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Original article

In-vitro examination and isolation of antidiarrheal compounds using five bacterial strains from invasive species *Bidens bipinnata* L.

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ABSTRACT

Bidens bipinnata is widely utilized medicinal plant for treatment of diseases like malaria, sore throat, acute nephritis and dysentery. However, despite its traditional uses *Bidens bipinnata* is not widely explored for its antimicrobial effect. Thus, the current study is aimed to form antimicrobial activity report of *Bidens bipinnata* extracts, along with isolation and evaluation of antibacterial activity of the isolated compounds through bioassay-guided purification. Hexane extract of its leaves has appeared to be most active thus it is exposed to automated column chromatography. Further purification using High-performance liquid chromatography has led to isolation of active peaks, identified by Gas Chromatography-mass spectrometry, as 16-Pregnenolone and 9-Octadecenoic acid (Z)-, methyl ester. Their antimicrobial activity was confirmed via broth dilution procedure on *Staphylococcus aureus*, 16-Pregnenolone revealed a strong antimicrobial activity with MIC_{50} of 72 µg/mL whereas 9-Octadecenoic acid (Z)-, methyl ester display an MIC_{50} of >250 µg/mL. Present study is the first report on isolation of these compounds from *Bidens bipinnata*.

1. Introduction

In the present world, one of the main reasons of this raise in infectious diseases is development of antibiotic resistance in pathogens (Djeussi et al. 2013). Therefore in order to reduce antimicrobial resistance novel antibiotics must be developed. Natural products always act as a reservoir of novel chemical compounds with better pharmacological potential. Thus they must be explored for drugs candidate that may replace synthetic drugs (Adisa et al. 2011). Various approaches have been used to investigate pharmacological potential of ethno medicinally used plants. It has been understood that indigenous communities has remarkable understanding towards medicinal plants (Mahomoodally, 2013).

The genus *Bidens*, is a member of Asteraceae family, consist of almost 240 species (Silva et al. 2011). Plant is being utilized by conventional systems as a remedy of pyrexia, rheumatism and hepatitis (Bo et al. 2012). Genus *Bidens* has been reported to for various bioactive constituents i.e., polyacetylenes, flavonoids, alkalids, phenols and sesquiterpene lactones. Various pharmacological studies on this genus also revealed hypoglycaemic (Chien et al. 2009), anti-plasmodial (Oliveira et al. 2004) and anti-hepatic fibrosis (Yuan et al. 2008). The purpose of the current research was to separate the major antimicrobial ingredients from *Bidens bipinnata* via bioassay-guided purification.

2. Materials and methods

2.1. Reagents

High-performance liquid chromatography (HPLC) grade solvents were used i.e., Ethyl acetate, hexane, acetone, methanol, ace-

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tonitrile. Deionised water, Mueller–Hinton (MH) broth, DMSO, hydroquinone and ciprofloxacin hydrochloride.

2.2. Collection and identification of plants

Bidens bipinnata L. was collected from Azad Jammu and Kashmir, Pakistan during spring 2017 to spring 2018. The specimens were identified and for future references voucher specimens were deposited. Plants were fully desiccated, fine powder was prepared and stored.

2.3. Small-scale extraction

Fully desiccated plant material was ground to powder and Small-scale extractions were achieved using protocol designed by (Panda et al. 2017). Plant powder was taken with 10 mL solvent. The tubes were placed in sonicator bath for one hour after every four hour interval. From each extract, 1 mL aliquots were dried in Savant SpeedVac Concentrator 200H.

2.4. Antibacterial activity

Bacterial Strains: *Escherichia coli, Staphylococcus aureus, Shigella sonnei, Shigella Flexner, Shigella dysenteriae.*

Inoculation: Mueller Hinton (MH) agar was prepared and colonies were inoculated, allowed to grow over night and were stored at 4 °C.

Pre-culturing: Mueller Hinton Media was prepared and taken separately (5 mL) in reaction tubes. In each reaction tube a single colony of bacteria was taken and incubated overnight in shaker incubator at 37 °C.

Microdilution Broth Protocol: In a 96 well plate, test samples (10 µL) were taken. Ciprofloxacin was taken as positive control and dimethylsulfoxide (DMSO) and water are taken as blank control.

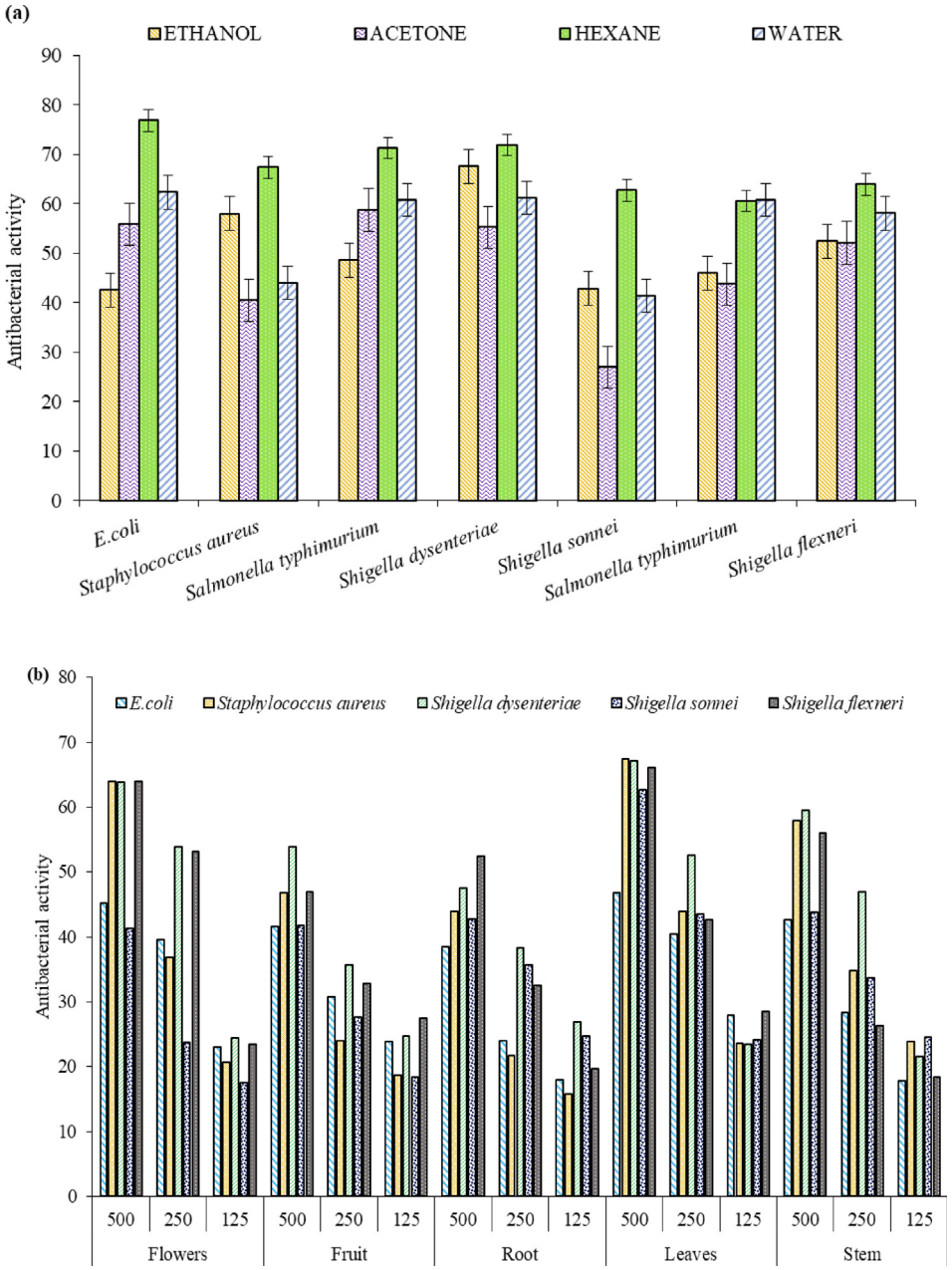


Fig. 1. Antibacterial activity (a) different extracts of *Bidens bipinnata*; positive control Ciprofloxacin. (b) Hexane extract of different parts of *Bidens bipinnata.*

Each well was then inoculated with 190 μL inoculum. Plates were incubated at 37 °C for 18 min.

Large-Scale Extraction: For large scale extraction 200 g plant powder was taken in a large container with the 2000 mL of n-Hexane HPLC grade from Sigma (Germany). The container was left standing for 24 h at ambient temperature. During this time, the container was placed in a water bath sonicator 4 times for 60 min each to maximize the yield of extraction. After each sonication, there was an interval of at least 6 hrs to let the suspension cool to ambient temperature. After 4th sonication the filtration of this material was done with the VWR® Grade 313, size of 5 μm filter paper to get the dry residue, filtrate was evaporated through rotary evaporator (BUCHI rotavapor R-100) and the weight of first dried extract was measured. The recover hexane was again used for second extraction with same procedure for 24 h, after that again the filtrate was evaporated with rotary evaporator to get the dried extract. The same procedure was repeated again and again to get all the compounds from the plant powder. The final weight of the dried material was calculated. Plant extract will be stored at 4 °C for further analysis. The powder of the plant was dried again to treat it with the polar solvent to get the polar compound with the same procedure.

2.5. Purification

The plant extract was bound with silica gel and silica column was prepared. Elutions were collected using a step gradient increasing in polarity of hexane, ethyl acetate, methanol, and acetic acid. At the end column was eluted with 100% acetic acid.

2.6. Reverse-phase high performance liquid chromatography analysis

High performance liquid chromatography (HPLC-DAD) analyses were conducted using a Shimadzu, LC-20AT system having a DGU20A3/DGU-20A5 on-line degasser, LC-20AT quaternary pump. The data was attained using Lab Solution software. A reverse-phase HPLC column was used. As a mobile phase the mobile phase acetonitrile and H_2O with 0.1% trifluoroacetic acid (TFA) was used.

2.7. Gas chromatography mass spectrometry

Collected peaks were subjected to a gas chromatography. A Restek RXi-5sil MS 20 m column was used (internal diameter: 0.18 mm, thickness: 0.18 μm) and the spectrum was searched using NIST 14 MS library.

3. Results

Initially prepared four different extracts of *Bidens bipinnata* were tested against five bacterial strains. The hexane extract showed a broadest-spectrum activity against tested pathogens followed by the aqueous extracts (Fig. 1a). This extract of *Bidens bipinnata* was again tested for antimicrobial activity in parallel from leaves, stem, root, flower and achene. The results showed that leaves hexane extract of *Bidens bipinnata* are the most active

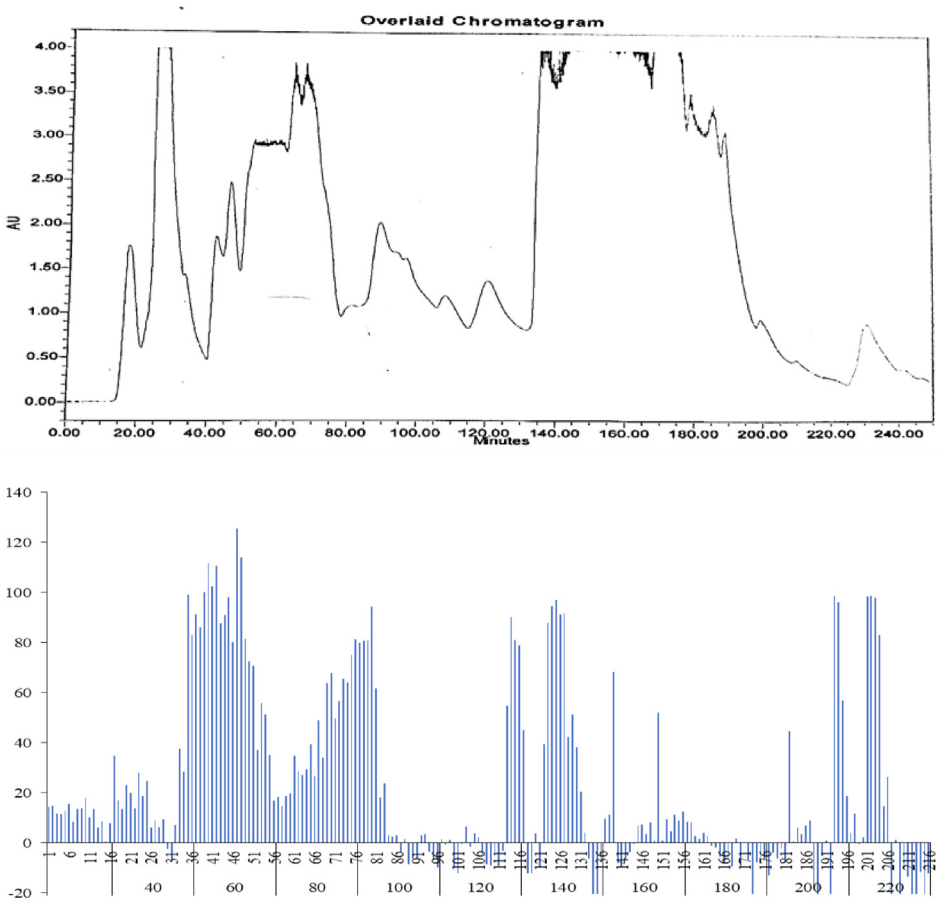


Fig. 2. Top panel: Overlaid chromatogram of from a silica gel column of Hexane extract of *Bidens bipinnata leaves;* fractions were collected per minute and tested for activity (percentage inhibition of *Staphylococcus aureus*) (bottom panel); positive control ciprofloxacin.

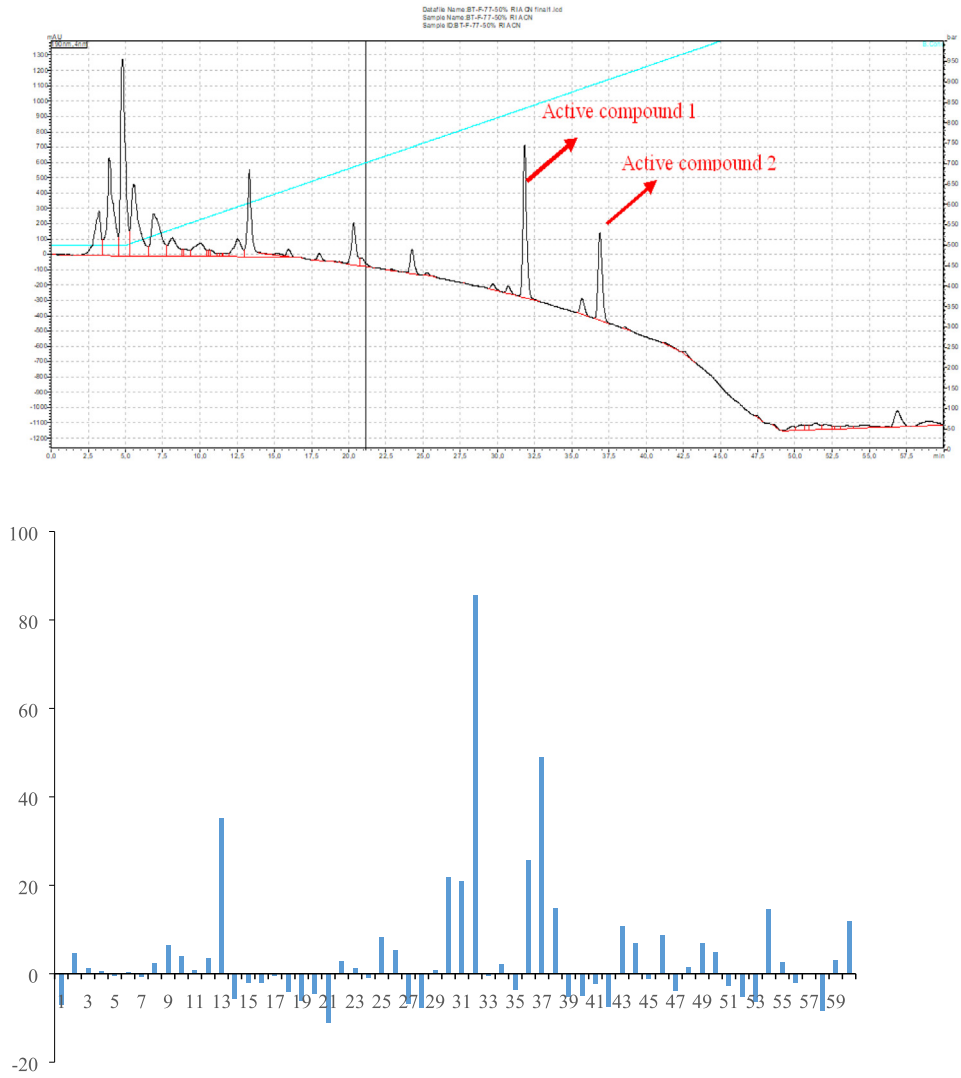


Fig. 3. Top panel: HPLC chromatogram of fraction 77 of silica gel column; fractions were collected per minute and tested for activity (percentage inhibition of *Staphylococcus aureus*) (bottom panel).

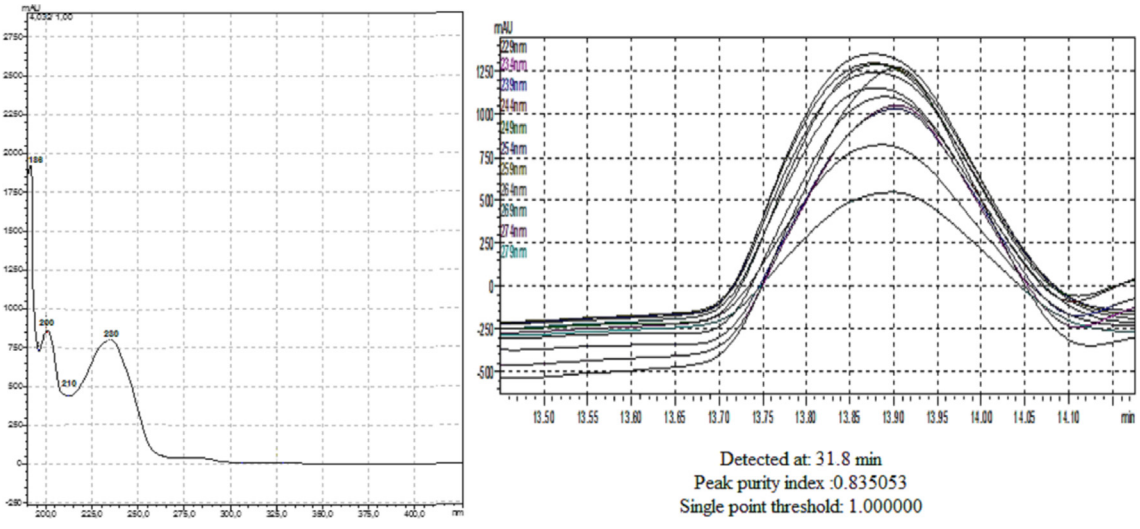


Fig. 4. UV–vis apex absorption spectra of Compound 1 (16-Pregnenolone).

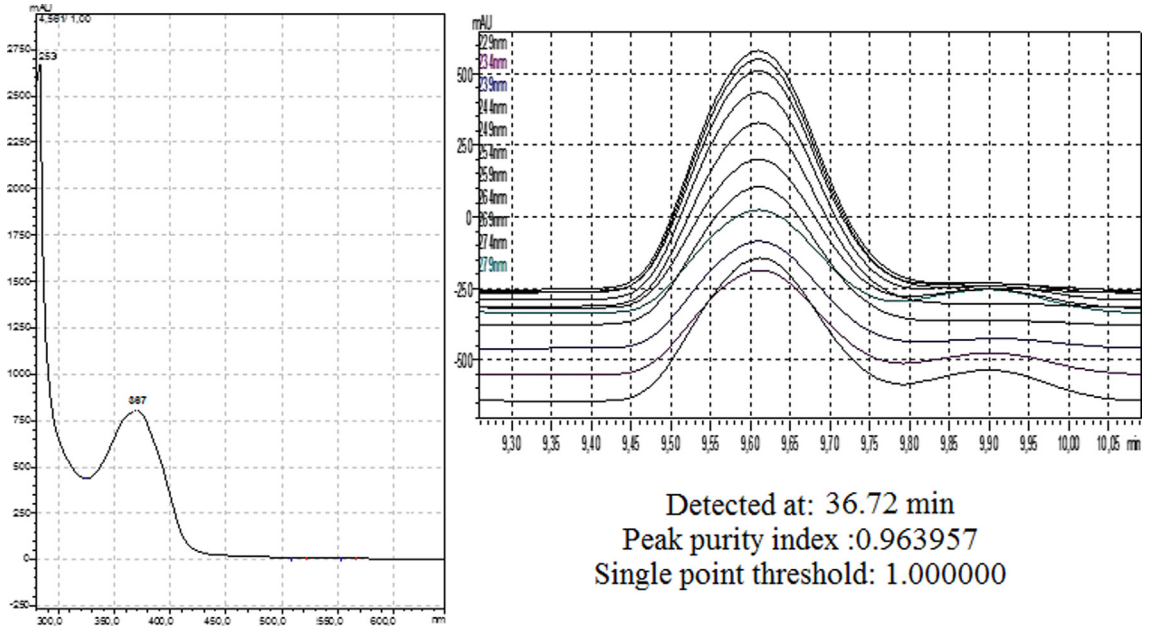


Fig. 5. UV–vis apex absorption spectra of Compound 2 (9-Octadecenoic acid (Z)-, methyl ester).

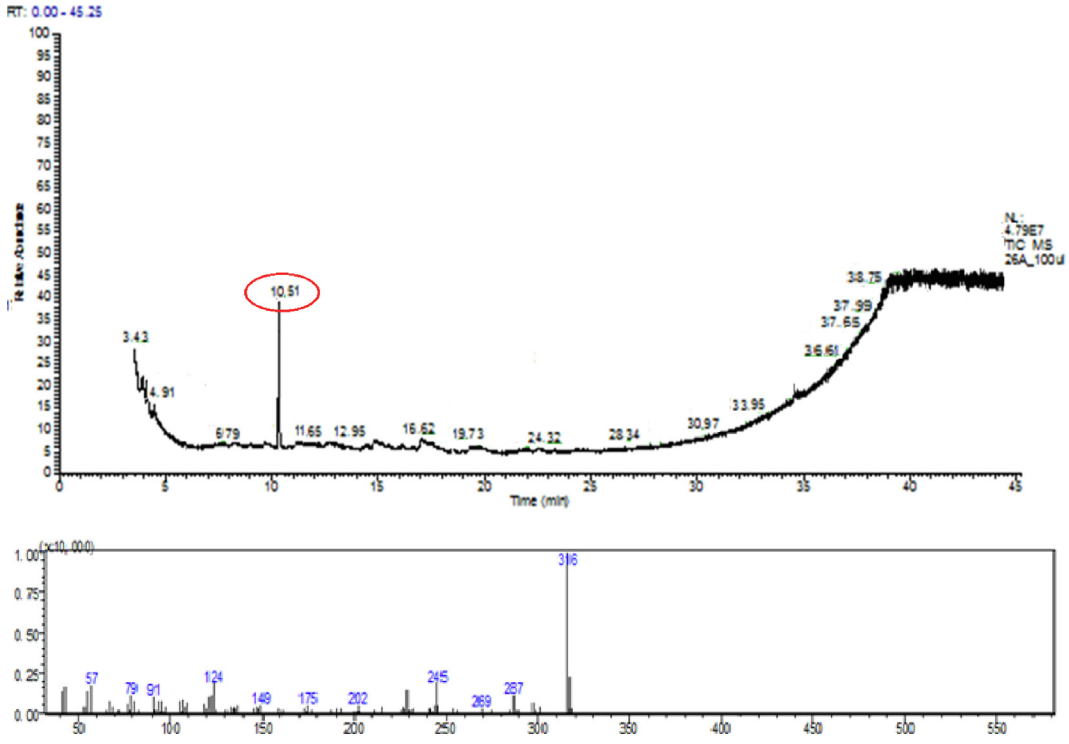


Fig. 6. Mass spectra of Compound 1 (16-Pregnenolone).

against all tested strains followed by flowers and stem (Fig. 1b). Hence, a large-scale extraction was performed using hexane and obtained extract was further separated into 220 fractions using column chromatography.

All fractions were tested against *Staphylococcus aureus*, fraction 35–51, 75–80, 114–127 and 193–204 found to be active (Fig. 2). The active fractions were dried and weighted. The active fractions were again tested against *Staphylococcus aureus* using a serial dilution protocol and fraction 75–80 showed a consistent antibacterial activity, indicating presence of some strong active antimicrobial compound. Therefore fraction 77 was further separated using a mobile phase starting with acetonitrile and Water. All 60 sub fractions were again analyzed using *Streptococcus aureus*, indicating two active sub fractions (Fig. 3).

Active peaks were exposed to UV-vis apex absorption spectrum as well as GCMS for identification (Fig. 4, 5, 6 & 7). Compound-1 was identified to be 16-Pregnenolone) and Compound 2 is identified as 9-Octadecenoic acid (Z)-, methyl ester (Fig. 8). The antibacterial properties of both compounds were evaluated where 9-Octadecenoic acid (Z)-, methyl ester had a greater activity with MIC_{50} of 72 mg/mL compared to 16-Pregnenolone (MIC_{50} > 250-mg/mL).

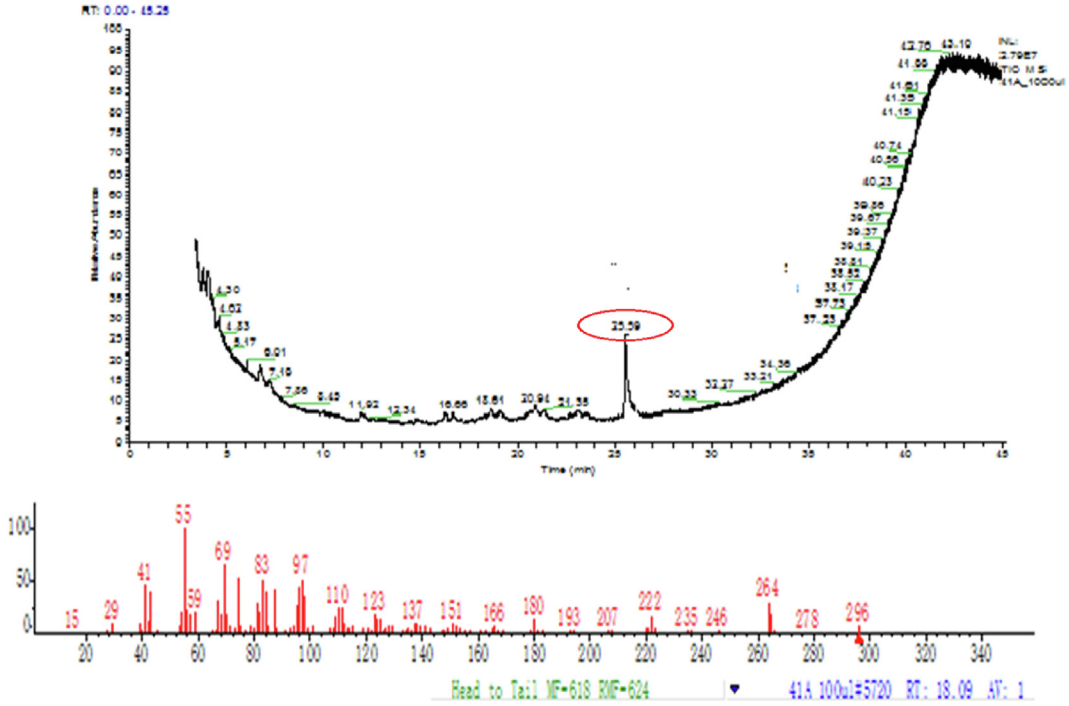
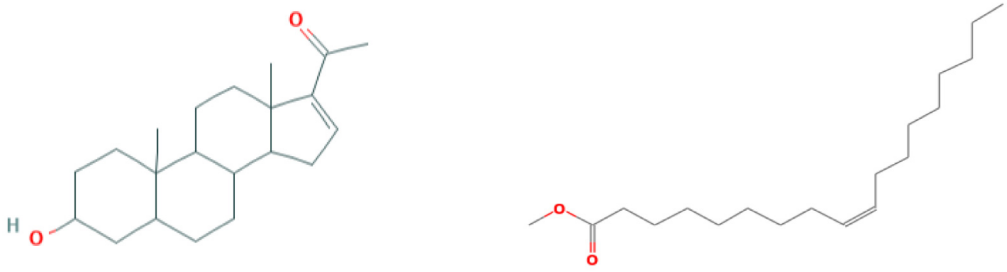


Fig. 7. Mass spectra of Compound 2 (9-Octadecenoic acid (Z)-, methyl ester).



16-Pregnenolone
Formula: $C_{21}H_{32}O_2$
Molecular weight: 316.5

9-Octadecenoic acid (Z)-, methyl ester
Formula: $C_{19}H_{36}O_2$
Molecular weight: 296.4879

Fig. 8. (A) Compound-1 (16-Pregnenolone). (B) Compound-2 (9-Octadecenoic acid (Z)-, methyl ester). Compounds 1 and 2 in the most active peaks were analyzed by mass spectrometry.

4. Discussion

Bidens bipinnata is traditionally used to treat a variety of infectious diseases especially in traditional Chinese medicine. The whole plant is used against malaria, sore throat, acute nephritis and dysentery. Pharmacological studies of this plant has been investigated before i.e. anti-arthritis (Shen et al. 2015) and anti-hepatoprotective activity (Wang et al. 2014). However its antibacterial activity is not well studied.

Different other species of genus *Bidens* has been proved to have considerable antibacterial activity i.e. antibacterial activity of leaf methanol extract showed an MIC of 2.0 against *Staphylococcus aureus*, 8.0 against *Staphylococcus epidermidis* and 4.0 against *Bacillus subtilis* (Rabe and Staden, 1997). *Bidens tripartita* root extract showed a moderate inhibition against *Neisseria gonorrhoeae* with MIC of 3.1 mg/Ml (Tomczykowa et al. 2011). In our present study among all tested extract of *Bidens bipinnata*, hexane extract is appeared to be the most active. The hexane extract of leaves showed highest activity with percentage inhibition of 46.89 (*Escherichia coli*), 67.40 (*Staphylococcus aureus*), 67.15 (*Shigella dysenteriae*), 62.72 (*Shigella sonnei*) and 66.09 (*Shigella flexneri*). Thus indicate the presence of some active antibacterial compounds in it. This significant bactericidal activity of this plant is might be due to the presence of alkaloids and tannins in a very high amount. Ravipati et al. (2012) explained a significant relationship between antimicrobial activity and alkaloid contents of plant. Current research was planned to identify the antibacterial compounds from *Bidens bipinnata*.

To the best of our knowledge the chemistry of *Bidens bipinnata* is not well studied. Li et al. (2004) indicated the occurrence of acetylenic glucosides. Shen et al. (2015) reported the presence of major flavonoids in *Bidens bipinnata* i.e. catechin, hyperin, isoquercitrin, astragalin and quercetin. Later on, Hu et al. (2018) conducted a phytochemical study and indicate the presence of nine ceramides,

thirteen flavonoids, one triterpenoid and one polyacetylene. However bioactivities of these isolated compounds has not been investigated. The current research led to the isolation of two antibacterial compounds: 16-Pregnenolone (compound 1) and 9-Octadecenoic acid (Z)-, methyl ester (compound 2).

16-Pregnenolone is a 20-oxo steroid that is pregnene substituted by a beta-hydroxy group at position 3 and an oxo group at position 20. Previously many other steroids has been identified from plants i.e. *Oryza sativa* L. (Macías et al. 2006), *Chresta exsucca, Grevillea scapigera* and *Centaurea sphaerocephala* (Schinor et al. 2004) and *Mammea siamensis* (Subhadhirasakul and Pechpongs, 2005).

16-Pregnenolone has been isolated previously from *Halorrhena curtisii*, *Habenaria floribunda, Xysmalobium undulatum* and *Trachycalyma fimbriatum*. From *Adonis aleppica* three sulfated pregnenolones has been isolated (Pauli et al. 2010). It is also observed to be a precursor of cardenolides in *Digitalis lanata* and *bufadienoid* in *hellaborus atroruben*. However there are no reports of this compound from genus *Bidens*. Valverde et al. (2008) conducted a study and pregneolone is appeared to be a very strong antibacterial compound.

In the present study the compound Pregnenolone has shown a moderate antibacterial activity with MIC_{50} > 250 mg/mL. It is suggested that this steroid-derivative, generally, assume cationic, facially amphiphilic conformations, which appears to be a main requirement for antibacterial activity. These structures allow them to interrupt bacterial membranes at comparatively different concentrations by recognizing some factors in the bacterial membrane as effective targets (Figueroa et al. 2008).

The second most active compound isolated in present study is 9-Octadecenoic acid (Z)-, methyl ester. Oleic acid is an octadec-9-enoic acid in which the double bond at C-9 has Z (cis) stereochemistry. In chemical terms, this compound is classified as a monounsaturated omega-9 fatty acid. It has the formula CH_3 (CH_2) 7CH = CH (CH_2)7COOH. The biosynthesis of 9-Octadecenoic acid (Z)-, methyl ester involves the conversion of stearic acid into monounsaturated derivative, oleic acid due to dehydrogenation by enzyme stearoyl-CoA 9-desaturase (Ntambi and Miyazaki, 2003).

Oleic acid has previously been identified from asteraceae members i.e. *Carthamus oxycantha* (Rafiq et al. 2017), *Anthemis mixta* and *Aristolochia tomentosa* (Formisano et al. 2012), *Ageratum conyzoides* (Mihigo et al. 2015) and *Vernonia nigritiana* (Senatore et al. 2004). This compound has also been identified from genus *Bidens* i.e. *Bidens pilosa* (Silva et al. 2011). But from *Bidens bipinnata* this is the first report of this compound.

It was observed in the present study that oleic acid has highest antibacterial activity i.e. with MIC_{50} of 72 mg/mL. The antibacterial effect of oleic acid has previously been reported (Speert et al. 1979; Muthamil et al. 2020, Zheng et al., 2005; Zhu et al. 2020). The exact mechanism of action of oleic acid is still unclear but the main mark appears to be the bacterial cell membrane and the numerous necessary processes that happen within and at the membrane (Desbois and Smith, 2009). It is also suggested that oleic acid causes distresses of the plasma membrane, comprising depolarization and consequent breaching and activates an influx of calcium into the cells.

5. Conclusion

In conclusion, *B. bipinnata* leaves hexane extract displayed antibacterial activity because of the occurrence of 16-Pregnenolone and 9-Octadecenoic acid (Z)-, methyl ester. This is the first report of these two compounds from *B. bipinnata*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics Approval

This research does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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