, quercetin, ellagic acid and kaempferol were reported for the first time in n-butanol fraction of *P. fulgens* and act as potential antioxidative and cancer chemopreventive agents (Choudhary *et al*., 2014).

Mass spectrometry (MS) coupled to high-performance liquid chromatography (HPLC) provides an adequate tool for all the phytoanalytical investigation. Mass spectrometry offers great selectivity and sensitivity and with the separative power of high-performance liquid chromatography enables simultaneous structural analysis of compounds present in complex matrices.

HPLC-MS is the method of choice for phytochemical analysis, since it provides a powerful, robust, versatile and available technique. High-performance liquid chromatography (HPLC) is by far the most relevant and important separation technique in phytochemical analysis.

Mass spectrometric (MS) detection coupled to HPLC has become the dominating technique in phytochemical analysis. Different mass analyzers: quadrupole (Q), triple-quadrupole (QQQ), time-of-flight (TOF), ion trap (IT), Orbitrap and Fourier-transform ion cyclotron resonance (FT-ICR), as well as hybrid, e.g. quadrupole – time-of-flight (Q-TOF) instruments are applied. Single-stage MS is usually used in combination with UV detection to confirm identity of known components in plant extracts with the help of literature data and reference compounds.

In the LC-MS of phytochemical compounds, atmospheric pressure ionization (API) interfaces, such as ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization) are almost exclusively applied. They complement one other

regarding polarity and molecular mass of analytes, as ESI is suitable for polar/very polar compounds with high molecular weights, while APCI is suitable for non-polar compounds with molecular weights up to several thousands Da (Steinmann, 2011). Other ionization techniques employed include fast atom bombardment (FAB), atmospheric pressure photo ionization (APPI) and matrix-assisted laser desorption ionization (MALDI). Ambient ionization techniques, e.g. direct analysis in real time (DART) and desorption electrospray ionization (DESI) have been proposed for the direct evaluation of molecules present in food without using HPLC and with a minimal or no sample preparation (Stefano, 2012).

LC is usually performed in the reversed-phase mode, on C8- or C18-bonded silica columns. Most frequently, the solvents are usually slightly acidified to prevent ionization of the phenolics, which could reduce their retention (De Rijke *et al*., 2006).

Both isocratic and gradient elution systems are applied. Gradient elution is generally performed with binary solvent systems, i.e. with water containing acetate or formate buffer, and methanol or acetonitrile as organic modifier (Stalikas, 2007).

Sample preparation is the initial and crucial step of both qualitative and quantitative analyses. Sample pretreatment steps may vary to a great extent, depending on the matrix of the particular sample. Several liquid samples such as urine, serum, plasma, and some beverages can be injected directly into the separation system after filtration and / or centrifugation (Praisan, 2004), while solid samples are usually first air-dried or freeze-dried then subjected to milling or grinding and homogenization before further extraction and purification steps (Stalikas, 2007).

Despite of the wide range of information provided by mass spectrometry, unambiguous structural identification can only be carried out by combining nuclear magnetic resonance spectroscopy (NMR) with LC-DAD and LC-MS/MS. On-line coupling of HPLC and NMR is also gaining in importance in the analysis of plant-derived compounds (Careri, 1998).

As mentioned above, application of ESI and APCI ionization is dominating in the analysis of phenolics. For both techniques negative ionization (NI) mode provides

better sensitivity, however – despite the stronger background noise – positive ionization (PI) mode can also be employed.

Mauri and Pietta (2000) analyzed different plant extracts without prepurification and chromatographic separation, by direct infusion into an ESI-MS apparatus with the aim to obtain their finger-prints. Gioacchini *et al.* (1996) concluded also that use of the ESI-MS system with selected ion monitoring mode (i.e., when only selected ions pass through the first analyzer and are then analyzed by the second one) did not required complete HPLC separation of the phenolic acids and aldehydes studied, unless isomers were to be identified. Nevertheless, sample purification and chromatographic separation should not be neglected, since co-eluting components may cause problems with the MS response due to ion suppression and other matrix effects. HPLC-ESI-MS/MS methods are adversely affected by matrix effects in terms of sensitivity, accuracy and precision. Consequently, almost all mass spectrometric methods in phenolic analysis include a high-performance separation method, as adequate chromatographic resolution improves LC-MS sensitivity.

It should be kept in mind that in all four operation modes: (–)-ESI, (+)-ESI, (–)-APCI and (+)-APCI, composition and pH of the LC eluent and the nature of the buffer components added can have a distinct influence on analyte responses and ionization efficiency. The most common additives in LC-MS are acetic acid, formic acid, ammonium-acetate and ammonium-formate. Trifluoroacetic acid is also used, despite its ion suppressing effects due to ion-pairing with basic analytes and its adverse effect on efficient spray formation due to its high surface tension. Phosphate buffers are not employed, because of contamination of the ion source. However, sensitivity of ESI is improved when the organic content in the mobile phase exceeds 20% (v/v) (Ryan, 1999).

Sample preparation is the initial and crucial step of both qualitative and quantitative analyses. Sample pretreatment steps may vary to a great extent, depending on the matrix of the particular sample. Several liquid samples such as urine, serum, plasma, and some beverages can be injected directly into the separation system after

filtration and / or centrifugation, while solid samples are usually first air-dried or freeze-dried then subjected to milling or grinding and homogenization before further extraction and purification steps (Stalikas, 2007; De Rijke *et al*., 2006).

5.2 Materials and Methods:

5.2.1 LC-ESI-MS Instrumentation and Experiments on *P. lineata* methanol extract

The methanol extracts of *P. lineata* was subjected to LCMS analysis. The UPLC/MS was performed on Waters ACQUITY UPLC-TQD Mass spectrometer model ACQ-TQD#QBB1152. The ACCUCORE C18, 150 X 3mm, 2.6um column, maintained at temperature of 30° C was used for separation. The solvents used were of LC-MS grade. The sample injection volume was 3.0 µl. A continuous gradient system was followed using mobile phase composed of acetonitrile (ACN) and formic acid (0.1 % FA, v/v in water) for 30 mins. For the investigation of the extract, the following eluent mixtures were used at a flow rate of 0.250 mL/min:

1) 0-6 min 5:95 → 30:70 (ACN : FA, v/v)
2) 6-12 min 30:70 → 60:40 (ACN : FA, v/v)
3) 12-20 min 60:40 ---80:20 (ACN : FA v/v)
4) 20-26 min 80:20---5:95 (ACN : FA, v/v)
5) 26-30 min 5:95 → 5:95 (ACN : FA, v/v)

The capillary temperature was 350℃ and the ESI voltage was 3.5 Kv. All analyses were carried out in the full scan mode from 150.0 to 2000.0 m/z using an ESI source in both positive-ion mode and negative-ion mode.

5.2.2 LC-ESI-MS Instrumentation and Experiment on *G. pedunculata* extract

The methanol extract of *G. pedunculata* (MEGP) was subjected to LCMS analysis. The LC/MS was performed on Waters ACQUITY UPLC-TQD Mass spectrometer model XEVO-TQD#QCA1232 with Waters Acquity PDA detector. The HPLC Column SUNFIRE C18 (100Å pore size, 5 µm particle size, 250 X4.6 mm),

maintained at temperature of 35° C was used for separation. The solvents used were of LC-MS grade. The sample injection volume was 20.0 µl. A continuous gradient system was followed using mobile phase composed of acetonitrile (ACN) and formic acid (0.1 % FA, v/v in water) for 40 mins. At a flow rate of 1.5 mL/min, the extract was investigated using the following eluent mixtures:

1) 0-10 min 5:95 → 30:70 (ACN : FA, v/v)
2) 10-16 min 30:70 → 60:40 (ACN : FA, v/v)
3) 16-24 min 60:40 ---80:20 (ACN : FA, v/v)
4) 24-35 min 80:20---5:95 (ACN : FA, v/v)
5) 25-40 min 5:95 → 5:95 (ACN : FA, v/v)

The capillary temperature was 350˚C and the ESI voltage was 2.5 Kv. The detection was carried out in negative ion mode over a mass range of 150.0-750.0 m/z.

5.3 Results

5.3.1. LC-ESI-MS Result of methanol extract *of P. lineata* (MEPL)

Methanol extract of *P. lineata* (MEPL) was selected for LCMS analysis over ethanol extract (EEPL) since it was observed that it gives larger zone of inhibition against the bacteria tested. It implies that methanol may be a better organic solvent for extraction of antibacterial compounds from *P. lineata* root.

Bioactive compounds present in the methanol extract of *P. lineata* were analysed. The ESI- ve and ESI+ve base peak ion (BPI) chromatogram of the MEPL by UPLC/TQD MS is shown in Fig. 10(a) & 10(b). A total of 16 compounds (ESI-ve) and 17 compounds (ESI+ve) were characterized. Among them, 7 compounds viz. gallic acid, catechin/epicatechin, epiafz/afz-epicat/cat dimer, epicat/cat-epicat/cat dimer, epiafz/afz-epiafz/afz-epicat/cat trimer, epiafz/afz-epicat/cat-epicat/cat trimer, and epiafz/afz-epiafz/afz-epiafz/afz-epicat/cat tetramer were tentatively identified (peaks 72, 10, 9, 8, 13, 11 and 15; Fig.10 (c-p) & Table 9). For unambiguous identification, retention time (t_R), UV and mass spectral data of compounds were compared to literature data and to those of authentic standards, where available.

Table 9: Selected List of compounds Identified by LC-MS in the Methanol Extract of *P. lineata*.

SN	Peak ID	Retention Time ($t_{R)}$	Name of Compounds	Molecular Formula	Molecular Weight	m/z ESI +ve	m/z ESI-ve
1.	8	4.32	Epicat/cat-epicat/cat dimer	-	578	579	577
2.	9	4.59	Epiafz/afz-epicat/cat dimer	-	562	563	561
3.	10	5.00	Catechin / epicatechin dimer	$C_{15}H_{14}O_6$	290	291	289
4.	11	5.31	Epiafz/afz-epicat/cat-epicat/cat trimer	-	850	851	849
5.	13	5.82	Epiafz/afz-epiafz/afz-epicat/cat trimer	-	834	835	833
6.	15	6.57	Epiafz/afz-epiafz/afz-epiafz/afz-epicat/cat tetramer	-	1106	1107	1105
7.	72	24.45	Gallic acid	$C_7H_6O_5$	170	171	169

Abbreviation: cat - catechin; epicat - epicatechin; afz - afzellechin; epiafz – epiafzellechin

CSIR
Openlynx Report SAIF, CSIR-CDRI, LUCKNOW
Sample: 12
File:18JANLCMS12
Description:ACCUCORE C18, 150 X3, 2.6um
Printed: Thu Jan 03 14:20:27 2019
Vial:1:B,3
Date:03-Jan-2019
ID:ZLN01 [SAIF517]
Time:10:13:58
Page 2

Sample Report (continued):

1: MS ES- :BPI

3.6e+006

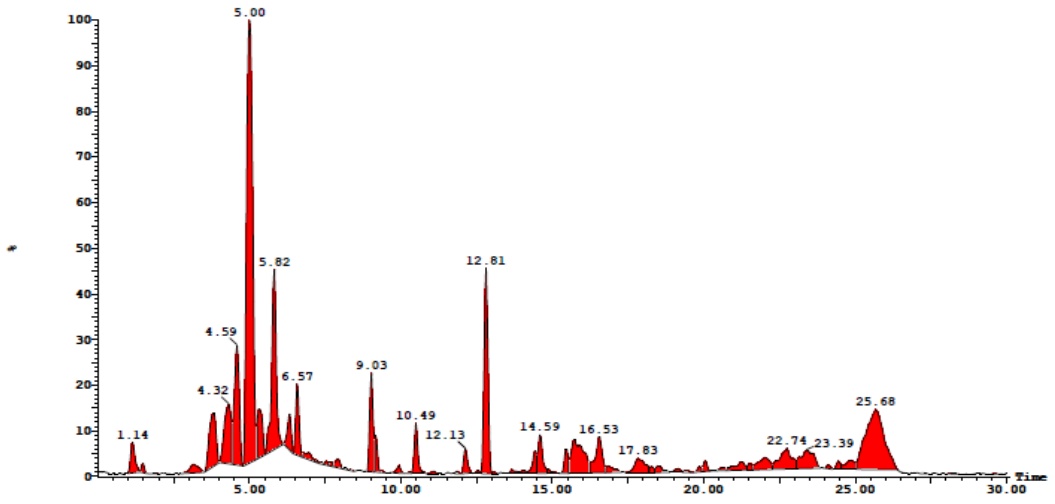


Figure 10(a): BPI chromatogram of MEPL (ESI - ve)

CSIR
Openlynx Report SAIF, CSIR-CDRI, LUCKNOW
Sample: 12
File:18JANLCMS12
Description:ACCUCORE C18, 150 X3, 2.6um
Printed: Thu Jan 03 14:20:27 2019
Vial:1:B,3
Date:03-Jan-2019
ID:ZLN01 [SAIF517]
Time:10:13:58
Page 3

Sample Report (continued):

2: MS ES+ :BPI

4.2e+006

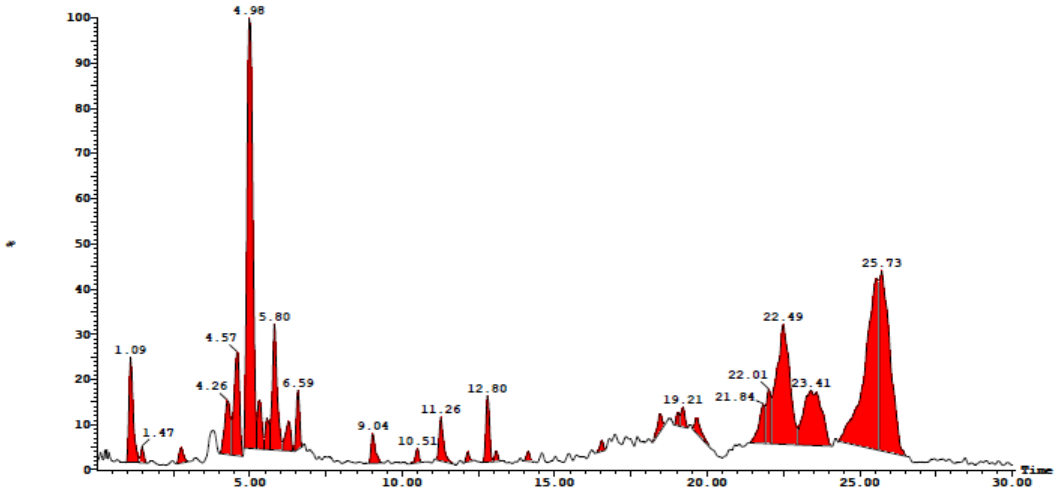


Figure 10(b): BPI chromatogram of MEPL (ESI + ve)

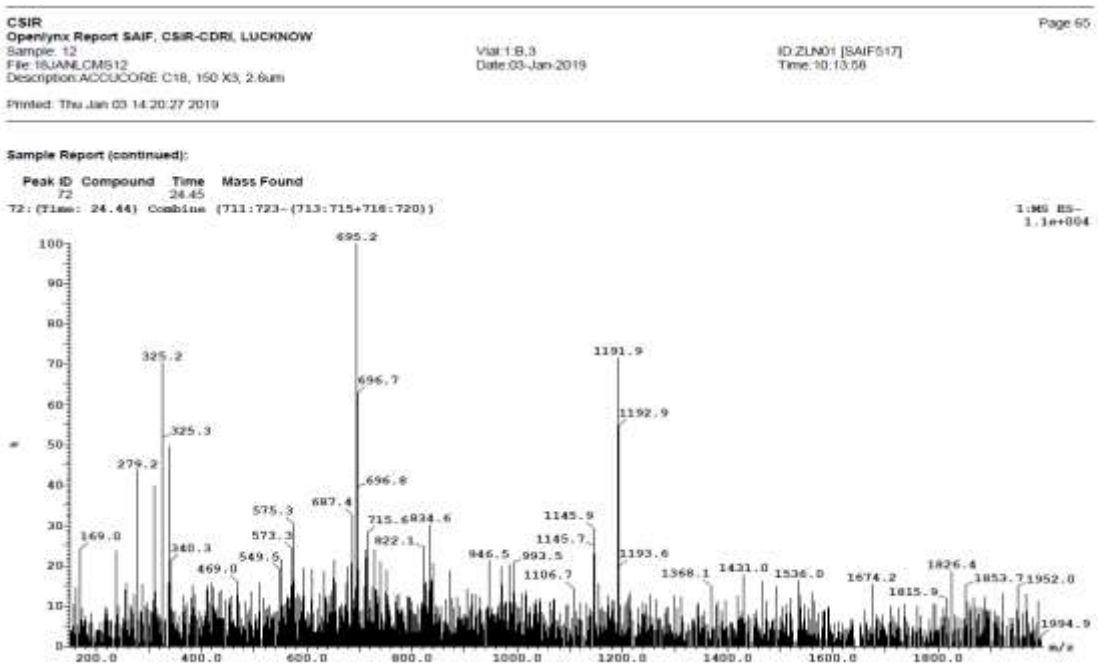


Figure 10(c): LC-MS spectrum of MEPL (Peak ID 72, t_R – 24.44, ESI - ve)

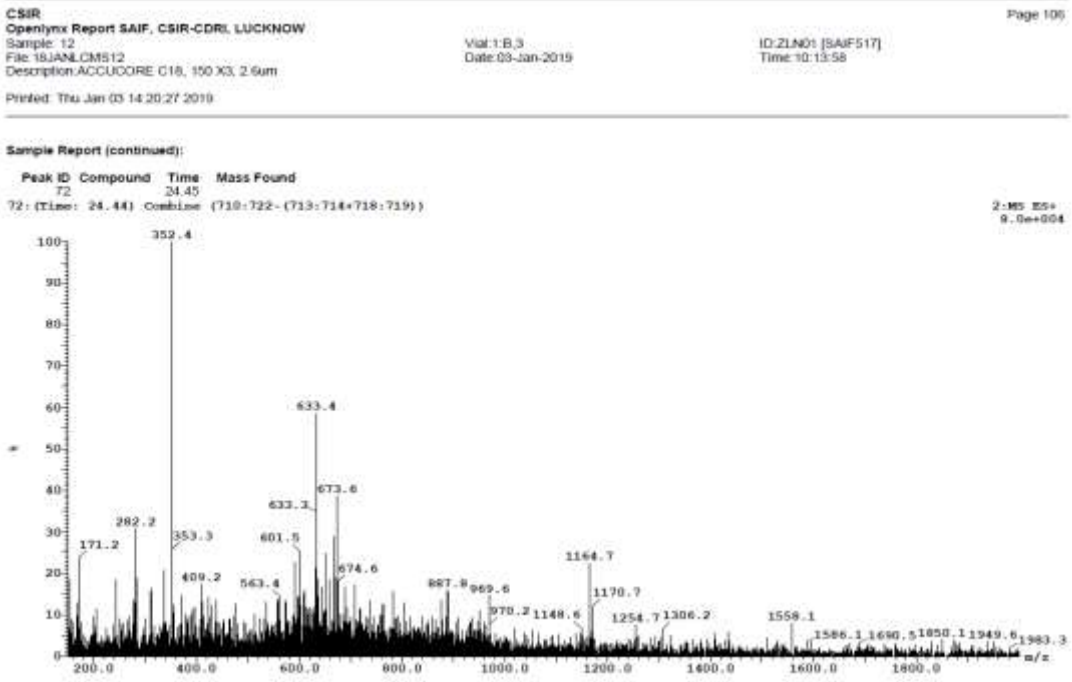


Figure 10(d): LC- MS spectrum of MEPL (Peak ID 72, t_R – 24.44, ESI + ve)

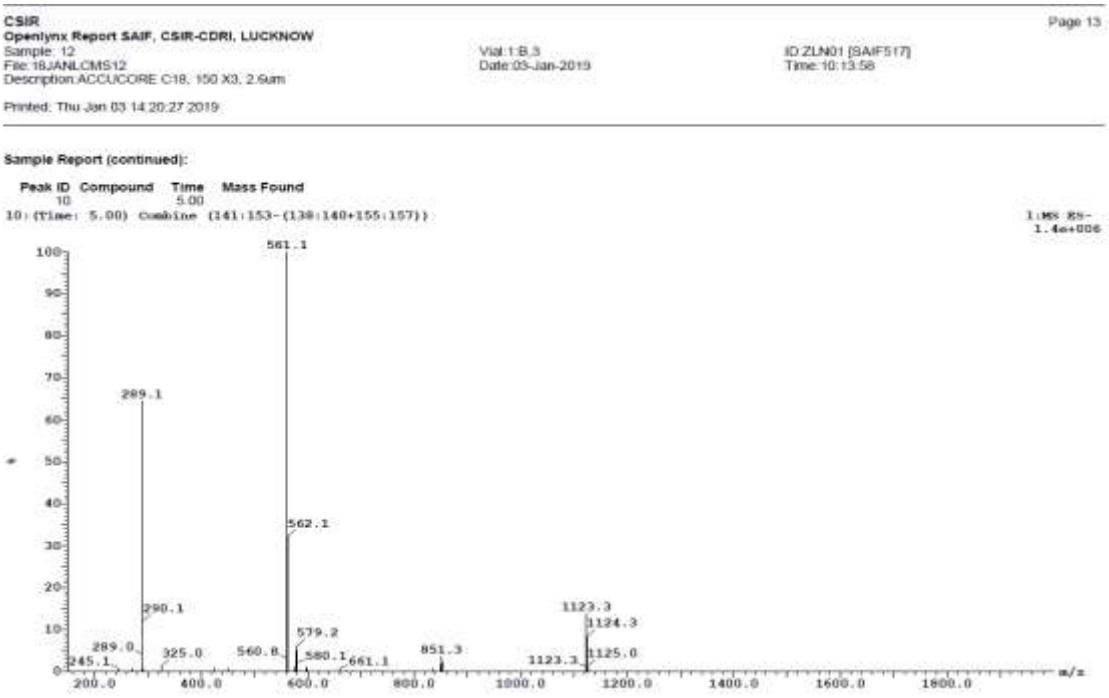


Figure 10(e): LC-MS spectrum of MEPL (Peak ID 10, t_R- 5.00, ESI - ve)

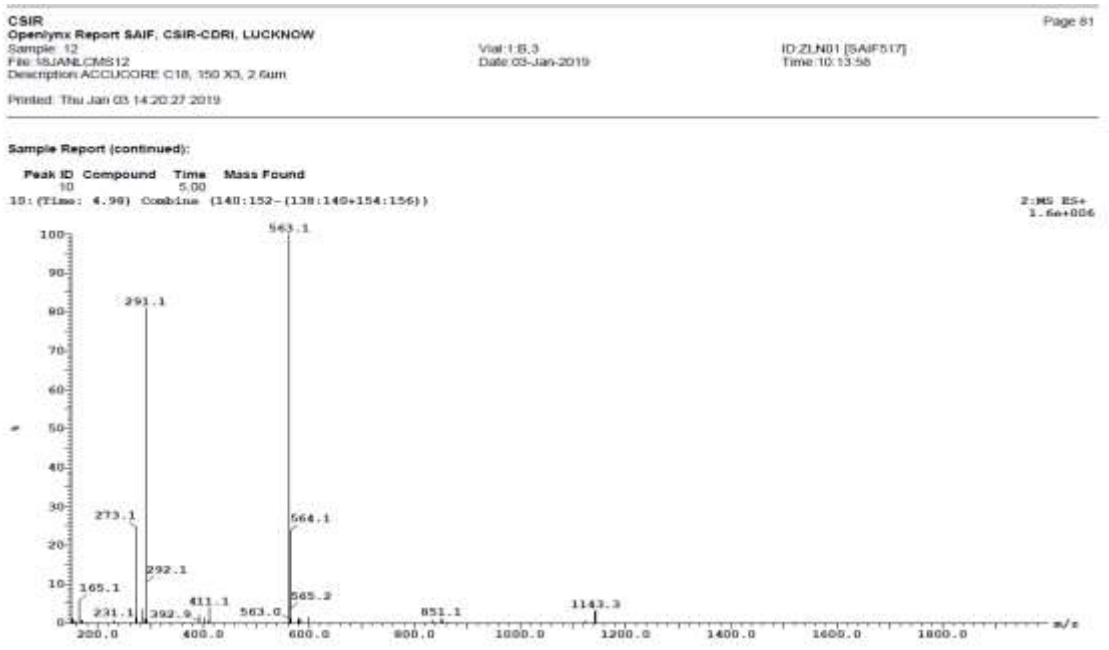


Figure 10(f): LC- MS spectrum of MEPL (Peak ID 10, t_R – 5.00, ESI + ve)

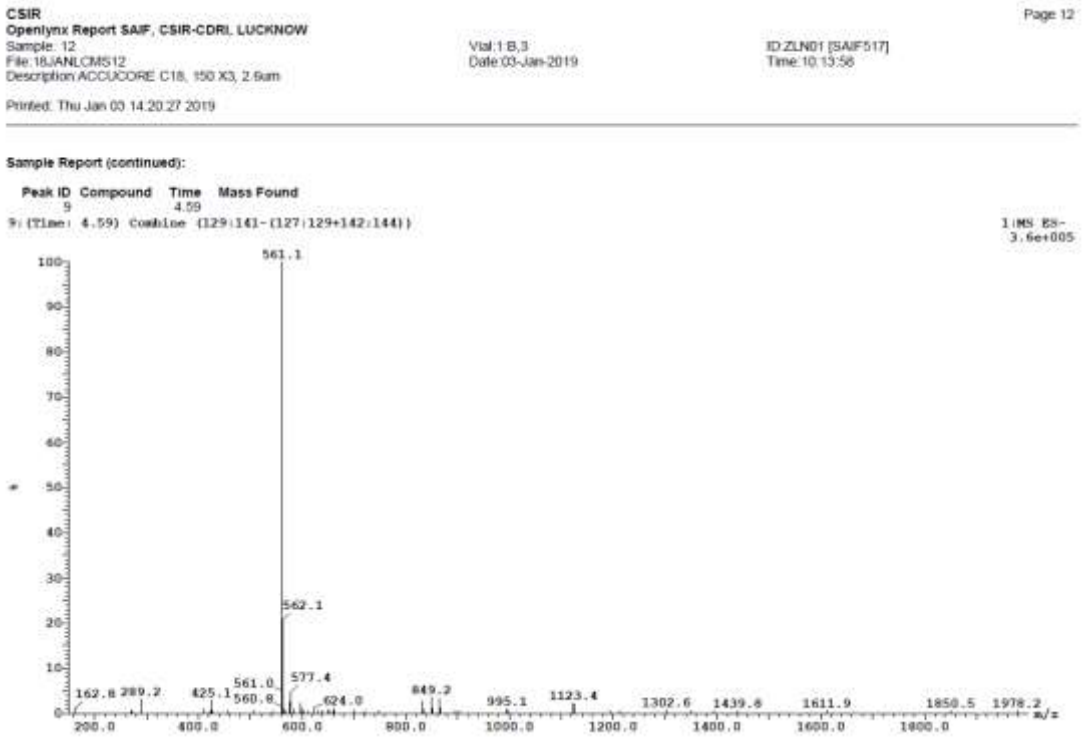


Figure10(g): LC – MS spectrum of crude MEPL (Peak ID 9, t_R- 4.59, ESI –ve)

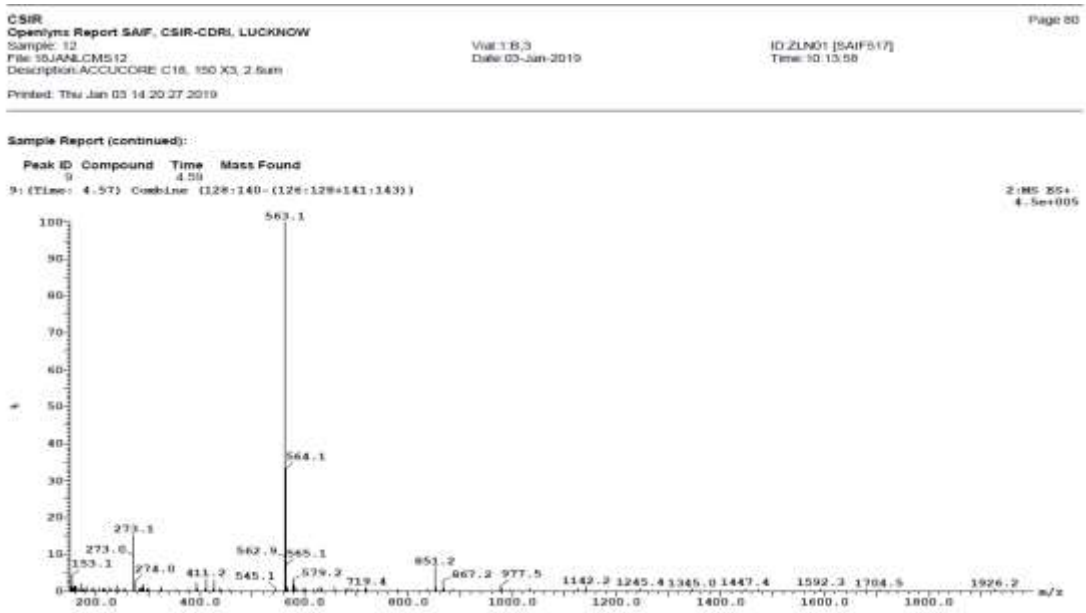


Figure 10(h): LC – MS spectrum of MEPL (Peak ID 9, t_R- 4.57, ESI +ve)

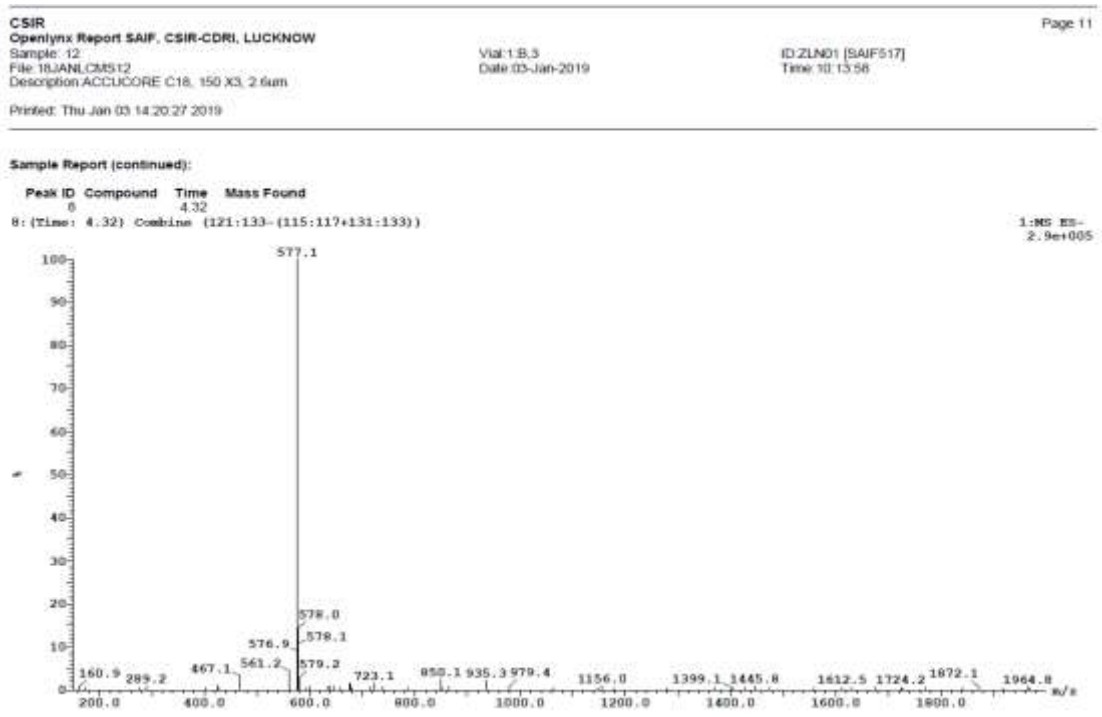


Figure 10(i): LC-MS spectrum of MEPL (Peak ID 8, t_R- 4.32, ESI - ve)

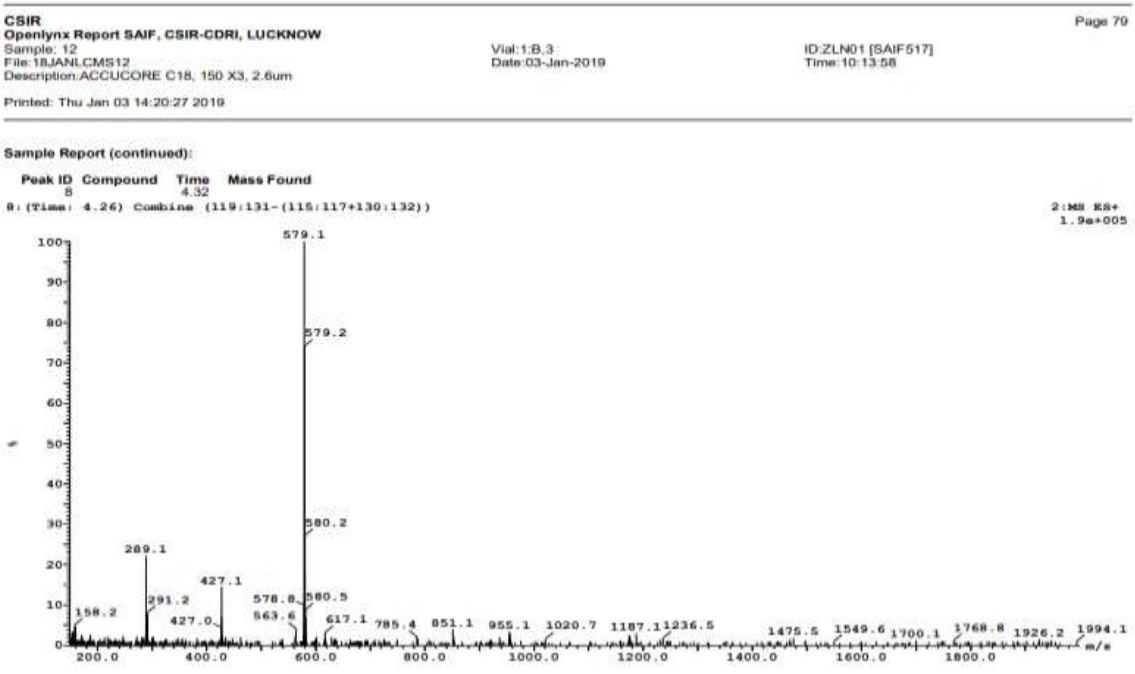


Figure 10(j): LC-MS spectrum of MEPL (Peak ID 8, t_R- 4.26, ESI + ve)

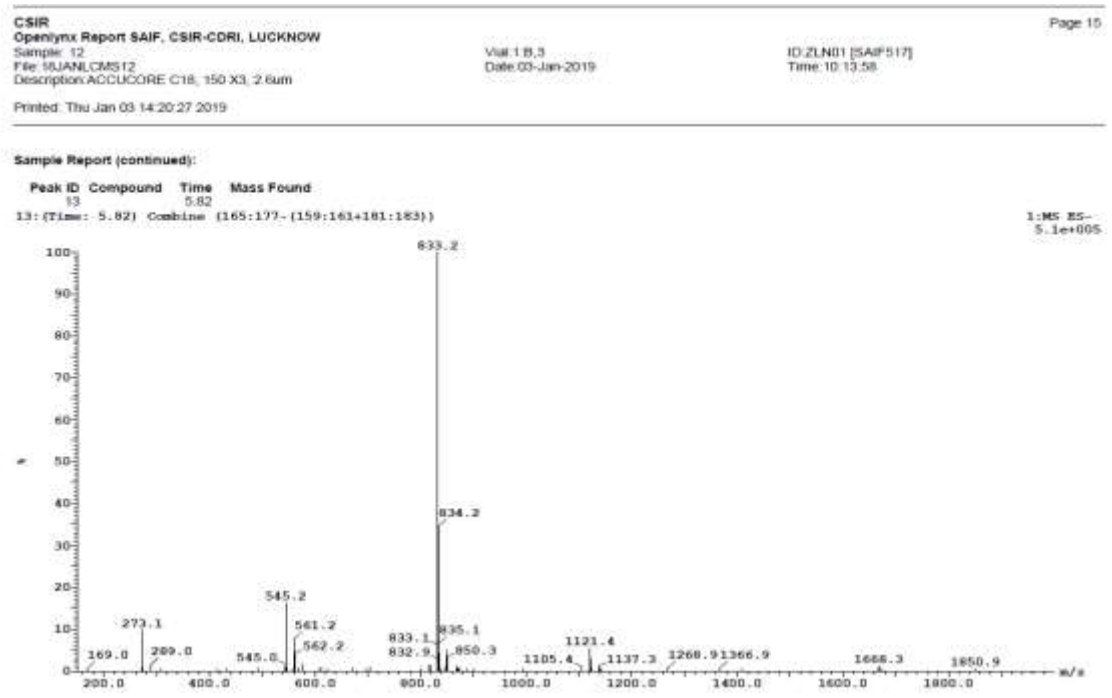


Figure 10(k): LC – MS spectrum of MEPL (Peak ID 13, t_R- 5.82, ESI – ve)

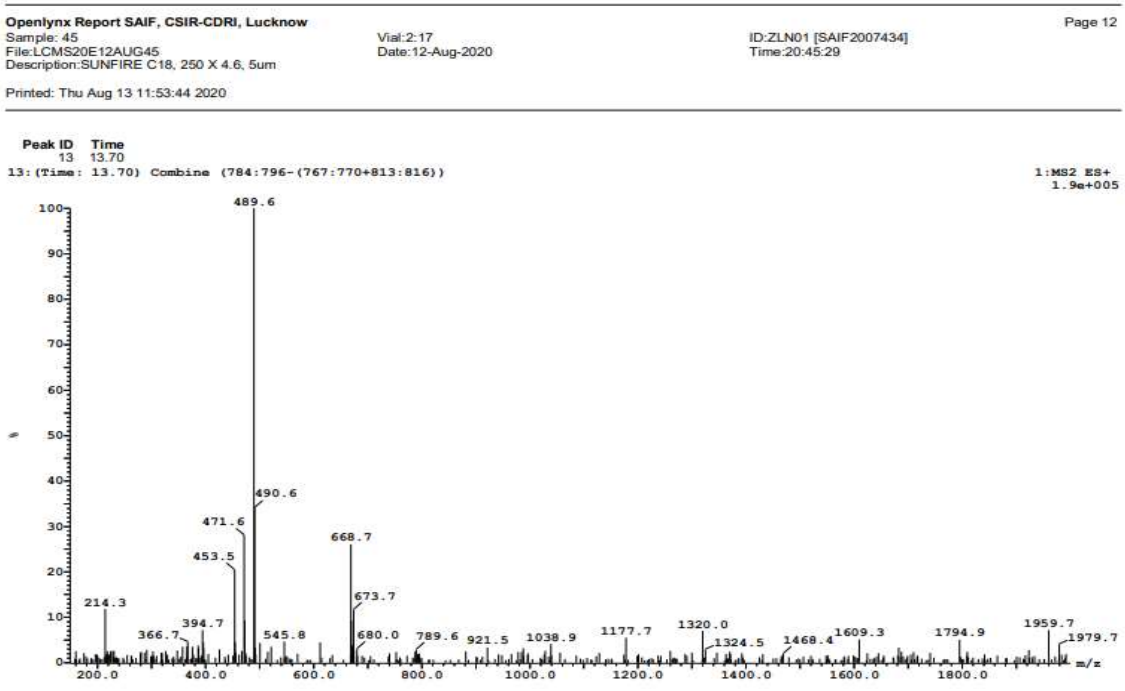


Figure 10(l): LC – MS spectrum of MEPL (Peak ID 13, t_R- 13.70, ESI +ve)

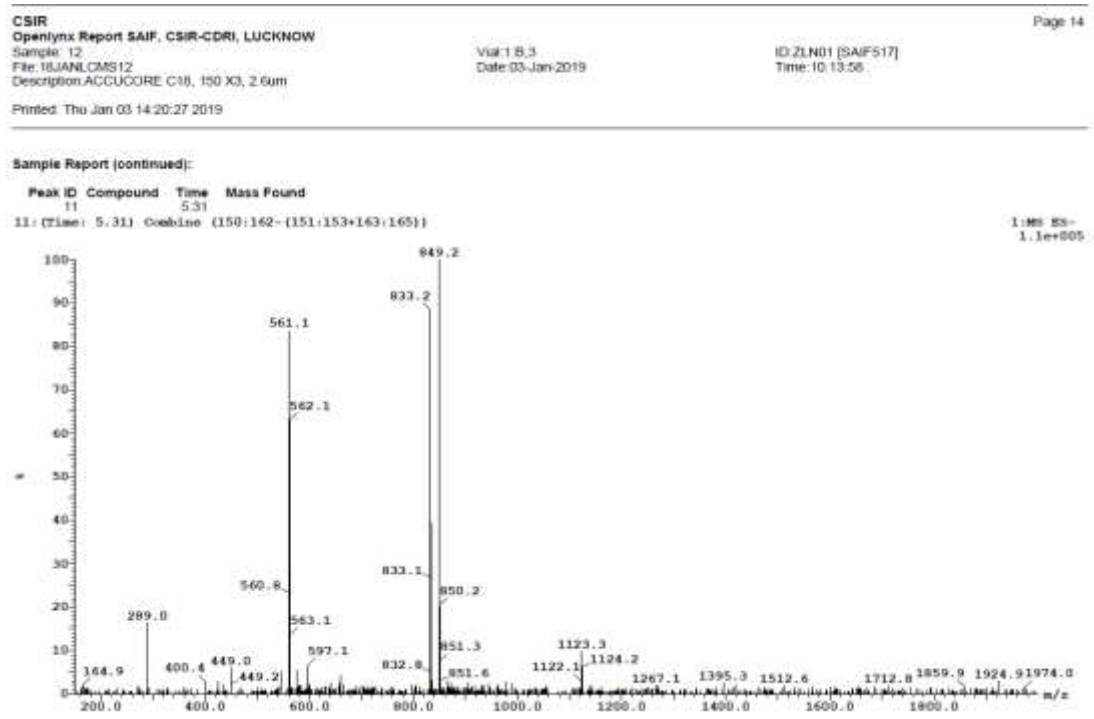


Figure 10(m): LC – MS spectrum of MEPL (Peak ID 11, t_R- 5.31, ESI – ve)

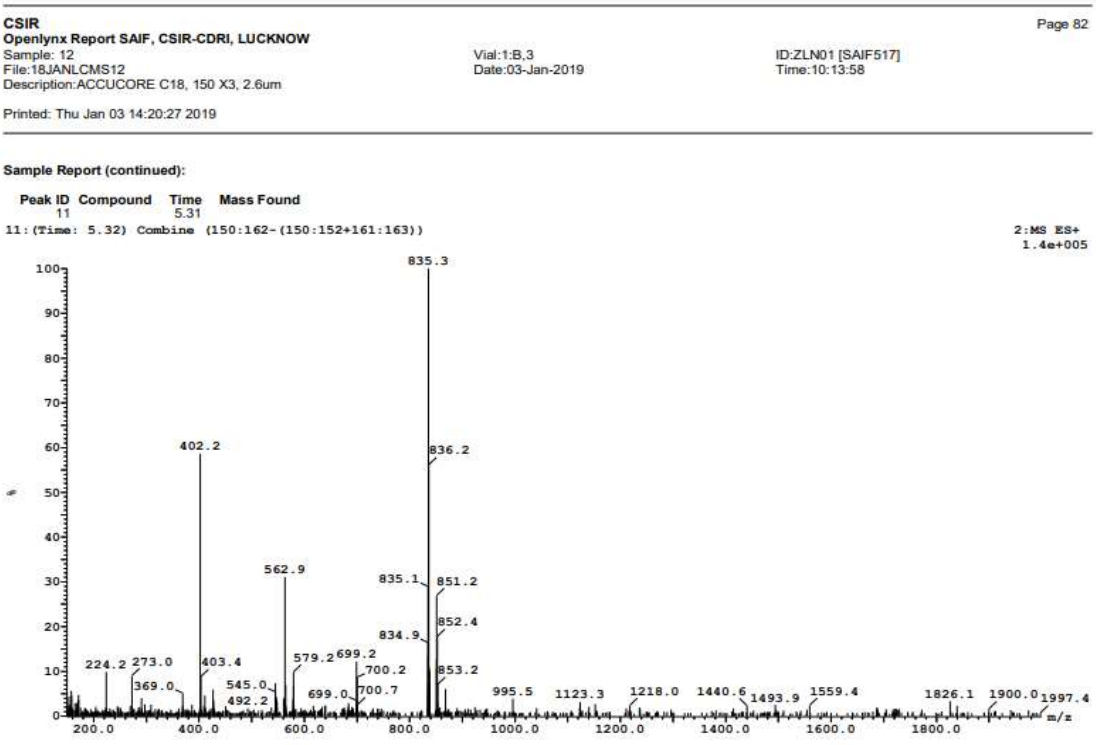


Figure 10 (n): LC-MS spectrum of MEPL (Peak ID 11, t_R- 5.31, ESI + ve)

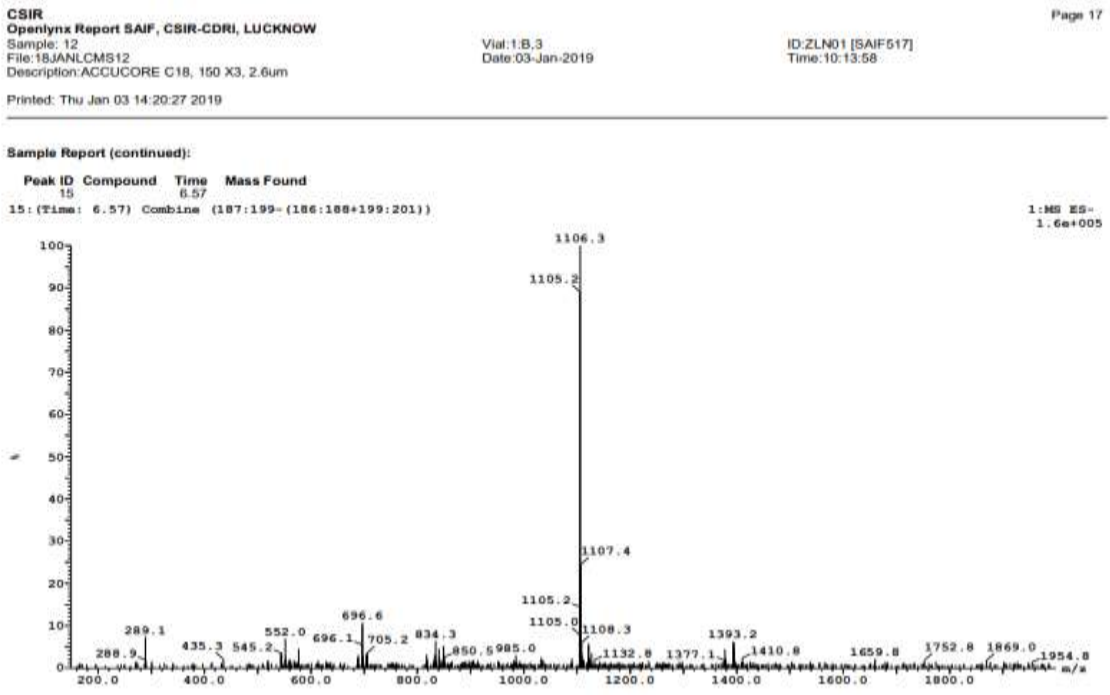


Figure 10(o): LC-MS spectrum of MEPL (Peak ID15, t_R- 6.57, ESI - ve)

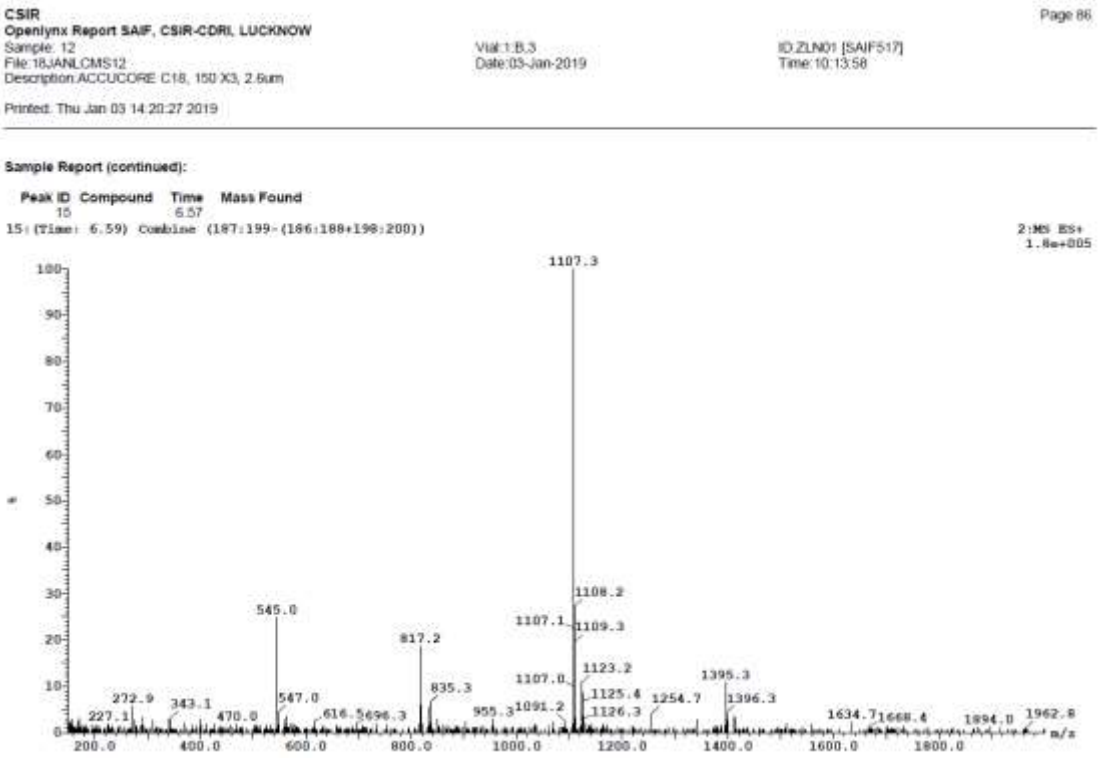


Figure 10(p): LC-MS spectrum of MEPL (Peak ID 15, t_R- 6.57, ESI + ve)

5.3.2. LC-ESI-MS Result of Methanol extract of *G. pedunculata* (MEGP)

LC-MS of the methanol extract of *G. pedunculata* fruit revealed presence of several compounds. The ESI- ve and ESI+ve base peak ion (BPI) chromatogram of the MEGP by UPLC/TQD MS is shown in Fig.10 (a) & 10(b). A total of 23 compounds (ESI-ve) and 17 compounds (ESI+ve) were characterized. Among them, 5 compounds viz. Hydroxy Citric Acid Lactone (MW-190), Garcinone E (MW-464), α-Mangostin (MW-410), β–Mangostin (MW-424), and γ-Mangostin (MW-396) were tentatively identified (peaks 2, 36, 46, 69 and 72, Fig. 11 (c-i) & Table 10).

Identification was carried out based on molecular weight, using the mass spectra of the LC-MS. Retention times (tR), UV and mass spectral data of compounds were compared to literature data and to those of authentic standards, where available, for unambiguous identification.

Table 10: Selected List of compounds Identified by LC-MS in the Methanol Extract of *G. pedunculata* fruit.

S N	Peak ID	Retention Time (t_R)	Name of Compounds	IUPAC Name	Mol. Formula	Mol. Weight	m/z ESI-ve
1	2	1.87	Hydroxy Citric Acid Lactone	(2S,3S)-3-hydroxy-5-oxooxolane-2,3-dicarboxylic acid	$C_6H_6O_7$	190	189.3
2	36	24.17	γ-Mangostin	1,3,6,7-tetrahydroxy-2,8-bis(3-methylbut-2-enyl)xanthen-9-one	$C_{23}H_{24}O_6$	396	395.8
3	46	26.55	α-Mangostin	1,3,6-trihydroxy-7-methoxy-2,8-bis(3-methylbut-2-enyl)xanthen-9-one	$C_{24}H_{26}O_6$	410	409.5
4	69	30.48	Garcinone E	2,3,6,8-tetrahydroxy-1,4,7-tris(3-methylbut-2-enyl)xanthen-9-one	$C_{28}H_{32}O_6$	464	463.6
5	72	31.92	β–Mangostin	1,6-dihydroxy-3,7-dimethoxy-2,8-bis(3-methylbut-2-enyl)xanthen-9-one	$C_{25}H_{28}O_6$	424	423.6

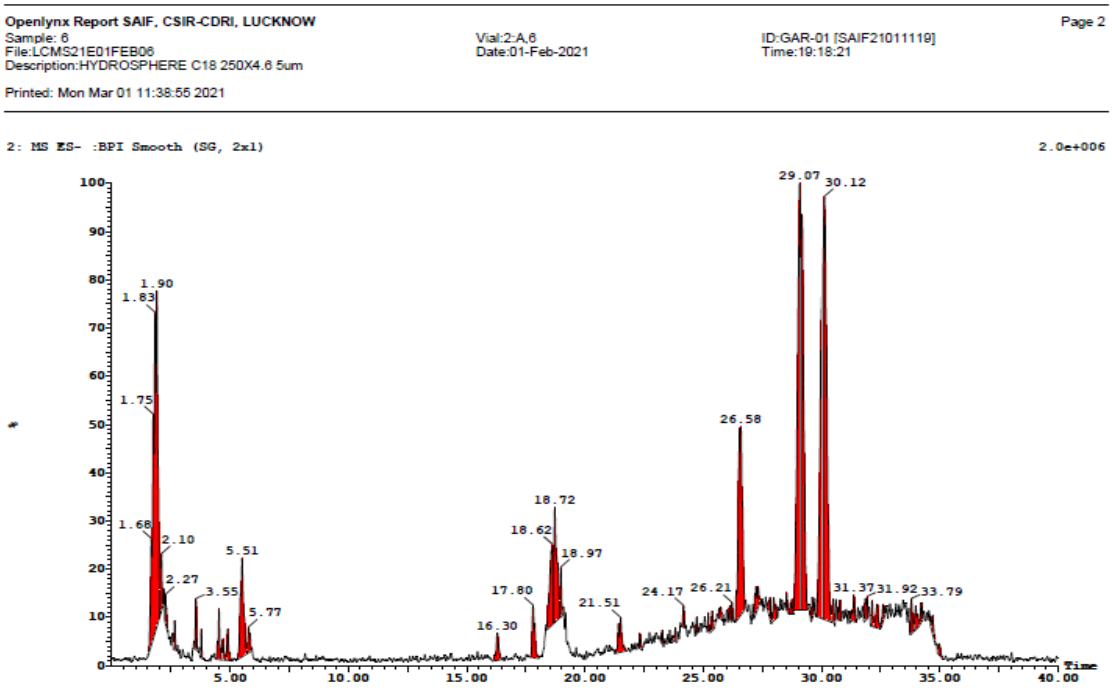


Figure 11(a): BPI chromatogram of MEGP (ESI - ve)

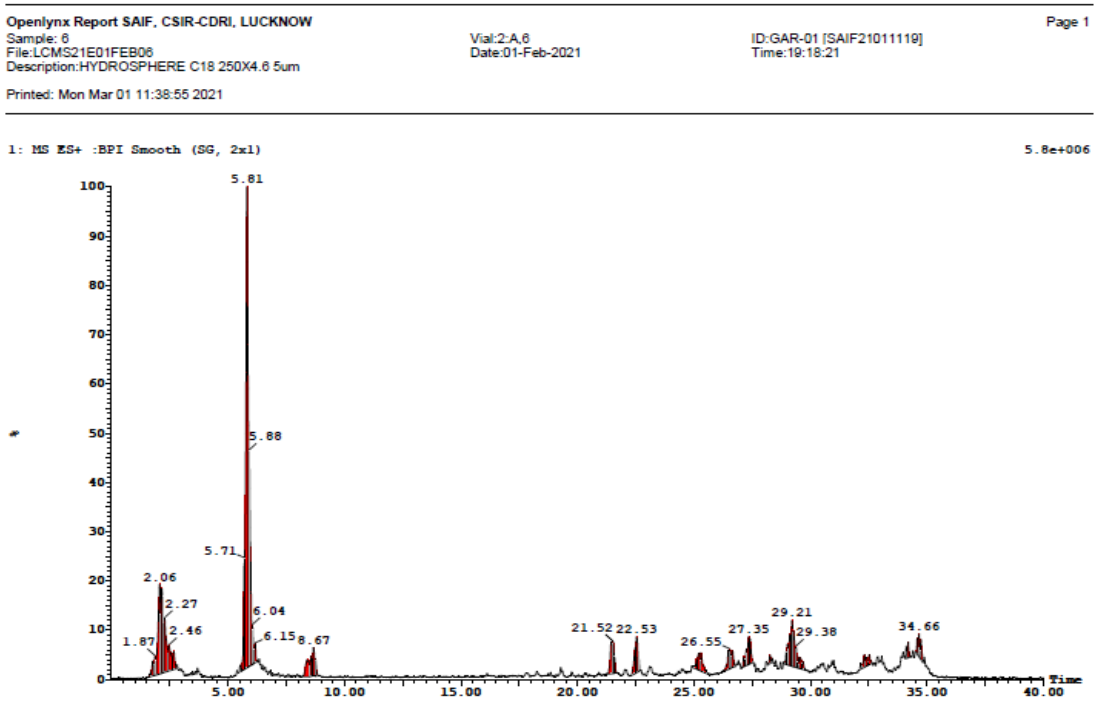


Figure 11(b): BPI chromatogram of MEGP (ESI + ve)

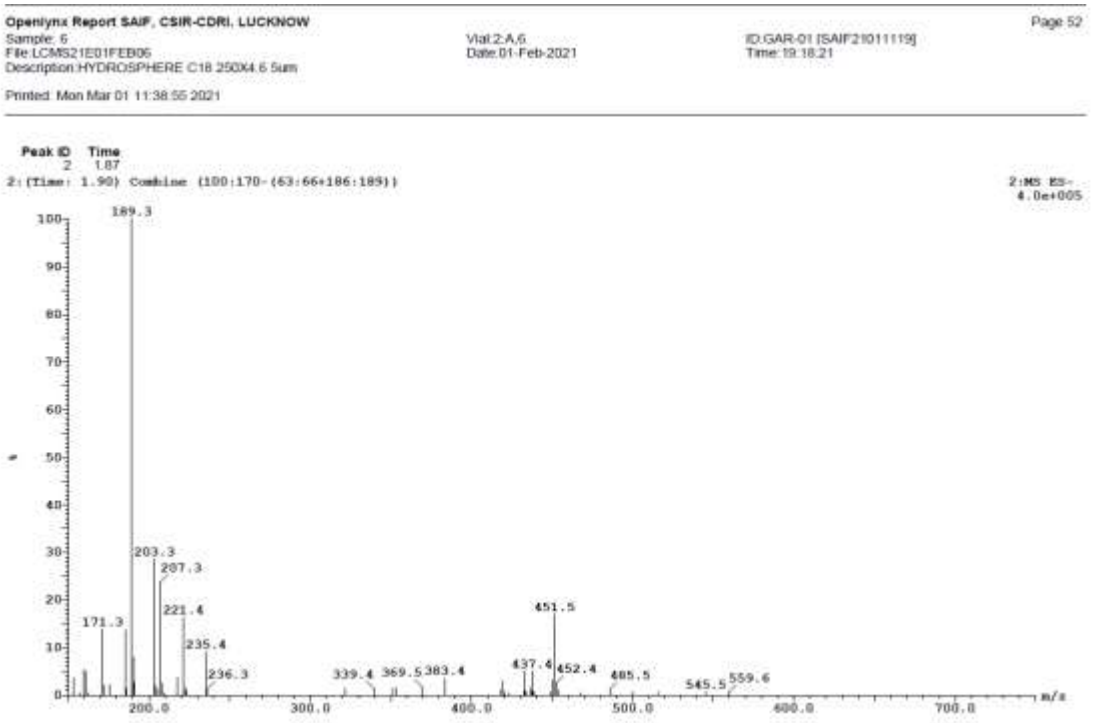


Figure 11(c): LC-MS spectrum of MEGP (Peak ID 2, t_R- 1.87, ESI - ve)

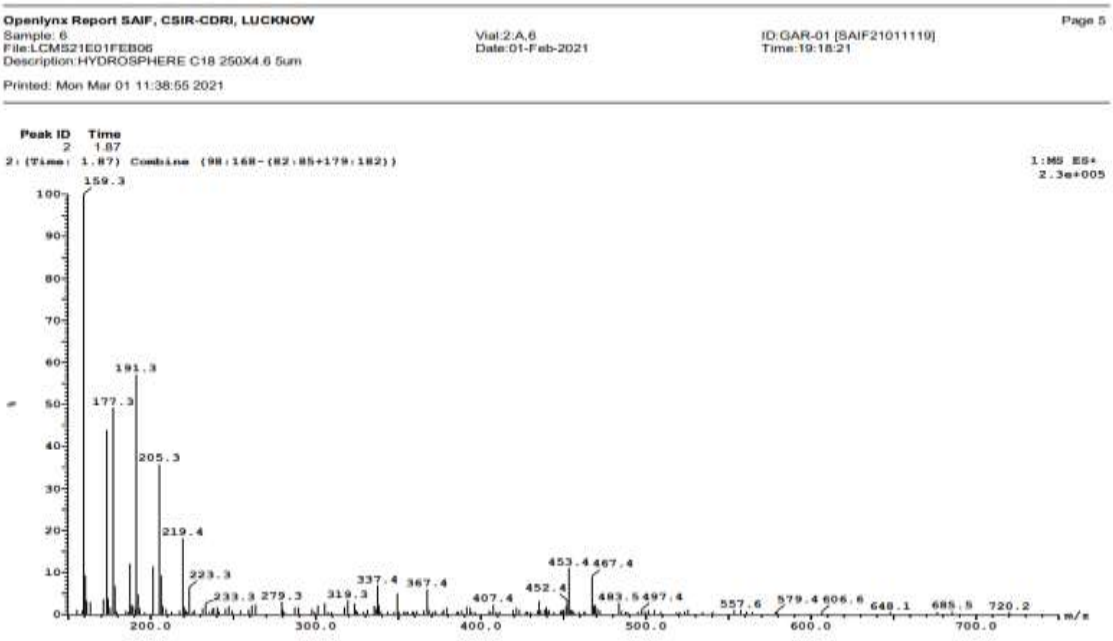


Figure 11(d): LC-MS spectrum of MEGP (Peak ID 2, t_R- 1.87, ESI + ve)

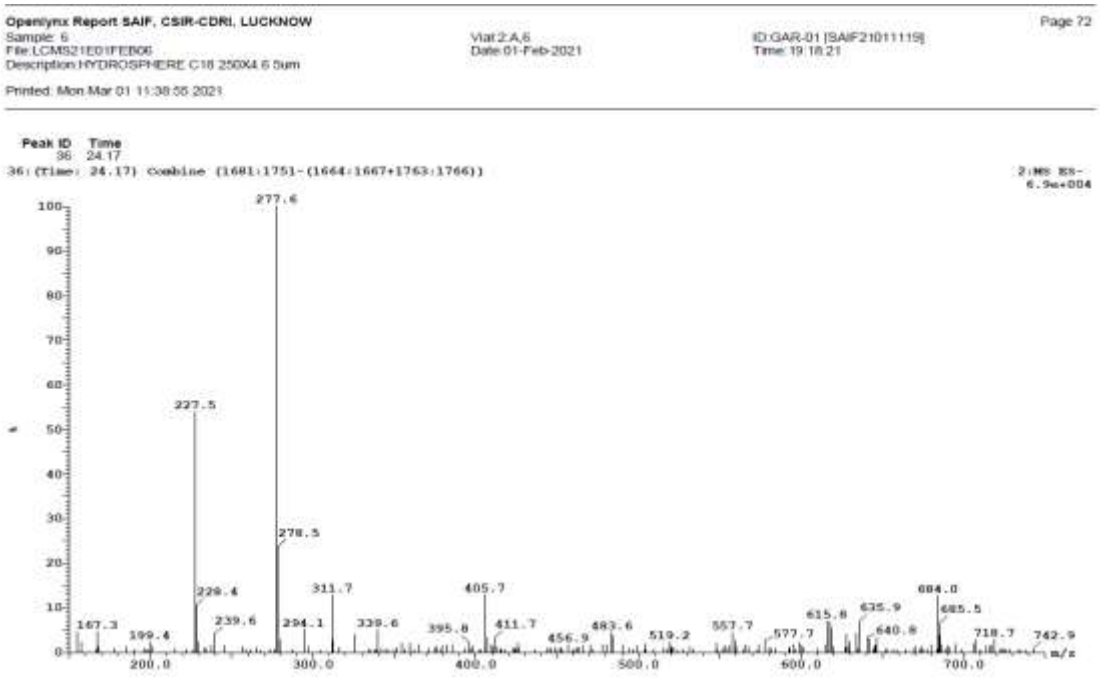


Figure 11(e): LC-MS spectrum of MEGP (Peak ID 36, t_R- 24.17, ESI - ve)

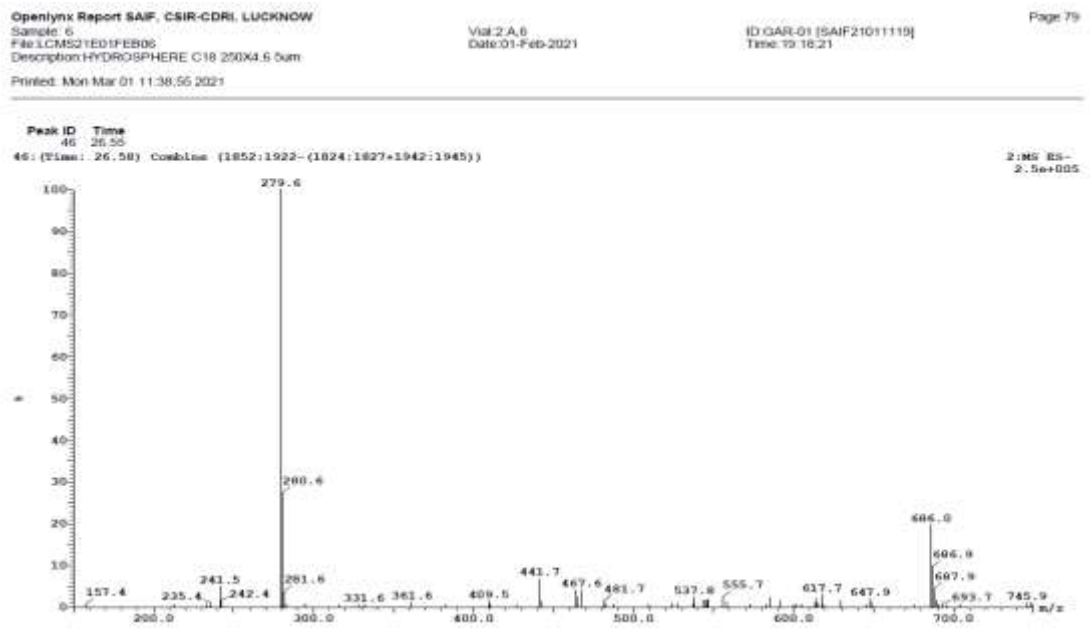


Figure 11(f): LC-MS spectrum of MEGP (Peak ID 46, t_R- 26.55, ESI - ve)

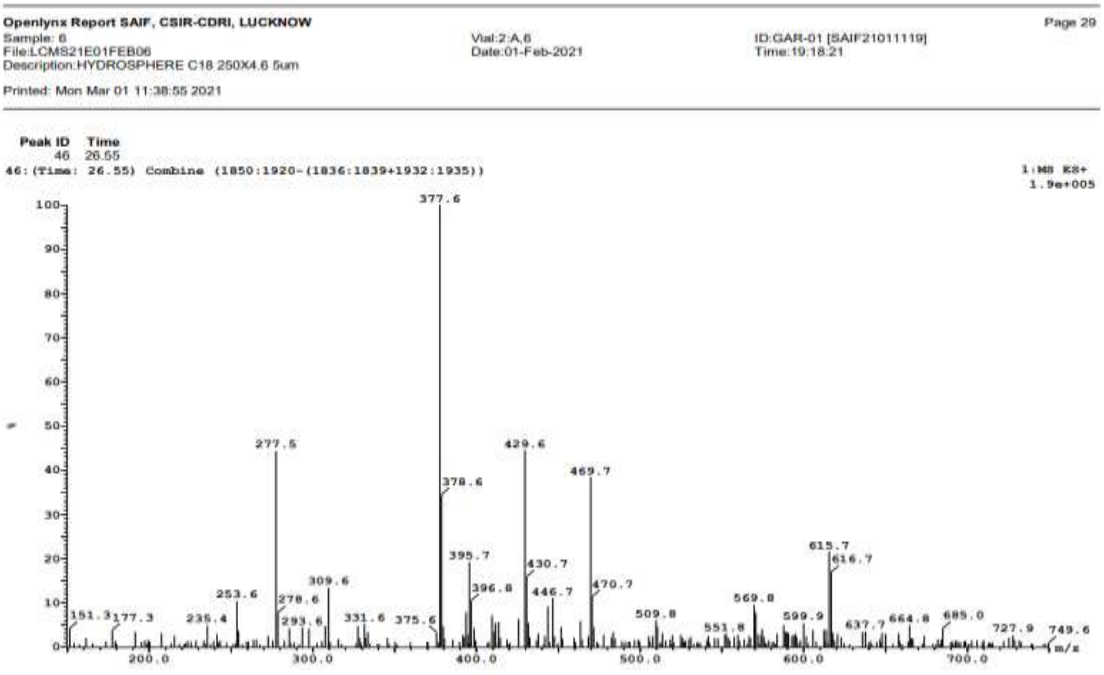


Figure 11(g): LC-MS spectrum of MEGP (Peak ID 46, t_R- 26.55, ESI + ve)

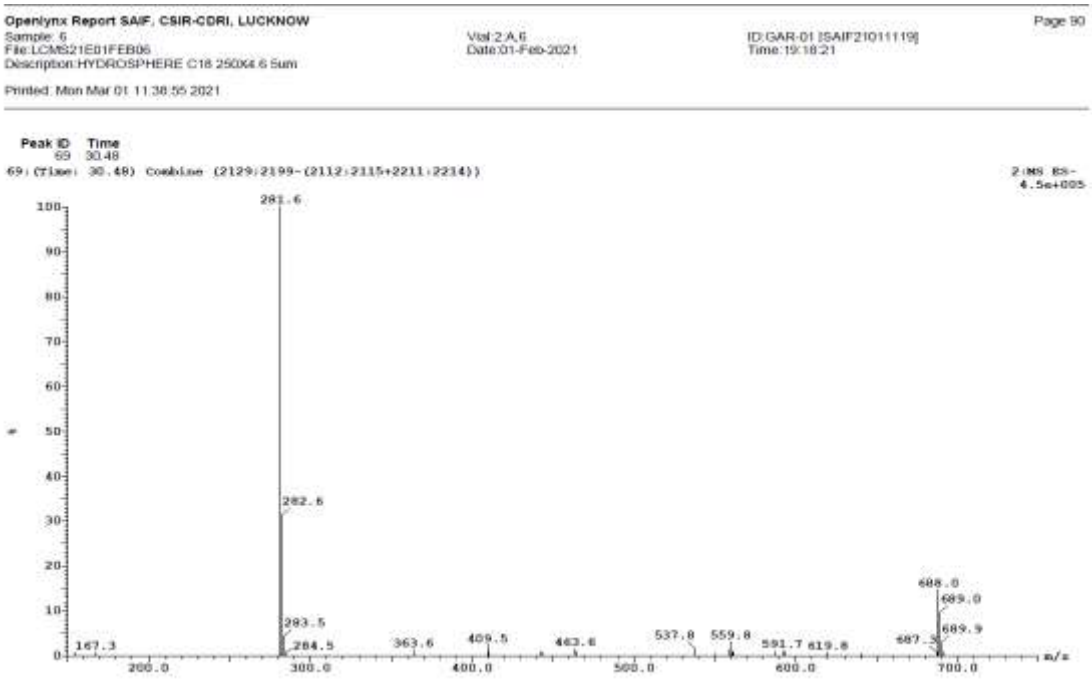


Figure 11(h): LC-MS spectrum of MEGP (Peak ID 69, t_R- 30.48, ESI - ve)

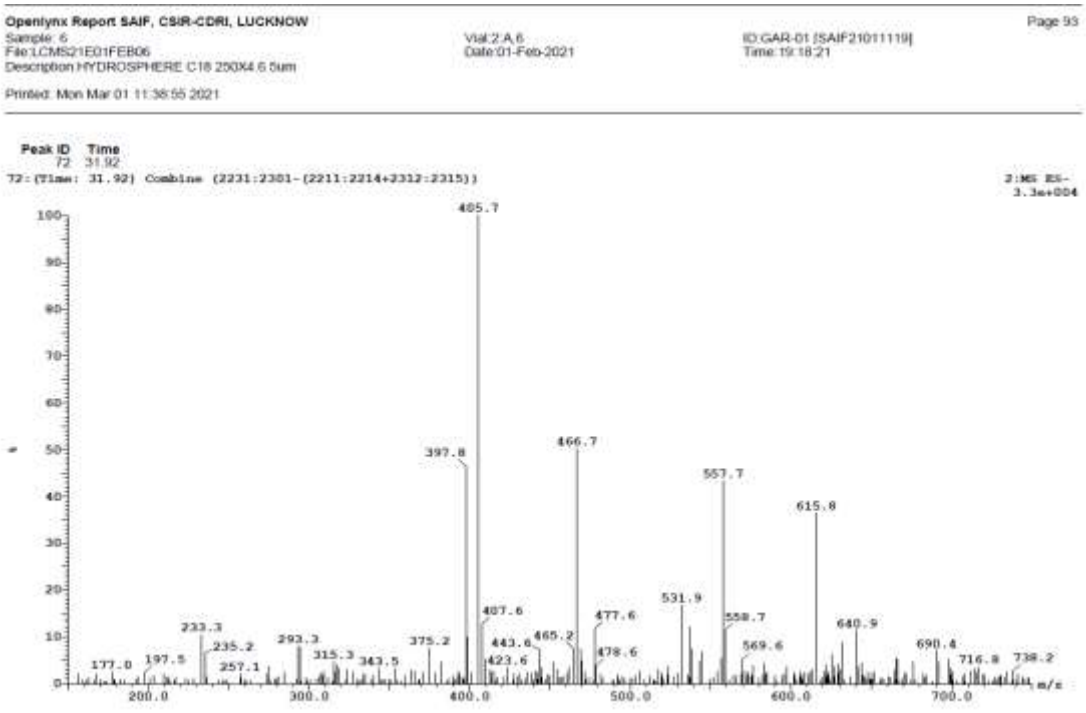


Figure 11(i): LC-MS spectrum of MEGP (Peak ID 72, t_R- 31.92, ESI - ve)

5.4. Discussion

LC-ESI-MS study of methanol extract of *P. lineata* detected the presence of gallic acid, catechin/epicatechin, epiafz/afz-epicat/cat dimer, epicat/cat-epicat/cat dimer, epiafz/afz-epiafz/afz-epicat/cat trimer, epiafz/afz-epicat/cat-epicat/cat trimer and epiafz/afz-epiafz/afz-epiafz/afz-epicat/cat tetramer, as detected in previous report (Choudhary *et al.*, 2014). Both gallic acid and catechin polyphenols are usually part of the complex composition of plants.

Gallic acid is a non-flavonoid polyphenol whose concentrations may reach 220 mg/kg in some plants (Obreque-Slier *et al*., 2010). Roberto Díaz-Gómez *et al.* (2013) observed that gallic acid provokes a dose-dependent decrease in the CFU/mL value and also a dose-dependent increase in the diameters of the inhibition halos in agar plate cultures of *H. pylori*, suggestig that gallic acid has a significant inhibitory effect on the growth of *H. pylori*. They also made an observation that the CFU/mL value decreased with increasing times of exposure of the *H. pylori* strains to gallic acid, thus confirming

that time of exposure to the polyphenol has a major influence on the viability of these microorganisms (Roberto Díaz-Gómez *et al.*, 2013).

Catechin is a flavonoid phenolic compound occurring in a large number of plants, whose concentrations may reach levels of 3000 mg/kg (Obreque-Slier *et al*., 2010). Roberto Díaz-Gómez *et al.* (2013) observed that the inhibitory effect of catechin on *H. pylori* growth had similar behaviour to that observed for gallic acid. Their observations indicated that catechin inhibits the growth of *H. pylori*, which fully coincides with reports by other authors (Tombola *et al*., 2003). It is important to note that, except for the maximum concentration of catechin in the measurement of the inhibition halo diameter, there were no statistically significant differences in the sensitivities of the two *H. pylori* strains to catechin and gallic acid. This observation suggests that the antibacterial effects of catechin and gallic acid are not dependent on the bacterial strain.

Yanagawa *et al*. (2003) and Roberto Díaz-Gómez *et al.* (2013) reported that both gallic acid and catechin display growth inhibitory effects in bacteria and those effects are partially additive in a dose-dependent manner over the range of doses in the study and growth inhibition is dependent on contact time and type of polyphenol.

LC-ESI-MS of the methanol extract of *G. pedunculata* fruit revealed that Hydroxy Citric Acid Lactone (MW-190), Garcinone E (MW-464), α-Mangostin (MW-410), β–Mangostin (MW-424), and γ-Mangostin (MW-396) are an important phytochemical content of *G. pedunculata*. All these phytochemical compounds have been reported from several *Garcinia* species including *G. indica*, *G. cambogia*, *G. atrovirdis*, *G. cowa*, *G. travancorica* and *G. mangostana* (Heymsfield *et al*., 1998; Kumar *et al*., 2013; Aravinda *et al*., 2016; Ibrahim *et al*., 2016). Garcinone E, α-Mangostin, β–Mangostin and γ-Mangostin are important phytochemical compound belonging to the group of xanthones, which are well known for their antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory and antitumor activities. From microbiological perspective, α-Mangostin, β-Mangostin and γ-mangostin had attracted all the attention and are under investigation for their exploitation as antibacterial agent.

α-Mangostin (α-MG) elicits in vitro rapid bactericidal activity against several Gram-positive pathogens (Nguyen and Marquis, 2011; Koh *et al.*, 2013; Sivaranjani *et al*., 2017). As reported by Koh *et al*. (2013), α-MG rapidly disintegrates the cytoplasmic membrane integrity of methicillin resistant *Staphylococcus aureus* (MRSA), which results in loss of cytoplasmic components. The multi-step resistance selection assay from previous studies suggested that Gram-positive pathogens do not develop resistance against α-MG (Koh *et al*., 2013; Sivaranjani *et al*., 2017). Sivaranjani *et al*. (2017) reported that α-MG effectively inhibits the onset of biofilm formation as well as disrupts the immature and mature biofilms of S. epidermidis RP62A biofilms, though the highest concentration of vancomycin was inefficient in killing the sessile cells of S. epidermidis RP62A (Sivaranjani *et al*., 2017). Similarly, Nguyen *et al*. (2014) reported that topical application of α-MG can effectively disrupt the development and structural integrity of *Streptococcus mutans* biofilm, which facilitates the mechanical clearance of cariogenic biofilms. Besides, several studies have demonstrated efficient methods to synthesize α-MG derivatives that also reflects the importance of α-MG and its derivatives in biological research (Matsumoto *et al*., 2004; Ha *et al*., 2009; Xu *et al*., 2013; Zou *et al*., 2013; Fei *et al*., 2014; Koh *et al*., 2015; Li *et al*., 2015; Koh *et al*., 2016). The potential bottleneck to develop α-MG as an effective antibacterial agent is the very limited understanding of the molecular mechanism of action of α-MG. Indeed, several studies have used omics techniques to elucidate the antibacterial mode of action of plant-derived compounds (Reddy *et al*., 2015; Dos Santos *et al*., 2016). The rapid antibacterial mode of action of α-MG has been already investigated through in vitro and in silico approaches (Koh *et al*., 2013). Sivaranjani *et al*. (2017) investigated the molecular mechanism of antibacterial activity of α-MG through an integrated transcriptomic and proteomic approach and concluded that α-MG targets *S. epidermidis* through multifarious mechanisms, and especially prompts that loss of cytoplasmic membrane integrity leads to rapid onset of bactericidal activity.

β-Mangostin are xanthones and they have anticancer activity against several types of cancer cells, including hepatocellular carcinoma cells (Huang *et al*., 2017), melanoma cells (Lee *et al.*, 2017) and cervical cancer cells (Lin *et al.*, 2020).

γ-mangostin showed antibacterial activity against the phytopathogen *R. solanacearum*, aetiological agent ofbacterial wilt, *in vitro*. γ-Mangostin, therefore, might have the potential to be developed as a natural bactericide to control plant bacterial wilt in the future (Ping *et al*., 2020).

Chapter 7

Summary and Conclusion

The use of medicinal plants for the treatment, prevention and cure of diseases accompanies man since the earliest civilizations (Firmo *et al*., 2011). Between 1981 and 2002, of the 877 new molecules introduced into the pharmaceutical market, 49% were substances isolated from natural products (Newman *et al.,* 2003). In 2010, herbal medicines accounted for approximately 15% of the capital of the world pharmaceutical industry (Niero, 2010).

The bioactive compounds present in the medicinal plant are responsible for the medicinal properties of the plant (Bargah, 2015). The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents (Nostro *et al.,* 2000). Indigenous people are generally very knowledgeable about the wild medicinal plants around them, many of which have local names and are important to the people medically or are featured in folklore (Kharkongor and Joseph, 1981).

Meghalaya is a small state in North-east India but it is one of the richest states of India in terms of vegetation and flora. This is due to the large variation in the altitude, topographical features, soil characteristics and climatic factors which has favoured the growth and luxuriance of rich flora here. The forests of Meghalaya are rich in biodiversity and endowed with rare species of orchids and medicinal plants.

According to Hynniewta (2010), the use of medicinal plants extract is common among the people of Meghalaya and found to be a significantly higher among rural people due to poor transportation and medical facilities in the rural areas. Modern medicinal facilities are scanty and could not reach these inaccessible areas inspite of government's best efforts.

However, analytical experimental studies like phytochemical screening and antimicrobial activities of medicinal plants of Meghalaya are lacking which is required to establish and confirm the benefit and effectiveness of medicinal plants as a treatment

for various diseases. Scientific analysis and evaluation of the ethnomedicinal plants used by the Khasi tribe in Meghalaya is the need of the hour and research work in this context is very scarce and limited. Hence, efforts are made in this direction in the form of phytochemical screening and antibacterial activity detection. It is a fact that justifies this research of evaluation of the antibacterial activity and phytochemical screening of the selected ethnomedicinal plants used by the Khasi tribe in Meghalaya.

All the selected potential ethno-medicinal plant were identified and authenticated at the Botanical Survey of India, Eastern Region, Shillong, Meghalaya and voucher specimens (herbarium) were deposited at the Assam Herbarium, BSI, Shillong, Meghalaya.

The medicinal plants selected and documented during the present work are *Achyranthes aspera* L. (Amaranthaceae), *Acmella paniculata* (Wall. ex DC.) R.K. Jansen (Asteraceae), *Ageratum conyzoides* L. (Asteraceae), *Bidens pilosa* L. (Asteraceae), *Centella asiatica* (L.) Urb. (Apiaceae), *Garcinia pedunculata* Roxb. ex Buch.-Ham. (Clusiaceae), *Gaultheria fragrantissima* Wall. (Ericaceae), *Hibiscus sabdariffa* L. (Malvaceae), *Houttuynia cordata* Thunb. (Saururaceae), *Lantana camara* L. (Verbenaceae), *Piper attenuatum* Buch.-Ham. ex Miq. (Piperaceae), *Potentilla lineata* Trevir. (Rosaceae), *Prunella vulgaris* L. (Lamiaceae), *Sonchus oleraceus* L. (Asteraceae) and *Sonchus palustris* L. (Asteraceae).

Out of the 15 plants selected, 5 plants belong to the family of Asteraceae, one plant each belong to the family of Amaranthaceae, Apiaceae, Clusiaceae, Ericaceae, Lamiaceae, Malvaceae, Piperaceae, Rosaceae, Sauraceae and Verbenaceae.

The specimens collected were as complete as possible and healthy. They are collected from its typical habitat. Flowers and fruit were included, as well as vegetative parts wherever possible. Clearly, in most cases, this is impossible since ripe fruit and flowers do not usually occur at the same time.

The randomly collected plants were brought to the laboratory and thoroughly washed in running water to remove debris and dust particles and then rinsed using distilled water and finally air dried at room temperature before grinding them to

powdered form using pestle and mortar / mechanized grinder. The powdered medicinal plants were extracted using water, methanol, ethanol and chloroform. The extract was then used for antibacterial activity screening and phytochemical analysis.

The test microorganisms used in the study were clinical isolates of *Escherichia coli* AF06, *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24, *Enterobacter cloacae* AM03, *Pseudomonas aeruginosa* CM07 and *Klebsiella pneumoniae* CF09. The microorganisms were isolated from urine samples collected from patients. The collected samples were streak on selective media and characteristic colony morphological study was conducted. The isolated colonies were sub – cultured to obtain pure culture and then the pure culture were then subjected to a biochemical test for identification using Vitek Automated Machine in the laboratory of Department of Microbiology, Woodland Hospital, Shillong.

The pure cultures of the clinical isolates of the test microorganisms were maintained on different agar slants - *Staphylococcus aureus* AM12 (MSA), *Enterococcus faecalis* AF24 (CLED), *Enterobacter cloacae* AM03 (McConkey), *Escherichia coli* AF06 (EMB), *Pseudomonas aeruginosa* CM07 (Cetrimide), *Klebsiella pneumoniae* CF09 (McConkey).

The susceptibility of the six (6) reference strain of test bacteria to twelve (12) commonly employed different antibiotics viz. Amikacin AK 30 mcg / disc, Aztreonam AT 30 mcg / disc, Cefepime CPM 30 mcg / disc, Cefoperazone CPZ 75 mcg / disc, Ceftazidime CAZ 30 mcg / disc, Ciproflaxacin CIP 5 mcg / disc, Gentamicin GEN 10 mcg / disc, Imipenem IPM 10 mcg / disc, Levoflaxacin LE 5 mcg / disc, Meropenem MRP 10 mcg / disc, Piperacillin PI 100 mcg / disc, and Piperacillin / Tazobactam PIT 100 / 10 mcg / disc was assessed by disc diffusion method using Hi - Media's Dodeca Disc for easy and relevant comparision cum to ascertain the relative effectiveness of the 15 medicinal plant extracts. The different cultures of the clinical bacterial isolates responded to standard antibiotics in a variable manner resulting in various size of zones of inhibition. The size of zone of inhibition ranges from 10.3 ± 0.5

(Piperacillin/Tazobactam PIT 100/10mcg against *K. pneumoniae* CF09) to 59.7±0.5 (Levoflaxacin LE 5mcg against *S. aureus* AM12).

The test bacteria were grown on Mueller-Hinton Agar (MHA, HiMedia) plate for 16-18 hr. at 37 ± 2°C. Well-isolated colonies were suspended in sterile Mueller-Hinton Broth (MHB, HiMedia) and the turbidity was adjusted against 0.5 McFarland standard to comprise approximately 1.5 x 10^8 CFU/ml.

Antibacterial activity of the methanol extract of the 15 medicinal plants towards different clinical isolates were measured in terms of zone of inhibition using disc diffusion method as stated by Kirby - Bauer *et al*. (1966). It is the recommended method of European Committee on Antimicrobial Susceptibility Testing (EUCAST) and National Committee for Clinical Laboratory Standard (NCCLS), USA.

The susceptibility of different bacterial clinical isolates to the methanol extract of the selected 15 ethnomedicinal plants were also measured in terms of zone of inhibition using agar well diffusion assay as well. All the tested bacteria were susceptible to *Garcinia pedunculata* while none of the extract of *A. aspera, A. paniculata, A. conzyoides, C. asiatica, H. sabdarifa, H. cordata, P. attenuatum, P. vulgaris, S. oleraceus and S. palustris* were active against any of the tested bacterial species.

Staphylococcus aureus AM12 was susceptible to *B. pilosa, G. pedunculata, G. fragrantissima* and *P. lineata* while both *S. aureus* AM12 and *E. faecalis* AF24 were susceptible to *G. pedunculata, G. fragrantissima* and *P. lineata*. *E. cloacae* AM03, *E. coli* AF05 and *K. pneumoniae* CF09 were inhibited by only *G. pedunculata* while *P. aeruginosa* CM07 was susceptible to only *G.pedunculata* and *L. camara*.

Comparative study of the Antimicrobial Activity of methanol and ethanol extract of *P. lineata* and *G. pedunculata* against the UTI clinical bacterial isolates were also investigated involving MIC and MBC analysis and Time-Kill studies.

The antibacterial activity of the methanol extract and ethanol extract of the selected 2 ethnomedicinal plants viz. *P. lineata* and *G.pedunculata* were determined by the disc and well diffusion method. It was observed that both the organic solvent extract

of *P. lineata* produced very effective and notable antimicrobial activities against the two tested Gram positive bacteria (*Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 but Gram negative bacteria (*Enterobacter cloacae* AM03, *E. coli* AF05, *Pseudomonas aeruginosa* CM07, *Klebsiella pneumoniae* CF09) were not susceptible to the extract.

On the other hand, both the organic solvent extract of *G. pedunculata* were inhibitory to all the six bacterial isolates and are more potent than that of the extract of *P. lineata*. All the methanol and ethanol negative control discs did not produce any zone of inhibition against any of the tested clinical isolates. The results were compared with those of ampicillin and methicillin as standard antibiotics. The solvent extract (50 mg/ml) of both *P. lineata* and *G.pedunculata* were not as potent as ampicillin (10 mcg) but are quite effective as methicillin (5 mcg) antibiotics or even better. It is noteworthy that *G. pedunculata* shows antibacterial activity against *E. cloacae* AM03, *P. aeruginosa* CM07 and *K. pneumoniae* CF09 which are not susceptible to the standard antibiotics namely, Ampicillin and methicillin.

The minimum inhibitory concentrations (MIC) of *P. lineata* plant extracts were determined by broth microdilution method, as described previously by Wiegand *et al.* (2008) supplemented with Resazurin dye (Elsinkh, 2016) based on Clinical Laboratory Standard Institute. The MICs of only methanol extract of *P. lineata* for the susceptible bacterial isolates was determined, though both the methanol and ethanol extracts showed antimicrobial activities against Gram-positive bacteria, *Staphylococcus aureus* AM12 and *Enterococcus faecalis* AF24. The extract is approximately equally potent against *Enterococcus faecalis* AF24 and *Staphylococcus aureus* AM12 with MICs being 25 mg/ml.

The minimum bactericidal concentration (MBC) of *P. lineata* was determined by plating directly the content of Eppendorf tubes with concentrations higher than the MIC value. The lowest concentration of the extract that did not permit any growth was taken as the MBC. The MBC values of methanol extract is 100 mg/ml for both *Staphylococcus aureus* AM12 and *Enterococcus faecalis* AF24.

The MICs of the methanol extract of *G.pedunculata* for the susceptible bacterial isolates were also determined. The extract is approximately equally potent against *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 *and Pseudomonas aeruginosa* CM07, with MIC being 12.5 mg/ml. Against *Enterobacter cloacae* AM03, *E. coli* AF05 and *Klebsiella pneumoniae* CF09, the MIC of the extract was observed to be approximately 25 mg/ml.

The MBC of the methanol extract of *G. pedunculata* was found to be approximately 25 mg/ml against *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 and *Pseudomonas aeruginosa* CM07 while 50 mg/ml was the observed MBC against *Enterobacter cloacae* AM03, *E. coli* AF05 and *K. pneumoniae* CF09.

Time-kill assays were performed by broth macro-dilution method in accordance with the CLSI guidelines. All the susceptible exponentially growing susceptible test bacteria in mid-logarithmic growth phase were adjusted to the 0.5 McFarland standard. Then, each of the standardized bacterial suspension of all the susceptible test bacteria (50 µl) were inoculated into several Eppendorf tubes (0,1,2,3,4,5,6,7 and 8 h) of MHB containing 450 µl of MHB and 500 µl of the extracts with final concentrations corresponding to 1X MIC (i.e.25 mg/ml of MEGP for *Enterobacter cloacae* AM03, *E. coli* AF05 and *Klebsiella pneumoniae* CF09*;* and 12.5 mg/ml of MEGP for *Staphylococcus aureus* AM12, *Enterococcus faecalis* AF24 and *Pseudomonas aeruginosa* CM07*;* 50 mg/ml of MEPL for *Staphylococcus aureus* AM12 and *Enterococcus faecalis* AF24) and 2X MIC. The final density of bacteria was approximately $4 – 5 \times 10^5$ CFU / mL.

Time-kill assays have been widely used for in vitro investigations of new antimicrobial agents as these provide descriptive (qualitative) information on the pharmacodynamics of antimicrobial agents (Lewis, 2007).

With regards to MEPL, only MEPL with activity towards susceptible bacterial strains was selected for time-kill studies. The kinetic interaction between susceptible bacteria and MEPL was examined at the concentration of 1X MIC (25 mg/ml) and 2X MIC (50 mg/ml). The kill kinetic profiles of MEPL displayed rapid killing rate towards

both the susceptible Gram positive bacteria, S. aureusAM12 and *E. faecalis* AF24, showing complete destruction after 5 h exposure at 1X MIC and after 4 h exposure at 2X .

In the present study, the kinetic interaction between susceptible bacteria and MEGP was examined at the concentration of 1X MIC (12.5mg/ml for *S. aureus* AM12, *E. faecalis* AF24 & *P. aeruginosa* CM07 and 25 mg/ml for *E. cloacae* AM03, *E. coli AF05 & K. pneumonia* CF09), and 2X MIC. The kill kinetic profiles of MEGP displayed rapid bactericidal activity towards all susceptible strains, showing complete destruction after 7 h exposure at 1X MIC and after 5 h exposure at 2X MIC. As expected from the determined MBC/MIC ratios, the time-kill assays for MEGP towards *S. aureus* AM12, *E. faecalis* AF24, *E. cloacae* AM03, *E. coli* AF05, P. aeruginosa CM07 and K. pneumoniae CF09 were consistent with bactericidal characteristic. The kill kinetic profiles of MEGP exhibited varying degrees of bactericidal activities depending on the tested strains. The killing rate of MEGP at 1XMIC was slower against *E. cloacae* AM03 and *K. pneumonia*e CF09 (complete killing were only seen after 7 h interaction at 1x MIC) than against *E. coli* AF05 and P. aeruginosa CM07 (complete killing were only seen after 6 h interaction at 1 X MIC) but the killing rate of MEGP at 2 X MIC was found to be similar for all tested Gram negative bacteria - *E. cloacae* AM03, *E. coli* AF05, *P. aeruginosa* CM07 and *K. pneumoniae* CF09 (complete killing were seen after 5h at 2X MIC). MEGP exhibited a faster killing rate against *S. aureus* AM12 and *E. faecalis* AF24, showing bactericidal activity only after 4 h at both 1 X MIC and 2 X MIC indicating that increase concentration does not have any significance.

The time-kill studies have provided valuable information on the rate, concentration and potential action of MEGP and MEPL *in vitro*. As the antibacterial activities and bacterial killing rates of MEGP and MEPL were different from each other, it is likely that different mechanisms are involved. An intensive investigation and research are needed to determine the mechanism(s) of action of these extracts in order to strengthen their potential as therapeutic antibiotics. In particular, MEGP, with

its potent and specific antibacterial profile, deserved further scientific research and evaluation.

Another challenging prospect for future research revolves around the screening of a combination of various plants extract since our study accomplished only antibacterial screening of single plant extract alone, not in combination. It is well known and observable in traditional medicinal practices that the recipe (herbal drugs) includes a combination of many plants and plant parts for a good number of diseases, although a single plant is administered singly for majority of health-related problems. Hence, exploration in this direction is advisable.

Preliminary qualitative phytochemical analysis of the crude extract of the 15 plants collected was determined as as per standard methods described by Brain and Turner (1975) and Evans (1996). The result of the phytochemical group test revealed that there was great diversity in the phytochemical content of various species of plants. Phytochemical screening revealed variations and diversity in the content of phytochemical compounds, qualitatively and quantitatively, for the 15 selected medicinal plants investigated. The variations and diversity were observed not only between the family and genus, but also between the species.

Quantitative phytochemical analysis was also persued. The alkaloid content was determined gravimetrically by following Harborne (1973) Method. The flavonoids were estimated spectrophotometrically by the method of Zhishen *et al.* (2010) whereas saponins and tannins were estimated by the method of Hiai *et al.* (1976) and Van-Burden and Robinson (1981) respectively. The result of the phytochemical group test revealed that there was great diversity in the phytochemical content of various species of plants. *S. palustris* contain highest total alkaloid content viz.13.2 μg/ml while the least alkaloid content (2.6 μg/ml) was estimated for *A. aspera. S. oleraceus* contain highest total flavonoid content viz.155.97 μg/ml while the least flavanoid content (50.135 μg/ml) was estimated for *S. palustris. B. pilosa* contain highest total saponin content viz.74.73 μg/ml while the least saponin content (47.36 μg/ml) was estimated for *P.*

vulgaris. *C. asiatica* contain highest total tannin content viz.71.77 μg/ml while the least tannin content (15.04 μg/ml) was estimated for *S. oleraceus*.

Phytochemical analysis by TLC were carried out by following the method of Harborne (Harborne, 1998). TLC is a quick, sensitive, and an inexpensive technique, which separates the number of components present in any non-volatile complex mixture or plant sample using a suitable solvent for separation of different components.

Clear separated resolved bands/spots were observed in three solvent systems i.e., Chloroform: Methanol (6:1), Hexane : Ethyl acetate (4:1) and Ethyl acetate:Methanol:Water:Glacial acetic Acid (1.35:0.5:0.5:0.5) after being sprayed with reagent. Therefore, they are recommendable as a solvent system for further analysis.

The evaluations of various plants extract showed presence of different bioactive compounds as indicated by varying number of spots on a TLC plate and different Rf values.

The methanol extracts of *P. lineata* and *G. pedunculata* (MEGP) were subjected to LCMS analysis. Methanol extract of *P. lineata* (MEPL) and *G.pedunculata* (MEGP) was selected for LCMS analysis over ethanol extract of *P. lineata* (EEPL) and *G. pedunculata* (EEGP) since it was observed that it gives larger zone of inhibition against the bacteria tested. It implies that methanol may be a better organic solvent for extraction of antibacterial compounds. A continuous gradient system was followed rather than isocratic system.

All analyses were carried out in the full scan mode from 150.0 to 2000.0 m/z using an ESI source in both positive-ion mode and negative-ion mode for MEPL while the analysis for MEGP was carried out in negative ion mode over a mass range of 150.0-750.0 m/z.

LC-MS of the methanol extract of *G. pedunculata* fruit revealed presence of several compounds. A total of 23 compounds (ESI-ve) and 17 compounds (ESI+ve) were characterized. Among them, 5 compounds viz. Hydroxy Citric Acid Lactone (MW-190), Garcinone E (MW-464), α-Mangostin (MW-410), β–Mangostin (MW-424), and γ-Mangostin (MW-396) were tentatively identified (peaks 2, 36, 46, 69 and 72). On

the other hand, gallic acid, catechin/epicatechin, epiafz/afz-epicat/cat dimer, epicat/cat-epicat/cat dimer, epiafz/afz-epiafz/afz-epicat/cat trimer, epiafz/afz-epicat/cat-epicat/cat trimer, and epiafz/afz-epiafz/afz-epiafz/afz-epicat/cat tetramer were identified in the methanolic extract of *P. lineata*.

Identification was carried out based on molecular weight, using the mass spectra of the LC-MS. Retention times (tR), UV and mass spectral data of compounds were compared to literature data and to those of authentic standards, where available, for unambiguous identification.

PATRICULARS OF THE CANDIDATE

Name of Candidate	**:**	Zoliansanga
Degree	**:**	Ph.D.
Department	**:**	Botany
Title of thesis	**:**	Phytochemical Diversity and Antibacterial Activity of Selected Medicinal Plants Used by Khasi Tribe in Meghalaya
Date of Admission	**:**	16th August, 2016
Approval of Research Proposal		
1. DRC	**:**	24th April, 2017
2. BOS	**:**	1st May, 2017
3. School Board	**:**	26th May, 2017
MZU Registration No.	**:**	1600835
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Appendix

A. Microbiological Media for Antibacterial Activity

(i) Nutrient agar

Sl.no.	Ingredients	Gram/Litre
1	Peptone	5.0 gm
2	Beef extract/yeast extract	2.0 gm
3	NaCl	5.0 gm
4	Distilled water	1000 ml
5	agar	15 – 17 gm
	NB: pH 7.4 ± 0.1 at 25 °C.	

(ii) Muller-Hinton agar

Sl.no.	Ingredients	Gram/Litre
1	Beef Extract	2.00 gm
2	Acid Hydrolysate of Casein	17.50 gm
3	Starch	1.50 gm
4	Agar	17.00 gm
5	Distilled Water	1000 ml
	NB: pH 7.3 ± 0.1 at 25ºC	

(iii) C.L.E.D. (Cystine-Lactose-Electrolyte-Deficient) medium

Sl.no.	Ingredients	Gram/Litre
1	Lactose	10.0
2	Pancreatic digest of gelatin	4.0
3	Pancreatic digest of casein	4.0
4	Beef extract	3.0
5	L-Cystine	0.128
6	Bromothymol Blue	0.02
7	Agar	15.0
	NB: pH 7.3 ± 0.2 at 25°C	

(iii) Mc Conkey agar

Sl.no.	Ingredients	Gram/Litre
1	Peptone	17.00 gm
2	Lactose	10.00 gm
3	Bile salts	1.50 gm
4	Sodium chloride	5.0
5	Neutral red	0.03
6	Crystal violet	0.001
7	Agar	13.5
8	Distilled Water	1000 ml
	NB: pH 7.3 ± 0.1 at 25ºC	

(iv) Eosin Methylene Blue (EMB) Agar

Sl.no.	Ingredients	Gram/Litre
1	Peptic digest of animal tissue	10.00 gm
2	Dipotassium phosphate	2.00 gm
3	Lactose	5.0 gm
4	Sucrose	5.0 gm
5	Eosin – Y	0.04 gm
6	Methylene blue	0.065 gm
7	Agar	13.5 gm
8	Distilled Water	1000 ml
	NB: pH 7.2±0.2 at 25°C	

(vi) Cetrimide Agar

Sl.no.	Ingredients	Gram/Litre
1	Pancreatic digest of gelatin	20.00 gm
2	Magnesium chloride	1.4 gm
3	Dipotassium sulphate	10.0 gm
4	Cetrimide	0.3 gm
5	Agar	13.6 gm
6	Distilled Water	1000 ml
	NB: pH 7.2±0.2 at 25°C	

(v) Mannitol Salt Agar

Sl.no.	Ingredients	Gram/Litre
1	Peptone	10.0 gm
2	Beef extract	1.00 gm
3	Sodium chloride	75.0 gm
4	D-Mannitol	10.0 gm
5	Phenol red	0.025gm
6	Agar	15.0 gm
7	Distilled Water	1000 ml
	NB: pH 7.4±0.2 at 25°C	

B. Reagent preparation for Antibacterial activity

(i) **1 % Resazurin (aqueous):** Dissolve 1 gm of resazurin in 50 ml of distilled water, then dilute to 100 ml. Stir and filter if necessary.

(ii) **McFarland Standards:** are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland Standard. A McFarland Standard is a chemical solution of barium chloride and sulfuric acid; the reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. When shaken well, the turbidity of a McFarland Standard is visually comparable to a bacterial suspension of known concentration as indicated below:

McFarland Standard	1% $BaCl_2$ (mL)	1% H_2SO_4 (mL)	Approximate Bacterial Suspension / mL
0.5	0.05	9.95	1.5×10^8
1.0	0.10	9.90	3.0×10^8
2.0	0.20	9.80	6.0×10^8
3.0	0.3	9.7	9.0×10^8
4.0	0.4	9.6	1.2×10^9
5.0	0.5	9.5	1.5×10^9
6.0	0.6	9.4	1.8×10^9
7.0	0.7	9.3	2.1×10^9
8.0	0.8	9.2	2.4×10^9
9.0	0.9	9.1	2.7×10^9
10.0	1.0	9.0	3.0×10^9

Prior to using, the McFarland Standard should be shaken up well and aliquoted into test tubes identical to those used to prepare the inoculum suspension. Once aliquoted, the tubes should be tightly sealed to prevent evaporation from occurring. Before each use, shake well to ensure that the barium sulfate is distributed evenly throughout the solution. The standard most commonly used in the clinical microbiology laboratory is the 0.5 McFarland Standard, which is prescribed for antimicrobial susceptibility testing and culture media performance testing.

C. Reagent preparation for Phytochemical Analysis

(i) **1% ammonia**: 1ml of ammonia dissolved in 99ml of distilled water.

(ii) **1% aluminium chloride**: 1gm of aluminium chloride was dissolved in 100ml distilled water.

(iii) **5% Ferric Chloride (alcoholic)**: 5 gm of ferric chloride in 100 ml of ethanol.

(iv) **Lead acetate**: A 25% basic lead acetate solution is used for the detection of flavonoid.

(v) **10% acetic acid** (for alkaloid estimation): Mixture of 10ml of acetic acid and 90 ml of ethanol.

(vi) **10% aluminium chloride** (for flavonoid estimation): 10gm of aluminium chloride was dissolved in 100ml ethanol.

(vii) **10% sodium nitrite** (for flavonoid estimation): 10gm of sodium nitrite was dissolved in 100ml distilled water.

(viii) **1% sodium hydroxide** (for flavonoid estimation): 1gm of sodium hydroxide was dissolved in 100ml distilled water.

(ix) **8 % vanillin solution** (for saponin estimation): 8 gm of vanillin in 100 ml of ethanol.

(x) **72 % sulphuric acid** (for saponin estimation): Mixture of 72 ml of sulphuric acid and 23 ml of distilled water.

(xi) **0.1M Ferric Chloride in 0.1 N HCl** (for tannin estimation): 16.2 gm of ferric chloride in 0.1 N HCl.

(xii) **0.1 N Hydrochloric acid** (for tannin estimation): 0.83 ml in 100 ml of distilled water.

(xiii) **0.008 M Potassium Ferrocyanide** (for tannin estimation): 33.76 gm in 100 ml of distilled water.

D. Reagent preparation for Phytochemical Analysis

(i) **Dragendorff reagent** (For detection of nitrogen compounds, alkaloids, antiarrhythmic drugs, surfactants)

- Solution 1) 1.7gm basic bismuth nitrate and 20g tartaric acid in 80ml water
- Solution 2) 16 gm potassium iodide in 40ml water
- Stock solution (stable for several weeks in a refrigerator):
- Mix equal volumes of solutions 1 and 2
- Procedure: Spray with a solution of 10gm tartaric acid, 50ml water and 5ml stock solution

(ii) **Ferric chloride**(For detecting tannin, phenols and phenolic acids)

Procedure: 2.7gm of salt dissolved in 100ml 2M hydrochloric acid.

(iii) **Vanillin / phosphoric acid** (Used as a charring reagent for polymer bound TLC plates.)

- Spray plate with a solution of 1gm vanillin in a mixture of 50ml water and 50ml H_3PO_4. Heat 5-30min at 110°C.
- View frequently for colored or fluorescent spots (at 254 and 360nm) to appear.
- Charring can be continued until spots are brown, grey or black.

(iv) **Vanillin / sulphuric acid** (For detection of steroids: This charring visualization reagent can only be used with glass TLC plates in which a G (gypsum) binder has been incorporated)

- 0.5gm vanillin in 80ml sulphuric acid and 20ml ethanoland spray plates. Dry at 120°C until maximum color development.
- Another formulation of this reagent: Dissolve 1gm of vanillin in 100ml conc. sulfuric acid

(v) **Vanillin Sulphuric Acid Developer** :The vanillin sulphuric acid reagent was prepared by dissolving 1 gm of vanillin added with 2 ml of sulfuric acid in 100 ml of 95% ethanol. After spraying, the TLC plate was heated at 110 °C for about 5 minutes. Several colorations appear depending on the types of compounds.

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Research Publication

1. Zoliansanga & Lalfakzuala (2021). Antibacterial Activity and Phytochemical Screeningof Garcinia pedunculata Roxb. ex Buch. - Ham. Fruitextract by HPLC–ESI- MS. J Pure Appl Microbiol |Article 7233 |15 (4):2183-2194.

2. Zoliansanga & Lalfakzuala (2022). Comparative study of the antibacterial activity of Garcinia pedunculata and Potentila lineata against the UTI clinical bacterial isolates – Disc Diffusion Assay and Well Diffusion Assay. Echoes from the Hills. Vol. V. No. 1. 52 - 75

Paper Presented in National Seminars / Conferences

1. National Seminar on Conservation of Biodiversity, Microbial Diversity with potential for application in Agriculture and Sericulture in North East India. Organised by Department of Botany, University of Science & Technology, Meghalaya in collaboration with Central Silk Board, Assam. (25th to 26th March, 2022).
"Antibacterial Activity and Phytochemical Screeningof *Garcinia pedunculata* Roxb. ex Buch. - Ham. Fruitextract by HPLC–ESI- MS."

2. National Seminar on Conservation on Emerging Trends in Plant Sciences (ETPS). Organised by Department of Botany, North Eastern Hill University, Shillong, Meghalaya in collaboration with CSIR – National Botanical Research Institute, Lucknow, Uttar Pradesh. (29thto 30th March, 2022).
"Comparative study of the Antibacterial Activity of *Garcinia pedunculata* Roxb. ex Buch.- Ham and *Potentilla lineata* against the UTI clinical bacterial isolates – Disc Diffusion and Well Diffusion Assay."

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