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Antiplatelet aggregation and cytotoxic activity of betulinic acid and its acetyl derivative from *Melaleuca bracteata*

Foluso O. Osunsanmi¹*, Oluwagbemiga S. Soyingbe¹, Idiat B. Ogunyinka¹, Rebamang A. Mosa Monisola I. Ikhile², J. Catherine Ngila², Francis O. Shode³ and Andy R. Opoku¹

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Platelet dysfunctions are implicated in cardiovascular diseases. Management of abnormal platelet aggregations with natural products is a promising approach to the treatment of cardiovascular diseases. In this study, betulinic acid (BA) isolated from *Melaleuca bracteata* leaf extract, and its acetyl derivative (3-β acetylbetulinic acid) (BAA) were investigated for their antiplatelet aggregation and cytotoxic activity. Structures of the compounds were established and confirmed through spectral (nuclear magnetic resonance [NMR], infrared [IR], mass spectroscopy [MS]) data analysis. The antiplatelet aggregation activity of the compounds was separately evaluated on collagen, adenosine diphosphate [ADP], thrombin and epinephrine induced rat platelet aggregations. The 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay was used to determine the cytotoxic effect of the compounds against human embryonic kidney (HEK293) and hepatocellular carcinoma (HEPG2) cell lines. The triterpenoids exhibited significant (p<0.05) dose dependent antiplatelet aggregation activity. The highest inhibitory activity of BA and BAA was observed on epinephrine induced platelet aggregation with IC₅₀ values 0.78 and 0.85 mg/ml, respectively. BA and BAA showed less cytotoxicity effect on both HEK293 cell (IC₅₀ 1027 and 1051 µg/ml, respectively) and HEPG2 cells (IC₅₀ 448 and 672 mg/ml, respectively). The results suggest that the compounds could serve as potential templates for synthesis of new antiplatelet drugs.

Key words: Antiplatelet aggregation, thrombin, collagen, cytotoxicity, aspirin, triterpenes.

INTRODUCTION

Blood platelets are non-nucleated cells that are important for regulation of hemostasis and repair of damaged...
endothelium cells (Semple et al., 2011). Abnormal platelet aggregation has been implicated as the underlying causes of cardiovascular diseases (stroke, heart attack, pulmonary thrombosis and venous thrombosis). These diseases are among the leading cause of death worldwide (Dickneite et al., 1995). Platelets are activated by various physiological agonists such as thrombin, collagen, epinephrine and adenosine diphosphate (ADP), which result into among others, secretion of the content of platelet granules, adhesions and aggregation. The roles of thrombin in normal platelet functions and coagulation processes showed the link between cellular (platelet) and biomolecules (coagulation) responsible for blood hemostasis (Takahashi et al., 2007).

Various antiplatelet aggregation drugs such as aspirin are currently used to manage abnormal platelet functions. Despite the use of the current antiplatelet agents, the incidences of cardiovascular diseases are still increasing. Also these antiplatelet agents are associated with some adverse effects. Aspirin, a commonly used antiplatelet drug for the treatment of cardiovascular related disease has been implicated in mucosa irritation and gastrointestinal bleeding (Armani et al., 2009). Thus a search and development of new effective antiplatelet drugs with improved safety profile is necessary.

Medicinal plants have traditionally been used for treatments of various chronic diseases with reduced side effects. The therapeutic activity of these plants is attributed to the presence of a wide range of phytochemicals such as flavonoids, phenols, alkaloids, glycosides and terpenoids (George et al., 2001).

*Melaleuca bracteata* var. revolution gold (Figure 1) commonly known as Johannesburg gold is a myrtle species that is aborigine in Australia. The plant is widely cultivated in South Africa as ornament (Craven, 2008). *M. bracteata* has been reported for the treatment of stroke, heart attack, sickle cell anemia and fungal infection (Habila et al., 2011).

Various plant derived triterpenes such as oleanolic acid, masculic acid, sawamilletin and ursolic acid have been reported to possess wide range of bioactivities including antioxidant, anti-plasmodial, anti-inflammatory, anticoagulant and antiplatelet aggregation activities (Mthokozisi et al., 2013; Aster et al., 2004; Habila et al., 2013). Betulinic acid (BA), a naturally occurring pentacyclic triterpene have also been reported to possess various bioactivities such as antiretroviral (Huang et al., 2006; Qian et al., 2007), anti-angiogenesis (Mukherjee et al., 2004), antioxidant and anti-inflammatory activities (Amico et al., 2006; Huang et al., 2007). Anti-sickling and anticancer activity of 3-β acetylbetulinic acid have been reported (Faujan et al., 2010; Habila et al., 2012). In this study, the antiplatelet aggregation and cytotoxicity of BA from *M. bracteata* and its acetyl derivative have been reported.

**MATERIALS AND METHODS**

**Reagents**

Unless otherwise stated, all solvents and reagents were purchased from Sigma-Aldrich and were used as received.

**Plant**

The leaves of *M. bracteata* var. revolution gold were harvested from the trees growing on and around the University of Zululand Campus, KwaDlangezwa, South Africa. The plant was identified.
and confirmed by chief botanist at the Department of Botany, University of Zululand, voucher number 0256 and deposited at the university herbarium.

**Extraction and isolation of BA**

The method described by Habila et al. (2011) was adopted with some slight modification to extract and isolate the triterpene from the plant material. The fresh leaves of *M. bracteata* (400 g) were extracted with dichloromethane (4 L × 3) for 24 h using mechanical platform shaker (40 rpm, 36°C). The filtrate was concentrated with rotary evaporator at 40°C. The crude extract was air dried at room temperature to yield 5% of the plant extract. The crude extract was then defatted with hexane and 5 g of the extract was subjected to silica gel (60 to 120 Mesh) column chromatograph (20 × 5.5 cm) for isolation of the desired compound, n-hexane and ethylacetate solvent system (8:2 to 7:3) was used to elute the column. A total of 80 fractions (20 ml) were collected and analysed with thin-layer chromatography (TLC). Fractions with similar profile on TLC were combined. The combined fractions with the desired pure compound were concentrated and recrystallized with methanol to give a white amorphous powder.

**Synthesis of 3-β acetylbetulinic acid (BAA)**

The method of Adrine et al. (2012) was adopted with slight modification to synthesize the acetyl derivative of BA (Figure 2). BA (2 g) isolated from the *M. bracteata* was dissolved in a mixture of pyridine (10 ml) and acetic anhydride (12 ml) in a round bottom flask. The mixture was refluxed for 6 h at room temperature (25°C). The reaction was then terminated with addition of distilled water (25 ml). The mixture was further stirred with magnetic rod for 45 min. The filtrate was washed with HCl (12%) to remove excess pyridine, concentrated by suction and air-dried. The synthesized compound was further subjected to silica gel (60 × 120 mesh) column chromatography (20 × 5.5 mm) for purification, eluted with n-hexane and acetyl acetate solvent system (8:2 to 7:3). A total of 47 fractions (20 ml) were collected and similar fractions on TLC were combined. The combined fraction with desired compound was concentrated in vacuo at 40°C. BAA was recrystallized with methanol to obtain a white powder.

**Structural elucidation**

All NMR experiments were conducted on a 400 MHz Bruker Ultrashield spectrometer. BA was dissolved in a mixture of deuterated chloroform and methanol-d_4, whereas BAA was dissolved in deuterated chloroform. Infrared spectra were recorded with a PerkinElmer Spectrum FTIR spectrophotometer. Mass data were run on Agilent 1100 series LC/MSD trap system Electrospray ionization. All solvents and reagents were purchased from Sigma-Aldrich and were used as received. Melting points were recorded on an Electrothermal (thermoscientific) digital melting point apparatus and were uncorrected.

**Compound identification**

BA (Figure 3) Colourless crystal; mp 315-316°C; IR (KBr) \(\nu_{max}\) 3456, 2920, 2851, 1724 cm\(^{-1}\); m/z (ESI) 455.2 (M+-1); \(\delta\)H (400 MHz, CDCl\(_3\) and CH\(_3\)OD): 4.59 (1H, s), 4.46 (1 H, s), 3.10 (2H, d), 2.13 (2H, dd), 1.80 (2H, s), 1.45 (8H, m), 1.38 (11H, m), 0.80-1.17 (21H, m); \(\delta\)C (100 MHz, CDCl\(_3\) and CH\(_3\)OD) (Table 1).

BAA (Figure 4) white powder; mp 258-260°C; IR (KBr) \(\nu_{max}\) 3424, 2919, 2851, 1724 cm\(^{-1}\); m/z (ESI) 496.8 (M+-1); \(\delta\)H (400 MHz, CDCl\(_3\)): 4.71 (1H, s), 4.59 (1H, s), 4.45 (1H, m), 2.98 (1H, m), 2.25 (1H, d), 2.15 (1H, d), 1.94 (5H, d), 1.59 (9H, m), 1.43 (3H, s), 1.40 (3H, m), 1.24 (3H, d), 1.17 (2H, s), 1.00 (8H, m), 0.80 (10H, m); \(\delta\)C (100 MHz, CDCl\(_3\)) (Table 1).

**Experimental animals**

The ethic clearance (UZREC 171110-030 PGD 2014/53) was obtained from the Research Animal Ethical Clearance Committee (RAEC) of University of Zululand. Sprague Dawley rats (8 weeks, 220 to 250 kg) were collected from the animal house at the Department of Biochemistry and Microbiology, University of Zululand. The animals were acclimatized in the standard laboratory facility and maintained using standard ethic protocol with access to enough clean drinking water and pellet feeds.

**Preparation of plasma rich platelet (PRP)**

The method described by Tomita et al. (1983) was adopted to prepare and obtain the platelets. The rats were sacrificed by cervical dislocation and blood (5 ml) collected by cardiac puncture. The blood was immediately mixed (5:1 v/v) with an acid-dextrose anticoagulant. The blood was centrifuged at 1200 rpm for 15 minutes and 2200 rpm for 3 min consecutively using Eppendorf centrifuge 5804R. The sediment was discarded and the supernatant was further centrifuged at 3200 rpm for 15 min. The
supernatant was then discarded and the sediment (platelets) was suspended in 5 ml of washing buffer (pH 6.5). This was further centrifuged at 300 rpm for 15 min and the supernatant was discarded. The washed platelets were then suspended in a resuspending buffer (0.14 mM NaCl; 15 mM Tris- HCl; 5 mM glucose, pH 7.4). The working solution was prepared by further diluting (1:10) the platelets with the resuspending buffer and supplementing with calcium chloride (0.4 ml: 10 µl CaCl₂).

**Preparation of compound for the antiplatelet aggregation assay**

The compounds were dissolved in 2% tween 20 to make different concentration of 1, 3, 5 and 10 mg/ml.

**Evaluation of platelet aggregation inhibition activity**

The antiplatelet aggregation activity of the compounds was evaluated following the method described by Mekhfi et al. (2004) with slight modification. Platelet aggregation inhibitory activity of the compound was separately investigated in thrombin (5 µg/ml), collagen (10 µg/ml), ADP (10 µg/ml) and epinephrine (5 µg/ml) induced platelet aggregation. Platelets (200 µl) and 20 µl of various concentration (1, 3, 5 and 10 mg/ml) of the compound was pipetted into corresponding wells of a 96-wells, mixed well and incubated for 5 min at 37°C. Platelet aggregation was induced by addition of the
Figure 5. Percentage inhibition activity of the compounds on (a) collagen induced platelet aggregation, (b) ADP induced platelet aggregation, (c) thrombin induced platelet aggregation, and (d) epinephrine induced platelet aggregation. Data were expressed as mean ± SD. *P<0.05, **P<0.01.

agonist (20 µl). The aggregation was read at 415 nm for 20 min at 30 s interval using Biotek plate reader. Aspirin served as the positive control while DMSO (1%) was used as negative control.

Percentage inhibition of platelet aggregation was calculated by the formula:

\[
\text{Inhibition} (\%) = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where A₀=control, A₁=tested sample. The IC₅₀ values were calculated using statistical package Origin 6.1.

**Determination of cytotoxicity of BA and BAA**

The cytotoxicity of the triterpene and its derivative was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) cytotoxicity proliferation assay (Mosman, 1983). The cytotoxic effect of the compounds was evaluated against normal human embryonic kidney (HEK293) and cancerous human hepatocellular carcinoma (HepG2) cell lines. The cytotoxicity results were calculated by regression analysis using QED statistics program.

**Statistical analysis**

Unless otherwise stated, all the experiments were triplicated and values were expressed as mean ± standard deviation. One way analysis of variance (ANOVA) and Post Hoc Dunnett’s test were used to analyze the results using Graphpad prism version 5.03. Statistical significance was considered at P<0.05.

**RESULTS**

The results revealed that the compounds exhibited concentration dependent platelet aggregation inhibitory activity against the four platelet agonists (collagen, ADP, thrombin, epinephrine) (Figure 5a to d). BAA exhibited significantly (p<0.05) higher platelet aggregation inhibition at 10 mg/ml on aggregation induced with ADP and thrombin in comparison with BA (Figure 5b and c). The IC₅₀ values (1.72, 2.72, and 2.92 mg/ml, respectively) of BAA on collagen, ADP and thrombin induce platelet
Table 1. $^{13}$C-NMR (100 MHz) spectral data for BA and BAA.

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<tr>
<th>Position</th>
<th>BA</th>
<th>BAA</th>
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<tbody>
<tr>
<td>1</td>
<td>38.4 (CH$_2$)</td>
<td>38.1</td>
</tr>
<tr>
<td>2</td>
<td>26.5 (CH$_2$)</td>
<td>27.6</td>
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<tr>
<td>3</td>
<td>78.4 (CH)</td>
<td>80.7</td>
</tr>
<tr>
<td>4</td>
<td>38.5 (C)</td>
<td>37.5</td>
</tr>
<tr>
<td>5</td>
<td>55.0 (CH)</td>
<td>55.1</td>
</tr>
<tr>
<td>6</td>
<td>17.9 (CH$_2$)</td>
<td>17.8</td>
</tr>
<tr>
<td>7</td>
<td>33.9 (CH$_2$)</td>
<td>33.9</td>
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<td>8</td>
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<td>32</td>
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<td>23.4 (CH$_3$)</td>
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Data reported in ppm.

Table 2. The IC$_{50}$ values of betulinic acid and 3-β acetylbetulinic on platelet aggregation inhibition.

<table>
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<tr>
<th>Compound</th>
<th>Collagen</th>
<th>ADP</th>
<th>Thrombin</th>
<th>Epinephrine</th>
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<td>Betulinic acid</td>
<td>5.45</td>
<td>11.1</td>
<td>11.6</td>
<td>0.78</td>
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<tr>
<td>3-β Acetylbetulinic acid</td>
<td>1.72</td>
<td>2.72</td>
<td>2.92</td>
<td>0.85</td>
</tr>
<tr>
<td>Aspirin</td>
<td>2.58</td>
<td>2.72</td>
<td>2.72</td>
<td>2.98</td>
</tr>
</tbody>
</table>

aggregation were lower than that of BA (IC$_{50}$: 5.45, 11.1, and 11.6 mg/ml, respectively) (Table 2). Both BA and BAA showed significant inhibitory activity on epinephrine induced platelet aggregation with IC$_{50}$ of 0.78 and 0.85 mg/ml, respectively when compared to aspirin with IC$_{50}$ of 2.98 mg/ml (Table 2).

The cytotoxicity of the triterpenoids was evaluated and the results are presented in Table 3. BA and BAA showed weak cytotoxic effect on both HEK293 cells (IC$_{50}$ 1027 and 1051 µg/ml, respectively) and HEPG2 cell (IC$_{50}$
Table 3. The IC\(_{50}\) (µg/ml) of betulinic acid and 3-β acetylbetulinic acid on HEK293 and HEPG2 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) (µg/ml)</th>
<th>HEK 293</th>
<th>HEPG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betulinic acid</td>
<td>1027</td>
<td>448</td>
<td></td>
</tr>
<tr>
<td>3-β Acetylbetulinic acid</td>
<td>1051</td>
<td>672</td>
<td></td>
</tr>
</tbody>
</table>

448 and 672 µg/ml, respectively). However, a relatively higher activity was observed on the cancerous HEPG2 cell than on the normal HEK293 cells.

DISCUSSION

The structures of BA and BAA were established and confirmed with \(^1\)H and \(^13\)C NMR spectroscopy, IR and mass spectrometry. The melting points of the two compounds were also determined which is in agreement with previously reported values (Habila et al., 2013). The presence of hydroxyl groups in the compounds was indicated by the appearance of an absorption band between 3424 to 3456 cm\(^{-1}\) in the IR Spectra. The \(^1\)H NMR spectrum of BA revealed various peaks corresponding to the methyl groups at around 0.80 to 1.17 ppm and terminal methylene protons at 4.46 to 4.59 ppm, which is indicative of the presence of 48 hydrogen atoms in BA. As expected, the \(^1\)H NMR spectrum of BAA showed the presence of 50 hydrogen atoms, which agrees with literature for previously reported values (Habila et al., 2011). In \(^13\)C NMR spectra, the appearances of two additional carbons assigned as C-31 and C-32 (Table 1) further confirmed the formation of BAA. The carboxylic acid carbon assigned as C-28 (Table 1) appeared as the most deshielded around 178.8 and 182.2 ppm for both BA and BAA, respectively, which is also in agreement with literature (Habila et al., 2013). Further evidence for the isolation of BA and BAA was provided by the ESI-MS spectra which showed intense molecular ions corresponding to M\(^+\)-1 at 455.2 and 496.8, respectively.

Blood platelets are crucial for hemostatic system and repair of damaged endothelium. However, aberrant platelet aggregations have been reported as the major cause of cardiovascular diseases (Dickneite et al., 1995). Targeting the abnormal platelet aggregation could be a good strategy to combat the ever increasing cardiovascular events. Medicinal plants have always served as rich sources of diverse bioactive compounds vital to human health. Triterpenes, due to their diverse potential pharmacological activities, are now targets for new drugs development. The results obtained from this study showed that BA and BAA (Figure 5a to d) inhibited platelet aggregation regardless of the agonists (thrombin, collagen, ADP and epinephrine). Antiplatelet aggregation activities of some other pentacyclic triterpenes against the platelet agonists (ADP, thrombin and epinephrine) have previously been reported (Jin et al., 2004; Kim et al., 2010; Xuemei et al., 2010). The higher antiplatelet aggregation activity (Table 2) exhibited by BAA than BA is consistent with the results reported by Habila et al. (2013) on the antiplatelet activity of BA and its derivative. Targeting carbon positions 3 and 28 are new pharmacophores for increasing biological activity (Ban et al., 2010). Thus, the relatively higher antiplatelet aggregation activity of BAA could be attributed to the acetyl modification at carbon-3 (C-3) position. This compound has potential to serve as a template for antiplatelet drug development or synthesis.

Depending on the intended biological activity, a good antiplatelet drug has to be active with no cytotoxic effects on normal cells. While reports of some triterpenes indicate their strong cytotoxicity effect (Lee et al., 2007; Pteros and Uy, 2010), the results from this study (Table 3) indicated weak cytotoxic effect of BA and BAA on normal cells (HEK293) and cancerous cell (HEPG2). The American National Cancer Institute guidelines consider a pure compound as cytototoxic with IC\(_{50}\) < 30 µg/ml (Suffness and Pezzuto, 1990). Despite the weak cytotoxic effect exhibited by the two triterpenes, a relatively higher activity on HEPG2 than HEK293 implies the compound could selectively inhibit the proliferation of cancer cells at higher concentration. Betulinic acid has previously been reported to selectively inhibit tumour cells (Pisha et al., 1995). Faujan et al. (2010) reported a selective cytotoxic effect of BA and BAA from Melaleuca cajuput on myeloid leukemia (HL-60) cell line. The weaker cytotoxic effect of BAA on the two cells used in this study could also be attributed to the acetyl modification of C-3 position. Therefore, the compound could be potential safer antiplatelet agent.

Conclusion

The present study revealed that betulinic acid and its acetyl derivates (3-β acetylbetulinic acid) have antiplatelet aggregation activity regardless of the agonist. In addition to efficacy, the weak cytotoxic effect showed by the compounds indicated their potential use as templates for synthesis of safe pharmacologically active antiplatelet agents. For further study, elucidation of the
mechanism of action of the compounds is recommended.

ACKNOWLEDGEMENTS

The authors thanks the University of Zululand Research Committee and South Africa Medical Research Council (MRC) for their financial support.

Conflicts of interest

The authors declare that they have no conflicts of interest.

REFERENCES


Antituberculous, antimicrobial, cytotoxicity and phytochemical activity study of *Piliostigma thonningii* extract fractions

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World Health Organization studies have demonstrated that 80\% of the world's population depends on medicinal plants for their primary health care. This has prompted increased efforts to the adoption and integration of herbal practices in health systems. This study soughts to answer the question whether *Piliostigma thonningii* has antitubercular, antibacterial, antifungal and cytotoxic activity. Antimicrobial activity was investigated by disc diffusion and micro dilution techniques. Antituberculous activity was investigated using the BACTEC MGIT 960 system while cytotoxicity was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on Vero cells. Phytochemicals were profiled using standard chemical procedures. A major output of our study is the methanolic fractions which yielded the best antituberculous activity (minimum inhibitory concentration [MIC] of 12.5 µg/ml), the highest antibacterial activity with zones of inhibition of 20.3 mm and MIC of 31.25 µg/ml (*Staphylococcus aureus*), 18.3 mm and MIC of 62.5 µg/ml (Methicillin Resistant *S. aureus*, MRSA), 14 mm and MIC of 125 µg/ml (*Escherichia coli*), 13.3 mm and MIC of 31.25 (*Shigella sonnei*) and 13 mm against (*Candida albicans*), all within the acceptable toxicity limit (CC\(_{50}>500\) µg/ml). The activity could be attributed to various phytochemicals that tested positive especially terpenoids. Important is its high activity against MRSA, *S. aureus*, *E. coli*, *S. sonnei*, *C. albicans* and *Mycobacterium tuberculosis* which are health challenge due to drug resistance and sources of community and nosocomial infections. To the best of our knowledge, this is the first report exploring the antituberculous activity of *P. thonningii* and thence a major addition in search of new safe antituberculous drug leads.

**Key words:** Antibacterial activity, cytotoxicity, antifungal activity, antibacterial activity, terpenoids, phytochemicals, *Piliostigma thonningii*, herbal medicine.

**INTRODUCTION**

There is an increased and concerted efforts to the adoption and integration of traditional medicine and medical practices referred to as complementary or/and alternative medicine in both developing and developed
countries in their health system (World Health Organization [WHO], 2005). This is after studies by WHO have demonstrated that 80% of the world’s population depends on medicinal plants for their primary health care (Mothana et al., 2008; Gupta et al., 2010).

Numerous studies have shown that medicinal plants are the oldest source of bioactive products with proven efficacy (Hemalatha et al., 2013; de Souza Nascimento et al., 2013; Yadav et al., 2010; Silva and Fernandes, 2010) serving as the basic components of several drugs (Rates, 2001), analgesics (Almeida et al., 2001), anesthetics (Dube et al., 2013), antibiotics (Cowan, 1999), anti-cancers (Gonzales and Valerio, 2006; Prakash et al., 2013), anti-parasitics, anti-inflammatory (Shilpi et al., 2012), oral contraceptives (Ogbuewu et al., 2011; Umadevi et al., 2013) and diuretic drugs (Dutta et al., 2014) among others.

*Pilostigma thonningii* (Schum.) Milne-Redh locally known as *mukuura* in Mbeere, Embu County in Kenya has been used and reported for different and varied medicinal purposes. For example, it has been reported that in many African countries, various parts of the plant (root, bark, seed, and fruit) are used to treat wounds, ulcers, gastric and heart pain, gingivitis, and as an antipyretic (Silva et al., 1997; Maroyi, 2011, 2013). In addition, a cough remedy is prepared from the root bark (Akinpelu and Obuotor, 2000). Its roots mixed with roots of *Elephantorrhiza goetzei* are used as bilharzia (schistosomiasis) medicine (Maroyi, 2011). Certain compounds (alkaloids, flavonoids, saponins and tannins) isolated from some of its parts have been reported to bear anti-inflammatory, analgesic and antibacterial activities (Paiva et al., 2010; Akinpelu and Obuotor, 2000).

However, reported scientific work is rather incomprehensive, in relating the phytochemicals present in the antibacterial activities and cytotoxicity of the same extracts from any part of *P. thonningii*. For example, the reported studies deal distinctly in quantification of active substances (Babajide et al., 2010; Deshi et al., 2014) or antibacterial and anti-inflammatory activity (Etsuyankpa et al., 2013; Aderogba et al., 2006; Ibewuike et al., 1997) or effects of ethanolic extracts on kidney function (Dasofunjo et al., 2012) and lipid profile (Ighodaro and Omole, 2012) in Wistar rats. Other studies independently show phytochemical evaluation, antibacterial activity (Tor-Anyiin and Anyam, 2013) and anti-malaria activity (Kwaji et al., 2010).

No literature was found on antitubercular activity of the plants extracts and therefore this study was designed to investigate anti-TB activity of the root extract. In addition, this study sought to investigate its antibacterial and antifungal activity, determine the phytochemicals present and relate these findings with the observed activities and cytotoxicity to Vero cells.

**MATERIALS AND METHODS**

**Plant**

The plant used in this study was identified through ethnobotanical approach. The information of its use and preparation in Mbeere community, Kenya was gleaned from local herbalist and confirmed from documentation by Riley and Brokensha (1988) in The Mbeere in Kenya (ii), Botanical identity and use.. This plant is not an endangered species and it was collected in open community field and therefore no prior permission was required. The location for collection was around 0°46’27.0’S 37°40’54.9’E; -0.774156, 37.681908 of GPS co-ordinates. The identity was also confirmed by a Botanist at Egerton University where voucher specimen number NSN4 was deposited.

**Plant extract preparation**

Root samples were chopped into small pieces of 2 to 3 cm and air-dried in dark at room temperature (23±2°C) to constant weight. Using a mechanical grinder, the dried root specimens were ground to powder. The powder (50 g) was cold extracted in water with intermittent shaking to mimic the traditional local method of extraction and later lyophilized to obtain a dry powder. Methanol extraction was done by macerating 50 g in 200 ml of methanol for 48 h and then filtered using a filter paper (Whatmann 1) and the residue obtained was further re-extracted using similar amount of methanol. The two volumes of filtrate were pooled together and concentrated in vacuo using a rotary evaporator. Afterwards, the product was allowed to air dry and the yields recorded.

Fractionation of powdered root part of *P. thonningii* was done using different solvents of increasing polarity. The root powder (50 g) was macerated in 200 ml of Petro ether with intermittent shaking for 48 h (two days) after which they were filtered using Whatman No. 1 filter paper. The residue was further re-extracted using the same fresh solvent for 24 h and thereafter the filtrates pooled together. The resulting residue was air dried and further extracted with dichloromethane followed by ethyl acetate and lastly methanol using the same procedure carried out for petrol ether. Using a rotary evaporator, the solvent was removed from each filtrate under conditions of reduced temperature and pressure. The resulting dry extract was weighed and stored in air tight sample bottles at -20°C until the next use (Njere et al., 2015).

**Antibacterial activity**

**Culturing of micro organisms**

One Gram positive: *Staphylococcus aureus* (ATCC 25923) strain and Methicillin Resistant *Staphylococcus aureus* strain (clinical isolate), five Gram negative: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (clinical isolate) and *Shigella sonnei* (clinical isolate) and two Fungi: *Candida albicans* (ATCC...
Disc diffusion test

The antibacterial activity was assayed by disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2007) and Mbaveng et al. (2008) with slight modifications. Fresh inoculum was prepared by suspending activated colonies in physiological saline water (0.85% NaCl). Using 0.5 McFarland turbidity standard, the bacteria and fungi suspensions were adjusted to $1.5 \times 10^8$ CFU/ml after which they were inoculated aseptically by swabbing the surfaces of the Muller-Hinton agar (MHA plates and Sabouraud dextrose agar (SDA) plates. Whatmann filter paper (No. 1) discs of 6 mm diameter were made by punching the paper, and the blank discs sterilized in the hot air oven at 160°C for 1 h. They were then saturated with 10 µl of various stock extract solution. The methanolic and water crude extracts stock solution was at 1.0 g/ml. For fractions, petro ether, dichloromethane, and methanol fractions stock solutions were made at 500 µg/ml, while ethyl acetate at 250 µg /ml. This afforded disc extract concentration of $1.0 \times 10^2$ µg/disc for water and methanol crude extracts, 5 µg/disc for petro ether, dichloromethane, and methanol fractions and 2.5 µg/disc for ethyl acetate. Three standard drugs were used as positive controls: Oxacillin 10 µg/disc (Oxoid Ltd, Tokyo-Japan) and Gentamycin 10 µg/disc (Oxoid Ltd, Tokyo-Japan) for Gram positive and Gram negative bacteria, respectively. Nystatin 100 µg/disc (Oxoid Ltd, Tokyo-Japan) was used as the standard drug for all fungi while discs loaded with 10 µl of DMSO was used as negative controls. Using a sterile forceps, the impregnated dry discs were carefully placed on the agar plates at equidistance points. A positive control as well as a negative control was incorporated in each plate and the plates incubated at 4°C for 2 h so as to allow the extract to diffuse into the media after which they were incubated at 37°C for 18 h. Antimicrobial activity was determined by measuring the size of the inhibition zone to the control was incorporated in each plate and the plates incubated at 4°C for 2 h so as to allow the extract to diffuse into the media after which they were incubated at 37°C for 18 h. Antimicrobial activity was determined by measuring the size of the inhibition zone to the nearest mm and the results recorded. Extracts fractions that gave an inhibition zone of more than 10 mm were considered to be active (CLSI, 2007) and therefore their Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined (Moithana et al., 2008).

Determination of MIC and MBC

The MIC and MBC of the plant extracts was determined for all the organisms in triplicates using broth micro-dilution assay. The petro ether, dichloromethane, and methanol fractions stock solutions were made at 500 µg/ml while ethyl acetate at 250 µg /ml with dimethyl sulfoxide (DMSO). To 100 µl of nutrient broth in a sterile 96 well plate, 50 µl of varying plant concentration (petro ether, dichloromethane, and methanol fractions at 500 to 3.91 µg/ml while ethyl acetate at 250 to 1.95 µg/ml) was added followed by 50 µl of test organisms previously diluted to the equivalent of 0.5 McFarland standard. Addition of the test organisms was done in all the wells except for wells of column 11 which contained neat DMSO and broth, this served as control to check for purity. The adequacy of the media to support the growth of the test organism was evaluated by putting the broth and the test organism in wells of column 12. The plates were then covered with a sterile "cling-on" seal and incubated for 24 h at 37°C. Bacterial growth was evaluated by addition of 40 µl of 0.2 mg/ml p-iodonitrotetrazolium chloride (INT, Sigma) to each well and incubated for 30 min. Growth of bacteria was detected by formation of a pink-red coloration while inhibition of growth was signalled by persistence of a clear coloration. The lowest concentration that exhibited color change was considered as the MIC. MBC was determined by streaking a loopful of broth from wells that exhibited no color change onto sterile nutrient agar and Sabouraud dextrose agar for bacteria and fungi, respectively and thereafter incubated at 37°C for 24 h. The lowest concentration that exhibited no growth was considered as the MBC (Lai et al., 2010).

Antitubercular activity

Prior to its use, the M. tuberculosis was revived on Lowenstein Jensen (LJ) slants for 14 days at 37°C following standard procedures (Gupta et al., 2010; Marita et al., 2010). The efficacy of the plant extracts against M. tuberculosis was carried out using the BACTEC MGIT 960 system (BD, New York-U.S.A). This is a fully automated, high volume, non-radiometric instrument that offers continuous monitoring of culture growth. The dry crude extract (water and methanolic) was first dissolved in DMSO to a final concentration of 1 g/ml for preliminary screening. Growth supplement (0.8 ml) containing a mixture of oleic acid, bovine albumen, dextrose and catalase (OADC) was added to five 7 ml BBL™ MGIT tube labeled growth control (GC), streptomycin (STR), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB) to provide essential substrates for rapid growth of Mycobacteria. 100 µl of BBL™ MGIT™ SIRE (streptomycin, isoniazid, rifampicin, ethambutol) prepared aseptically according to the manufacturer's instructions was added to corresponding labeled BBL™ MGIT tube followed by addition of 0.5 ml of 1% Mycobacterium suspension. Mycobacterium suspension was prepared by pipetting 0.1 ml Middlebrook 7H9 Broth containing Mycobacterium adjusted to 0.5 McFarland standard into 10 ml sterile saline aseptically. The BACTEC MGIT™ 960 system (BD, New York-U.S.A) was then loaded following the manufacturer's instructions and incubated at 37°C. Streptomycin at 1.0 µg/ml, isoniazid at 0.1 µg/ml, rifampicin at 1.0 µg/ml and ethambutol at 5.0 µg/ml served as the positive control whereas DMSO was used as a negative control. The procedure was repeated using plant water and methanolic crude extracts at 1.0 g/ml in place of SIRE. The process was also repeated with petro ether, dichloromethane, ethyl acetate and methanol solvent fractions. The fractions were tested at concentrations ranging from 50 to 6.25 µg/ml (petro ether, dichloromethane and methanol) or 25 to 3.125 µg/ml (ethyl acetate) to determine the MIC.

Cytotoxicity screening

MTT assay was used to determine the toxicity of the extracts obtained from the plant. This is a colorimetric assay hinged on the ability of mitochondrial enzyme (succinate dehydrogenase) to reduce yellow water soluble MTT to an insoluble colored substance, (formazan) that is spectrophotometrically measurable (Njeru et al., 2015). The level of formazan is directly proportional to the measure of cell viability because only metabolically active cells can reduce MTT. The test cell line used was Vero cells from African green Monkey Kidney cells (Cercopithecus aethiops epithelial cell line; ATCC CCL-81). The test cells were grown in growth media comprising of 100 ml DMEM, 10 ml fetal bovine serum (FBS), 1 ml penstrept, 1 ml amphotericin B, 1 ml L-glutamine. The test cells were incubated at 37°C in 5% CO2 until they attained confluency after which they were passaged by adding 2 ml of 0.25% trypsin and further incubated at room temperature until the cell were detached. Growth media (6 ml) was introduced to the test cells to inactivate trypsin off action, while the cell crumps formed were broken gently by sucking and releasing the cell suspension using a pipette. 2 ml of the harvested cells were then transferred into a 50 ml vial and topped up to 50 ml mark using growth media. A cell suspension of 100 µl ($1 \times 10^5$ cell/ml) was seeded into two rows of 90028), Cryptococcus neoformans (ATCC 66031) and acid fast Mycobacterium tuberculosis strain H37Rv (ATCC 27294) were investigated for antimicrobial activity. These organisms were sourced from Kenya Medical Research Institute (KEMRI), Nairobi.
wells in a 96-well micro-titer plate for one sample. The test cells were then incubated in 100 μl growth media at 37°C and 5% CO₂ for 48 h to form a confluent monolayer. The growth medium was then aspirated off and replaced with 100 μl of maintenance medium comprising of 100 ml DMEM, 2 ml fetal bovine serum (FBS), 1 ml penstrep, 1 ml amphotericin B, 1 ml L-glutamate and 0.1 ml gentamycin. Afterwards, cells were exposed to decreasing concentrations of respective plant extracts (from 250 to 0.24 μg/ml for petro ether and dichloromethane fractions and 500 to 0.49 μg/ml for ethyl acetate and methanolic fraction) and incubated at 37°C for 48 h. This was followed by a further incubation period of 4 h in 10 μl of 5 mg/ml MTT solution after aspirating off the plant extracts. This was followed by addition of 100 μl acidified isopropanol (0.04 N HCl in isopropanol). The well plate was gently shaken for 5 min to dissolve the formazan and then optical density measured using ELISA Scanning Multiwell Spectrophotometer (Multiskan Ex labssystems) at 562 nm using 690 nm as reference wave length. The last column containing medium without plant extracts was included to act as negative control. Cell viability (%) was calculated at each concentration as follows using the formula of Ngeny et al. (2013).

\[
\text{Cell viability} = \frac{(OD_{\text{sample}} \ 562 - OD_{\text{690}})}{(OD_{\text{control}} \ 562 - OD_{\text{690}})} \times 100
\]

**Phytochemical tests**

Phytochemical tests were done to determine the class of compounds present in the active fractions that could be responsible for activity and/or cytotoxicity. They were identified by characteristic colour changes based on standard procedures according to Houghton and Raman (1998), Edeoga et al. (2005), Ngoci et al. (2011) and Somboro et al. (2011). The results were reported as either (+) for presence, and/or (-) for absence.

**Alkaloids**

Six to eight drops of Dragendorf reagent was mixed with 2 ml of the extract. Formation of brownish-red precipitate indicated the presence of alkaloids. The Dragendorf reagent was prepared by mixing two reagents: reagent 1 and reagent 2 in equal parts. Reagent 1 was made by dissolving 8.5 g of Bismuth subnitrate in a solution of 10 ml acetic acid and 40 ml of distilled water while as Reagent 2 was prepared by dissolving 8 g of potassium iodide in 20 ml of water (Harborne, 1973; Somboro et al., 2011).

**Phenols**

Phenols were detected using ferric ferichloride which was prepared by dissolving 0.1 g of ferric ferichloride in 10 ml of water. Equal volumes (2 ml) of both ferric ferichloride and the plant extract were mixed. Formation of a violet-blue color or greenish color was evidence that phenols is present (Harborne, 1973; Somboro et al., 2011).

**Terpenoids**

One gram of Vanillin was mixed with 100 ml of concentrated sulphuric acid after which 2 ml of the resultant solution was mixed with 2 ml of the plant extract. Formation of a blue-green ring or pink-purple coloration signified the presences of terpenoids (Harborne, 1973; Somboro et al., 2011).

**Anthracquinones**

0.5 ml of the plant extract was mixed with 0.5 ml of 10% methanolic potassium hydroxide. Red coloration indicated the presences of anthroquinones. 10% methanolic potassium hydroxide was prepared by dissolving 0.5 g of potassium hydroxide pellets in 50 ml of methanol (Harborne, 1973; Somboro et al., 2011).

**Flavonoids**

Five milliliters of dilute aqueous ammonia solution was added to a portion of the aqueous filtrate of the plant extract, followed by concentrated sulphuric acid. A positive test result was confirmed by the formation of a yellow coloration that disappeared instantly (Edeoga et al., 2005; Ngoci et al., 2011).

**Statistical analysis**

Ms Excel 2010 data sheets and Graphpad Prism version 6 were used to analyze the data. The data on cytotoxicity was expressed as a percentage of the untreated controls. CC₅₀ values, which is the concentration that kills 50% of the Vero cells, was determined by regression analysis. A particular fraction’s extract was considered cytotoxic if it had CC₅₀ of less than 80 μg/ml (Irungu et al., 2007). Furthermore, unpaired student’s t-test was used to test for statistical significance in the differences between the treatments and the control in this study. A p value of less than 0.05 was considered to indicate statistical significance. Values were expressed as mean ± standard error of mean (S.E.M).

**RESULTS**

**Antibacterial, antifungal and antitubercular activity of water and methanolic crude extract**

This study first sought to identify whether the plant bark extract had any general antibacterial activity by mimicking the indigenous extraction methods. Traditionally, the plant bark extract was prepared in water or in combination with alcoholic beverages. It was found out that both water and methanolic crude extract had general antimicrobial activity. The diameter of inhibition zones was in all cases higher than 10 mm (Table 1) and therefore this formed the basis for further fractionation.

The test was however performed at higher concentration and we needed to bring this down as well as determine MIC and MBC. Unexpectedly, both crude extract had any general antibacterial activity by mimicking the indigenous extraction methods. Traditionally, the plant bark extract was prepared in water or in combination with alcoholic beverages. It was found out that both water and methanolic crude extract had general antimicrobial activity. The diameter of inhibition zones was in all cases higher than 10 mm (Table 1) and therefore this formed the basis for further fractionation.

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**Antibacterial and antifungal activity of fractions**

Further analysis on fractions demonstrated very weak activity on petro ether, dichloromethane and ethyl acetate fractions with all giving diameter of zones of inhibition of
Table 1. Antimicrobial activity results for the crude root extract.

<table>
<thead>
<tr>
<th>Extract</th>
<th>SA</th>
<th>EC</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>12.3±0.3</td>
<td>10±1.2</td>
<td>10.7±2.4</td>
</tr>
<tr>
<td>MOH</td>
<td>14.0±0.6</td>
<td>11.7±0.3</td>
<td>11.7±1.9</td>
</tr>
<tr>
<td>PC</td>
<td>24.0±1.3</td>
<td>22.0±0</td>
<td>16.3±0.9</td>
</tr>
<tr>
<td>NC</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

WT: Water crude extract at 1.0 ×10^4 µg; MOH: methanol crude extract at 1.0 ×10^4 µg; EC: E. coli; SA: S. aureus; CA: C. albicans; PC: positive control (Oxacillin 10 µg/disc for Gram positive, Gentamycin 10 µg/disc for Gram negative bacteria and Nystatin 100 µg/disc for fungi); NC: negative control (Discs loaded with 10 µl of DMSO); n=3; Values=mean±SEM.

Table 2. Antituberculous activity of crude extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>GU</th>
<th>R/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td>MOH</td>
<td></td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td>SIRE</td>
<td></td>
<td>0</td>
<td>S</td>
</tr>
<tr>
<td>NC</td>
<td></td>
<td>400</td>
<td>R</td>
</tr>
</tbody>
</table>

WT: Water crude extract at 1 g/ml; MOH: methanol crude extract at 1 g/ml; SIRE: positive control of streptomycin at 1.0 µg/ml, isoniazid at 0.5 µg/ml, rifampicin at 1.0 µg/ml and ethambutol at 5.0 µg/ml; GC: growth control; NC: negative control of media treated with DMSO; R: resistant; S: sensitive.

Table 3. Antimicrobial activity for fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Diameter of zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram positive</td>
</tr>
<tr>
<td></td>
<td>SA</td>
</tr>
<tr>
<td>PE</td>
<td>7.0±0.0</td>
</tr>
<tr>
<td>DCM</td>
<td>6.3±0.3</td>
</tr>
<tr>
<td>EA</td>
<td>0</td>
</tr>
<tr>
<td>MOH</td>
<td>20.3±0.3</td>
</tr>
<tr>
<td>PC</td>
<td>33.7±0.3</td>
</tr>
<tr>
<td>NC</td>
<td>0</td>
</tr>
</tbody>
</table>

PE: Petro ether fraction at 5 µg/disc; DCM: dichloromethane fraction at 5 µg/disc; EA: ethyl acetate fraction at 2.5 µg/disc; MOH: methanol fraction at 5 µg/disc; PA: P. aerogenosa; EC: E. coli; SA: S. aureus; KP: K. pneumoniae; MRSA: Methicillin Resistant S. aureus; SH: Shigella sonnei; ST: S. typhi; CA: C. albicans; CR: Cryptococcus; PC: positive control (Oxacillin 10 µg/disc and Gentamycin 10 µg/disc for Gram positive and Gram negative bacteria respectively. Nystatin 100 µg/disc for fungi); NC: negative control (Discs loaded with 10 µl of DMSO); n=3; values=Mean±SEM. *Indicates there is no significant difference between the test fraction and standard control (P>0.05).

less than 10 mm. Therefore, these 3 fractions were not considered for MIC and MBC determination. Interestingly, methanolic extract had broad spectrum activity inhibiting the growth of Gram positive bacteria (the highest was 20.3 mm against S. aureus), Gram negative bacteria (the highest was 14.0 mm against E. coli) and fungi (the highest was 13 mm against C. albicans). Activity of methanolic fraction against E. coli (14.0) was remarkable since there was no significant statistical difference in activity with that of positive control (p>0.05) (Table 3).

The MIC of methanolic fraction against 4 bacteria was less than 200 µg/ml. S. aureus and S. sonnei had MIC of 31.25 µg/ml; Methicillin resistant S. aureus had MIC of 62.5 µg/ml and E. coli had MIC of 125 µg/ml. However, all four had MBC >500 µg/ml and hence bacteriostatic in action (Table 4).
Table 4. MIC and MBC results for the Fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>SA</th>
<th>MRSA</th>
<th>EC</th>
<th>SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOH (µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC (µg/ml)</td>
<td>31.25</td>
<td>62.5</td>
<td>125</td>
<td>31.25</td>
</tr>
<tr>
<td>MBC (µg/ml)</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

MOH: Methanol fraction; EC: E. coli; SA: S. aureus; MRSA: Methicillin Resistant S. aureus; SH: Shigella. Sonnei.

Table 5. Antituberculous results for fractions.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Antituberculous testing</th>
<th>GU</th>
<th>R/S</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction</td>
<td>Concentration µg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>50</td>
<td>0</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>12.5</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>SIRE</td>
<td>50</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>DCM</td>
<td>12.5</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>50</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>SIRE</td>
<td>25</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>EA</td>
<td>12.5</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>25</td>
<td>400</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>SIRE</td>
<td>50</td>
<td>0</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>MOH</td>
<td>12.5</td>
<td>0</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>25</td>
<td>0</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>SIRE</td>
<td>50</td>
<td>0</td>
<td>S</td>
</tr>
</tbody>
</table>

PE: Petro ether fraction; DCM: dichloromethane fraction; EA: ethyl acetate fraction; MOH: methanol fraction; SIRE: positive control of streptomycin at 1.0 µg/ml, isoniazid at 0.5 µg/ml, rifampicin at 1.0 µg/ml and ethambutol at 5.0 µg/ml; GU: growth unit; NC: negative control of media treated with DMSO; R: resistant; S: sensitive.

Antituberculous, cytotoxicity activity and phytochemical profile of the fractions

When screening with crude water and methanolic extract revealed no antituberculous activity, we sought to investigate whether fractionation could improve on activity in some of the fractions. This is based on the knowledge that solvents of different polarities will retain certain compounds of equivalent polarity. It was also hypothesized that different bioactive compound combinations could bear antagonistic effects on each other once lumped together in crude extract, but their separation could render certain fractions active against M. tuberculosis (MTB). Interestingly, methanolic extract had MIC of 12.5 µg/ml and hence significant activity while petro ether fraction had MIC of >50 µg/ml, dichloromethane and ethyl acetate had MIC of >50 and 25 µg/ml (Table 5). All the fractions had CC50 > 90 µg/ml.
Table 6. Cytotoxicity results in µg/ml.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>CMOH</th>
<th>PE</th>
<th>DCM</th>
<th>EA</th>
<th>MOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC50 (µg/ml)</td>
<td>198.02</td>
<td>&gt;250</td>
<td>110.82</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

CMOH: Crude methanolic extract; PE: petro ether fraction extract; DCM: dichloromethane fraction extract; EA: ethyl acetate fraction extract; MOH: methanol fraction extract; CC50: Concentration that kills 50% of the cells (Vero cells with CMOH and HEp-2 cells with fractions).

Table 7. Phytochemical results of fractions.

<table>
<thead>
<tr>
<th>Extract Fraction</th>
<th>V-Ts</th>
<th>A-F</th>
<th>MK-A</th>
<th>D-A</th>
<th>F-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petro ether</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Methanol</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

V-T: Vanillin test for terpenoids; A-F: ammonia test for flavonoids; MK-A: methanolic potassium hydroxide test for anthraquenones; D-A: Dragendorff test for alkaloids; F-P: ferric chloride test for phenols; -: absent phytochemicals; +: low concentration of phytochemicals; +++: high concentration of phytochemicals.

DISCUSSION

Both crude extract and methanolic fraction extract had broad spectrum activity. They inhibited both Gram positive and Gram negative bacteria and fungi giving remarkable zones of inhibition. The activity against Gram positive was the highest. For example, methanolic fraction gave inhibition zone diameter of 20.3 mm and MIC of 31.25 µg/ml. This could be attributed to the cytoplasmic membrane of Gram-positive species which are simple lipid bilayer and hence not a barrier for most amphipathic compounds and can be readily traversed by antibacterials. In contrast, Gram-negative bacteria have evolved a sophisticated permeability barrier with an additional outer membrane comprising a highly hydrophilic lipopolysaccharide layer and this restricts penetration of hydrophobic and amphipathic compounds, which encompasses many drug compounds (Kim and Frederick, 2006). Difference in activity was also evidence among tested strains in both crude and fraction extracts. This could be due to genetic differences between different strains and this provides proof for the necessity of antibiogram prior to prescription as a precautionary measure in mitigating drug resistance development (Yimta et al., 2014).

Even though crude extract had no antituberculous activity, fractionation gave us two fraction (petro ether and methanolic fraction) which remarkably inhibited growth of MTB with MIC of 50 and 12.5 µg/ml, respectively. Moreover, the general antibacterial and antifungal activity was higher with methanolic fraction with four bacteria having MIC of less than 200 µg/ml; the set threshold for herbal extract activity (Kuete, 2010). This may suggest that fractionation enhanced antitubercular activity, implying either that there is higher concentration of active principle better at certain level of polarity or possibility of antagonism of various active compounds when lumped together as in crude extracts thus diminishing the activity. Equally important to note is that both crude and fraction extracts had CC50 >90 µg/ml and therefore were within the acceptable toxicity limit. The bioactivity in P.thonningii bark sample can to some extent be attributed to the tested phytochemicals, especially terpenoids which previous studies have associated with antibacterial, antifungal and antituberculosis activities. The mechanism of action of terpenoids is not entirely understood but is speculated to involve membrane disruption by the lipophilic compounds (Kuete, 2010).

Conclusion

A major output of the current study is the identification of the methanolic fractions which yielded the best antitubercular activity (MIC of 12.5 µg/ml) as well as the highest antibacterial activity (with zones of inhibition of 20.3 mm and MIC of 31.25 µg/ml (S. aureus), 18.3 mm and MIC of 62.5 µg/ml (MRSA), 14 mm and MIC of 125...
µg/ml (E. coli) and 13.3 mm and MIC of 31.25 (S. sonnei) which was within the acceptable toxicity limit (CC₅₀ >500 µg/ml). For us the fraction provides the best candidates for further investigation due to its selective activity. Of particular relevance is its high activity against MRSA, S. aureus, E. coli, S. sonnei, C. albicans and MTB which are currently posing great public health challenge due to drug resistance development and as major sources of community and hospital based infections. To the best of our knowledge, this is the first report exploring the antituberculous activity of P. thonningii and hence a major output in search of new safe drug leads to mitigate the global tuberculosis threat.

ACKNOWLEDGEMENTS

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Conflicts of Interest

Authors have not declare no conflict.

REFERENCES


Full Length Research Paper

Studies on Chemical and Biological properties of *Bryonia epigaea* (Rottler)

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To investigate the phytochemical, antimicrobial and antioxidant potentials of *Bryonia epigaea* (Rottler). The study was performed by using various in-vitro methods such as 1, 1 Diphenyl 2 picryl hydrazyl (DPPH), and Agar well diffusion method for different concentrations of methanolic extracts. Phytochemical constituents of the three extracts (Hexane, Acetone and Methanol) were determined. Total Phenol content was determined by Folin Ciocalteu Method. Yield of extract was determined by calculating the mass of plant material before minusing the mass of the plant material after the extraction process. Extracts of *Bryonia epigaea* (Rottler) contained saponins, flavonoids, steroids, tannins, alkaloids, coumarins, phenols and reducing sugars. The antimicrobial activity was moderate. Methanol extract showed good antibacterial activity at 100 mg/ml concentration while antifungal activity against four significant pathogens was moderate. *Bryonia epigaea* (Rottler) methanol extract exerted significant antioxidant activity and dose dependent effect. The Results showed that the methanol extract showed high amount of antioxidant compounds and exhibit significant antioxidant activity. The antioxidant activity was found to be increased with the concentration of the compound. In the present research work we tried to find out the bioactive properties of ancient medicinal plant *Bryonia epigaea* (Rottler). The phytochemical, antimicrobial and antioxidant properties have been discussed.

Key words: *Bryonia epigaea* (Rottler), phytochemical analysis, antimicrobial properties, antioxidant properties.

INTRODUCTION

Plants are the most reliable sources of phytochemical components in the world and have efficient and economically useful chemical compounds that are to be isolated and characterised. There is a dreadfull need for the human world to eradicate the much threat full diseases by utilising the natural components (Nostro
et al., 2000). *Bryonia*, a genus of perennial flowering and climbing plant belonging to the family Cucurbitaceae is most diversified with approximately 125 genera and 960 species. The Cucurbitaceae family is having pretty full of medicinal properties and is also having utilitarian chemical components. *Bryonia epigaea* (Rottler.) International plant name index (IPNI) is a perennial, tendril climber with a large, turnip shaped root and succulent commonly known as Muru Donda, Domma tendril climber with a large, turnip shaped root and succulent. It is having the rhizome as a root. The root has Antidiabetic (Kattamanchi et al., 2013), Anthelmintic activity (Shri Vijaya et al., 2011), Analgesic, Antipyretic and Anti-Inflammatory (Narendra et al., 2012). Anti-Arthritis (Patel et al., 2012) and Anti venom activities (Chandrakala et al., 2013). Leaves are having hepato protective activity (Rangu Mahesh et al., 2012). *Bryonia epigaea* (Rottler) is a well-known name in traditional medicine and folklore medicine. We tried to work with whole aerial parts of the plant and screened for Phyto active compounds, Antimicrobial activity and Anti-oxidant activity. The plant root has been used for snake bite in the tribal areas of Kurnool district and the Sriharikota island, Andhra Pradesh, India (Khalieel and Sudarsanam, 2012), (Bharath and Suryanarayana, 2011), the tribes of seshachalam forest use fresh roots in treating diabetes (Pavani et al., 2012). In Maruthamali hills of south Western Ghats of Tamilnadu dried roots are used for treating joint pains (Sarvalingam et al., 2011) and in the Salem region of Tamilnadu root powder is used for treating poisonous animal bites (Thirunarayanan, 2013). In tribes of Yavatmal District, Maharashtra, India: root powder is used for anorexia and snake bite (Borkar et al., 2012). The tribes of Buldhan Dist, Maharashtra, India use the decoction of the tuber in treating typhoid fever and its paste is used for treating swellings and poisonous strings. (Korpenwar, 2012). The tribes of Hoshangabad district, Madhya Pradesh, India use root powder decoction for treating chronic mucous enteritis and dysentery (Manish and Upadhyay, 2011). In folklore medicine of Meena community of Rajasthan, India, the root is used to cure stomach tumour, typhoid and diarrhoea. (Ajay and Rao, 2010). On Eastern Ghats of Peninsular India the root is a valuable remedy for rheumatism and also used in treating dysentery. Root paste is also a remedy for snakebites. (Sri Rama et al., 2013).

**MATERIALS AND METHODS**

**Plant collection**

Plant material was collected from the Nalamala Forest of Chittore district of Andhra Pradesh in the month of January to March. It was identified based on its floral characters, herbarium and including other pertinent taxonomic literature by Dr. S. M. Khasim, Botanist and Assistant Professor, Department of Botany, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India. It was shade dried and pulverized by the use of a grinder and stored in bags at room temperature.

**Collection of microorganisms**

The micro organisms used for the experiments were procured from MTCC, IMTECH, Chandighar and they were reconfirmed by gram staining, sub culturing in appropriate selective media and biochemical tests.

**Gram-positive organisms**

- *Staphylococcus aureus* (MTCC 3160), *Streptococcus mutans* (MTCC497), *Lactobacillus casei* (MTCC1423), *Lactobacillus acidophilus* (MTCC495) and *Bacillus megaterium* (NCIM2187).

**Gram-negative organisms**

- *Enterococcus faecalis* (MTCC439), *Xanthomonas campestris* (MTCC2286), *Escherichia coli* (ATCC35218) and *Pseudomonas aeruginosa* (ATCC 9027).

**Fungal strains**

- *Candida albicans* (ATCC10231), *Aspergillus niger* (ATCC1015), *Rhizopus oryzae* (MTCC262) and *Candida rugosa*.

**Extraction procedure**

The plant material was extracted by using the Soxhlet Extraction Apparatus. *Bryonia epigaea* (Rottler) was extracted with three solvents hexane, acetone and methanol. The Soxhlet extraction method is the most appropriate process to obtain the crude extracts and it was dependent on the boiling point of the solvents. The powdered plant material was weighed (50 g) and packed in the thimble. It was extracted with one litre of appropriate solvent (Hexane, Acetone, Methanol) and it was performed up to 30 to 40 cycles or till the solvent colour changes to orange red. The solvent in the round bottom flask was collected for the condensation with the rotary evaporator to minimise the solvent wastage and to concentrate the extract for the further usage. The crude extract was obtained from the rotary evaporator that it can be condensed in the vacuum at 50°C. The Yield of extract was determined by the calculation of weight before and after the extraction process.

**Phytochemical analysis**

This is according to Evans (1989), Gokhale et al. (1993), Trease and Evans (1996) and Harborne (1998). The phyto active compounds are analysed by conducting qualitative tests. The extracts are screened for alkaloids, steroidal compounds, flavonoids, saponins, phenolic compounds, tannins, coumarins and cardiac glycosides by using standard procedures.
Detection of alkaloids

Extract + Dil HCl + filtration.

Mayer’s test: Filtrate + Mayer’s reagent (Potassium Mercuric Iodide) = yellow colour precipitate.

Wagner’s test: Filtrate + Wagner’s reagent (Iodine in Potassium iodide) = brown/reddish precipitate.

Dragendorff’s test: Filtrate + Dragendorff’s reagent (solution of Potassium Bismuth Iodide) = Red precipitate.

Hager’s test: Filtrate + Hager’s reagent (saturated picric acid solution) = yellow colour precipitate.

Detection of phenols

Ferric chloride test: Filtrate + freshly prepared 1% Ferric Chloride and potassium ferrocyanide = bluish-green colour.

Filtrate + ferric sulphate = dark-violet colour.

Detection of flavonoids

Alkaline reagent test: Filtrate + NaOH solution = yellow colour + Dil HCl acid = Colourless.

Lead acetate test: Filtrate + lead acetate solution = yellow colour precipitate.

Detection of anthraquinones

Free anthraquinones test: (Borntrager’s test): Filtrate + 10 ml of benzene + filtered + 5 ml of 10% ammonia solution = pink, red, or violet colour in the colour (lower phase).

Modified borntrager’s test: Filtrate + Ferric Chloride soln + water bath + extracted with benzene + ammonia solution = rose-pink colour in the ammonical layer.

Detection of phytosterols

Salkowski’s test: Filtrate + 2 ml chloroform + Conc. Sulfuric acid = A reddish brown colour at the interface.

Libermann Burchard’s test: Filtrate + chloroform + acetic anhydride + Conc. Sulphuric acid = brown ring at the junction.

Detection of terpenoids

Filtrate + acetic anhydride + conc H₂SO₄ = blue, green rings.

Detection of fatty acids

Filtrate + 5 ml of ether + evaporation on filter paper = transparence on filter paper (Rangu et al., 2012).

Detection of tannins

Ferric chloride test: Filtrate + 10% ferric chloride solution = bluish black.

Lead acetate test: Filtrate + 10% Lead acetate solution = yellow precipitate.

Pot. dichromate test: Filtrate + strong potassium dichromate solution = yellow colour precipitate.

Detection of saponins

Froth test: Filtrate + shaken in a graduated cylinder for 15 min = 1 cm layer of “honey comb” froth.

Anthocyanins: Filtrate + 2 ml of 2N HCl and ammonia = pink-red turns blue-violet (Shri et al., 2011).

Leucoanthocyanins: Filtrate + 5 ml of isoamyl alcohol = Upper layer appears red in colour (Shri et al., 2011).

Coumarins: Filtrate + 10% NaOH = yellow colour (Skerget et al., 2005).

Emodins: Filtrate + 2 ml of NH₄ OH and 3 ml of Benzene = red colour (Skerget et al., 2005).

Detection of reducing sugars

Fehling’s test: Filtrate + dil. HCl + alkali + heated with Fehling’s A & B solution = red precipitate.

Keller - Kiliani test (for de-Oxy sugars in cardiac glycosides): Filtrate + 2 ml chloroform + conc H₂SO₄ = Brown ring at interphase.

Antimicrobial activity

Agar well diffusion method

The Antimicrobial sensitivity test was performed in vitro to find out the efficiency of the extract that can resist the growth of the microorganism. Nutrient broth (NB) was prepared and inoculated with the respective microorganism. They were incubated for 24 h with constant shaking by orbital shaker. The nutrient agar (NA) plates were cooled to above room temperature and to these plates 10 µl of cultured nutrient broth was added. Four wells of 10 mm diameter were prepared using a cork borer. 50 µl of plant extract at a concentration of 10 mg/ml were added to the each well by using the sterile micro pipette and they are allowed to diffuse at room temperature for 2 h. These plates were incubated at 37°C for 18 to 24 h. Antibiotic sensitivity was also studied with different concentrations of the extracts to find out their effective dosage response. Sabouraud Dextrose Agar (SDA) plates were swabbed (sterile cotton swabs) with 24 h old - broth culture of respective fungi. Four wells (10 mm diameter) were made in each of these plates using sterile cork borer. About 50 µl of different concentrations (25, 50, 75 and 100 mg/ml) of plant extracts were added using sterilized dropping pipettes into the wells and allowed to diffuse at room temperature for 2 h. The plates were incubated at 28°C/72 h for the growth of fungal pathogens. The respective
extracts were maintained and solvents were used as a control. The experiment was repeated thrice, and average values of zone of inhibition were recorded in mm for analyzing the antimicrobial activity. The antibiotic compound Streptomycin (10 mg/ml) was used as a Standard for the antibacterial study (Perez et al., 1990).

**Determination of total phenols**

**Folin Ciocalteu method**

The total phenol contents were determined by the Folin Ciocalteu procedure by Skerget et al., (2005). Briefly, 1 ml of different concentrations of the Methanol extracts were taken and to that 0.1 ml of Folin Ciocalteu reagent and 2.5 ml of 0.2 N Na₂CO₃ was added and incubated for 30 min at room temperature. Distilled water was used as a reference blank. Absorbance was measured at 760 nm using Thermo Fisher double beam spectrophotometer.

Gallic acid was used as standard and the results were expressed as mg of Gallic acid equivalents per gram dry mass of the extract (mg GAE gDM).

**Antioxidant properties**

1, 1- Diphenyl-2-Picrylhydrazyl radical (DPPH) scavenging activity: The free radical scavenging activity of the extract was measured by using 1, 1- Diphenyl-2-Picrylhydrazyl radical (DPPH) as described by (Brand-Williams et al., 1995) with some modifications. The methanol crude extracts were prepared at 1 mg/ml concentration with DMSO solution. The mixture was made uniform and working solutions were prepared at different concentrations. The 0.004% (W/V) solution of DPPH in methanol was added to the solution. The mixtures were shaken and incubated for 60 min in the dark at room temperature. The absorbance was measured at 517 nm against a blank (Distilled water). The DPPH scavenging activity (I%) was calculated as follows:

\[
I\% = \frac{(Ao - As)}{Ao} \times 100
\]

Where Ao, is the absorbance of the DPPH solution without sample extract and As, is the absorbance of sample with DPPH solution.

**RESULTS AND DISCUSSION**

The yield of the extract was measured and it was 21.048% of the methanol extract, the colour of the extract was dark green in colour. The other two extracts had less yield and hexane appeared dark brown in colour (Figure 1). The yield of extract was more for the high polar solvents as most of the bioactive constituents are dissolved in high polar organic solvents. The qualitative analysis is a preliminary study for detecting the new compounds having biological significance. A general analysis has done to characterise the chemical nature of Bryonia epigaea (Rottler.). Phytochemical analysis of the three extracts (hexane, acetone, methanol) have successive amounts of chemical constituents. Hexane extract was found to have more compounds like flavonoids, saponins and coumarines when compared with acetone and methanol extracts. Saponins are used in the medicine as an antioxidant, anti-inflammatory, anticancerous agent and for hyperglycaemia, weight loss and it is also a strong proa ctive agent of the plant. Saponins also contain antifungal property (De-lucca et al., 2005). Acetone extract contained alkaloids, flavonoids, steroids, tannins, saponins, coumarins and reducing sugars. The methanol extract showed the presence of alkaloids, phenolics, steroids, saponins, tannins and reducing sugars (Table 1). Plants are the major reservoirs of Alkaloids and Flavonoids are more admirable for their curative effect of various allergies and carcinogens. They also show anti-cancerous, anti-microbial and anti-inflammatory activities (Harborne, 1973; Aiyelaagbe and Osamudiamen, 2009). The antimicrobial efficiency of Bryonia epigaea (Rottler) aerial part extracts showed good results against various pathogens (Figure 2).

It was found that all the three extracts have significant activity against the nine pathogens and Pseudomonas aeruginosa was remained unreactive for all the extracts. The activity was not affected by the concentration of the extracts. Methanol extract has more number of phyto constituents and has higher phenol content. Based on these results methanol extract was subjected to study by taking four concentrations and it was shown that 100 mg/ml concentration of methanol extract showed significant activity against all the microorganisms and Pseudomonas aeruginosa remained ineffective to all concentrations. The lower concentrations showed almost similar inhibitory activity (Figure 3). Anti-fungal activity was found to be moderate against all four concentrations of methanol extracts (Figure 4). The total phenol content of methanol extract was 327.68 µg GAE/500 µg (Figure 5). The DPPH radical scavenging activity was due to the hydrogen donating ability of the reagent and more over a substance when it is mixed with the DPPH solution, it can donate a hydrogen atom converted into the reduced form of 1,1-diphenyl-2-picryl hydrazine non radical form (Molyneux, 2004). The antioxidant property of Bryonia epigaea (Rottler) was evaluated by using the 1, 1-diphenyl 2-picryl (DPPH) method of radical scavenging activity.

The concentrations of 100 µg/ml to 500 µg/ml showed a gradual increase in the percentage of scavenging activity of four extracts shown in Figure 6. The values were shown along with the positive control ascorbic acid. The methanol extract has shown the low IC₅₀ value of 177.28 µg when compared to the Hexane (429.46 µg) and Acetone extract (247.46 µg) (Table 2). For the last decade, there is an increased search of natural antioxidants from the new sources due to the ill effects
Table 1. Qualitative Analysis of Whole Arial Part Extracts of Bryoniaepigaea (Rottler).

<table>
<thead>
<tr>
<th>S/No</th>
<th>Tests</th>
<th>Hexane extract</th>
<th>Acetone extract</th>
<th>Methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Alkaloids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Mayers</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Dragon</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Wagners</td>
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Hexane Extract  Acetone Extract  Methanol Extract

Figure 2. Antibacterial Activity of Whole Arial Part Extracts of *Bryonia epigaea* (Rottler).
**Figure 3.** Anti-bacterial efficiency of methanol extracts with different concentrations.

**Figure 4.** Anti-fungal efficiency of Methanol extracts with different concentrations.
Figure 5. Total phenol content of methanol extract.

Figure 6. % of DPPH activity for different exteacts of Bryonia epigaea (Rottler).

Table 2. Antioxidant activity of Bryonia epigaea (rottler).

<table>
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<th>S/No.</th>
<th>Name of the Extract</th>
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of reactive oxygen species (ROS) production and oxidative stress. The oxidative stress has been linked to many diseases. The free radical is a molecule with an unpaired electron and is involved in bacterial and parasitic infections, inflammation, cardiovascular disorders, lung damage, reperfusion injury, atherosclerosis, neoplastic diseases and aging (Thomas and Kalyanaraman, 1997). The antioxidant activity of most of the plants is associated with their phenolic concentrations. There is a chance of a decrease in the antioxidant activity due to partial lacking of phenol constituents. These phenols and flavonoids have the functional groups with scavenging property (Kessler et al., 2003).

CONCLUSION

*Bryonia epigaea* (Rottler) is the most instinctive plant having wide range of activities. Wide range of compounds were analyzed in methanolic extract. The antimicrobial efficiency of *Bryonia epigaea* (Rottler) aerial part extracts showed good results against various pathogens Methanol extract showed good anti-oxidant activity. *Bryonia epigaea* (Rottler) aerial parts were analyzed for its biological activities and further isolation and characterization of the compounds with biological activities will certainly add a valuable invention in the field of drug discovery.

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Conflicts of interest

Authors have none to declare.

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