In vitro anti-inflammatory and phytochemical properties of crude ethyl acetate extract of *Baliospermum montanum* Leaf (Muell – Arg)

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**INTRODUCTION**

*Baliospermum montanum* (Muell – Arg) an aromatic medicinal plant belonging to the family Euphorbiaceae includes 280 genera with 730 species with the largest genes Euphorbia (Husain et al., 1980). Euphorbia plants are widespread in nature ranging from herbs and shrubs to trees in tropical and temperate regions all over the world (Johnson et al., 2003). Root, leaf and seeds of *B. montanum* are used medicinally and are documented from Asian countries including Nepal, Burma, Malay and India (Mali et al., 2008). Phorbol esters, include montanin, baliospermin, 12 - deoxyphorbol – 13 – palmitate, 12 – deoxy -16 – hydroxypherbol - 13-palmitate and 12 – deoxy - 5β - hydroxypherbol - 13 myristate. Leaves contain 8-sitosterol, and 8-D-glucoside and hexacosamol was observed from roots and 11, 13-dihydroxytetraicos-trans-9-enoic acid was reported from seeds of *B. montanum* (Johnson et al., 2010; Husain et al., 1980). The preliminary phytochemical analysis revealed the presence of flavonoids, glycosides, steroids and absence of alkaloids, saponins and terpenoids in the root and glycosides and terpenoids in the seeds of the plant (Mail et al., 2008; Johnson et al., 2010). *B. montanum* is known for its ethnobotanical and traditional use (Mali and Wadekar, 2008).

Inflammation is the protective mechanisms of local microcirculation responsible to fight against tissue injury caused by physical and chemical factors; immunological reactions, microbial infections, and tissue damage (Mahesh et al., 2011). Redness, swelling, heat, pain and loss of function are considered as symptoms of inflammation.
and are responsible for interruption and resolution of the infectious diseases. Persistence of inflammation leads to various diseases associated with chronic inflammation, including arthritis, atherosclerosis, and even cancer (Schett, 2006; Libby et al., 2002; Karin et al., 2005). Adverse effect of available anti-inflammatory drugs cause leads to search of novel curative agents of plant origin. Natural products are rich in novel bioactive secondary metabolites and it is important to identify natural products with pharmacological or biological activity for use in pharmaceutical drug discovery and design (Jang et al., 2013).

Roots of B. montanum are considered as purgative, anthelmintic, diuretic, diaphoretic, rubefacient, febrifuge and as tonic. Additionally, they are also reported to be useful in the treatments of dropsy, constipation, jaundice, leprosy and skin disease. The roots have long been used as Ayurvedic remedy for Jaundice (Ogura et al., 1978). The leaves are found to be good for asthma and bronchitis (Wadekar et al., 2008). The seeds of the plant are drastic, purgative, rubefacient, hydragogue and stimulant.

Based on the above review of available literature, it was noticed that there is need for considerable pharmacological research on the medicinal herbs B. montanum. Thus, in the present study, the anti-inflammatory potential of crude extract and phytochemical screening from B. montanum leaves were evaluated and the results were discussed in details.

MATERIALS AND METHODS

Chemical and reagents

RPMI 1640 medium, fetal bovine serum (FBS), trypan blue, Histopaque-1077, penicillin G, streptomycin, gentamycine, amphotericin B, 3-(4,5-Dimethylthiazolo-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethylsulfoxide (DMSO), Trypsin and phytohaemagglutinin (PHA) were purchased from Sigma. All other chemicals and solvents were purchased from Merck.

Collection of plant materials

The plant tissue material taken for investigation on anti-inflammatory studies was shade dried leaf of B. montanum (Muell – Arg). The plants were collected from their natural habitats in Pondicherry, India. The voucher specimen is available for reference (BST/WC/Tech 277).

Extraction with organic solvent

The dried plant leaf powders (100 g) of B. montanum (Muell – Arg) were extracted with different solvent with increasing polarity viz hexane, ethyl acetate, acetone, methanol, at room temperature. The extract was filtered with Whatman No 1 filter paper. Each of the extract was concentrated in a rotary evaporator under reduced pressure and temperature to prevent the extract. The compound thus obtained was re-suspended in appropriate volume of DMSO for the treatment of cells (Bhakuni et al., 1971).

Cell culture

PBMC was cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with glutamine (100 U/ml), streptomycin (0.75 µg/ml), penicillin (120 U/ml), amphotericin B (3 µg/ml) and gentamycine (160 µg/ml) and 10% FBS was maintained at 37°C with a humidified atmosphere of 5% CO2.

Isolation of PBMC

PBMC were isolated from heparinized venous blood by Histopaque-1077 (Sigma) gradient centrifugation. The cells were suspended in RPMI-1640 medium containing 1% penicillin, streptomycin and amphotericin B, supplemented with 10% fetal bovine serum. Ten milliliters (10 ml) of blood collected aseptically in a syringe was mixed gently with heparin and carefully layered over 5 ml of Ficoll gradient (2:1 ratio) and centrifuged at 1800 rpm for 30 min at room temperature (Souza-Fagundes et al., 2002). PBMC identified as a buffy layer at the interface were collected and washed twice with the RPMI medium without serum and centrifuged at 1500 rpm for 15 min. The pellet was suspended in RPMI with serum and 10 µl of the suspension was mixed with trypan blue and loaded in RPMI in the Neubauer’s chamber to check the viability. 0.2 x 10⁶ cells were dispensed in 200 µl of each well of 96-well plate (Bignold et al., 1987; Selvakkumar et al., 2007).

Cytotoxic studies by MTT assay

The isolated PBMCs (0.2 x 10⁶ /100 µl) were seeded into a 96 well plate. 10 µl of phytohaemagglutinin (PHA) (0.4 µg/ml) was to each well and appropriate control of the cells were incubated for 2 to 3 h at 37°C, 5% CO2 and 90% humidity. Compounds in the crude extract were added (2 µl) in various concentrations (0.1 to 100 µg/ml) to the wells. Negative control and positive control (Triton X was used as in case of MTT), were also maintained, un-induced control and a solvent control was used along with it. The cells were then incubated overnight at 24 h for 37°C, 5% CO2 and 90% humidity. Medium from the wells were removed and 10 µl of MTT (5 mg/ml re-suspended in PBS) was added to each well. Plates were incubated for 4 h at 37°C, floating cells were carefully removed and 100 µl of DMSO was added to each well to lyse the cells and the absorbance was measured at 570 nm. Finally, the percentage of cell viability was calculated using the formula: Cell viability (%) = (Absorbance of test sample/Absorbance of control) x 100 (Ashalatha et al., 2010).

Phytochemical screening

Chemical tests were carried out on the solvent extract and on the powdered specimens using standard procedures to identify the constituents as described by Sofowora (1993), Trease and Evans (1983) and Harborne (1998).

Test for alkaloids

Test sample (1 ml) was mixed with few drops of Mayer’s reagent and the formation of orange brown precipitate was recorded as indicator for the presence of alkaloids.

Test for anthraquinones

The Bornträger test was used for the detection of anthraquinones. Two milliliters (2 ml) of test sample with 4 ml of hexane was added and shaken well. The upper lipophilic layer was separated and treated with 4 ml of dilute ammonia. If the lower layer changed from violet to pink, it indicated the presence of anthraquinones.
Table 1. Inhibitory concentration of crude extracts from *Baliospernum montanum* leaf against PBMC cells.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Anti-inflammatory activity (% Inhibition) (24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexane</td>
</tr>
<tr>
<td>0.1</td>
<td>6.277±0.18</td>
</tr>
<tr>
<td>1</td>
<td>7.658±0.22</td>
</tr>
<tr>
<td>10</td>
<td>9.838±0.29</td>
</tr>
<tr>
<td>50</td>
<td>15.06±0.45</td>
</tr>
<tr>
<td>100</td>
<td>18.08±0.54</td>
</tr>
</tbody>
</table>

**Test for flavonoids**

Three methods were used to determine the presence of flavonoids in the plant sample (Sofowora, 1993; Harbrone, 1998). Five milliliters (5 ml) of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by the addition of concentrated H₂SO₄. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing. Few drops of 1% aluminum solution were added to a portion of each filtrate. A yellow colouration was observed indicating the presence of flavonoids. A portion of the powdered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration was observed indicating a positive test for flavonoids (Obianime and Uche, 2007).

**Test for cardiac glycosides (Keller-Killani test)**

5 ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayer with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer (Ekhaise et al., 2010).

**Test for phlobatanins**

Deposition of a red precipitate in extracts boiled with 1% aqueous hydrochloric acid was taken as the evidence for the presence of phlobatanins.

**Test for phenolic compound**

1 ml of test solution was treated with 10% ethanolic ferric chloride. Phenolic compounds were considered present when a colour change to blue green or dark blue was observed.

**Test for saponin**

About 2 g of the powered sample was boiled in 20 ml of distilled water in a water bath and filtered. Ten milliliter (10ml) of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion (Edeoga et al., 2005).

**Test for steroids**

200 µl of acetic anhydride was added to 0.5 ml ethyl acetate of each sample with 2 ml H₂SO₄. The colour changed from violet to blue or green in some samples indicating the presence of steroids (Akinpelu et al., 2008).

**Test for tannins**

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue – black coloration (Kubmarawa et al., 2007).

**Test for terpenoids (Salkowski test)**

5 ml of each extract was mixed in 2 ml of chloroform, and concentrated H₂SO₄ (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids.

**Statistical analysis**

All values were expressed as mean ± standard. The statistical significance was evaluated by one – way analysis of variance (ANOVA) using SPSS version. When there was a significant difference, Tukey’s multiple comparisons were performed by fixing the significance level at p ≤ 0.05.

**RESULTS**

The leaves of *B. montanum* were collected and shade dried and used for extraction of its active ingredients. A total of four different solvent extracts of *B. montanum* were tested with *in vitro* model for studying its anti-inflammatory activity using MTT assay. The optimum concentration of crude leaf's extract was evaluated with varying doses using PHA induced PBMC for 24 h and the inhibitory effect was studied (Table 1). Our study revealed that among the solvents used to prepare the crude extract, ethyl acetate provided the ingredients with notable anti-inflammatory activity. It was also noted that the ingredients of crude extracts from hexane and methanol were not effective even at higher dose. In hexane extract we noticed just 18.08±0.54% anti-inflammatory activity at the highest dose (100 µg/ml). Similarly, in methanol extract also, the highest dose used revealed just 21.26±0.64% anti-inflammatory activity. However, the crude extract with acetone revealed 49.01±1.47% anti-inflammatory activity at its higher dose (100 µg/ml). The ethyl acetate extract of *B. montanum* showed highest anti-inflammatory activity.
activity against PBMC even at low dose with IC_{50} values of 9.08 µg/ml (Figure 1A). However, on considering the ethyl acetate extract, inhibitions of proliferation were low and not significant in other crude extracts even at higher concentrations (Figure 1B).

Further phytochemical constituents were analysed for the active constituents involved in the anti-inflammatory activity from all four solvent extracts. Our phytochemical analysis revealed that ethyl acetate and acetone crude extracts showed the presence of flavonoids, tannins, and steroids. Hexane and methanol crude extracts did not show remarkable phytochemical activity (Table 2). Phytochemical constituents such as flavonoids, steroids, tannins, amino acids and carbohydrates were observed in the extract of ethyl acetate. Similarly, all these phytochemicals were observed in acetone. Our study reveals that the glycosides are exclusively observed only in acetone extract.

**DISCUSSION**

The present study revealed the anti-inflammatory activity of crude extracts like hexane, ethyl acetate, acetone, and methanol of *B. montanum* leaf. Our study revealed notable anti-inflammatory effect in crude extracts with ethyl acetate and acetone. It was also observed that the flavonoids detected in both extracts are known to be good anti-inflammatory agents. Studies of Raju et al. (2005) on anti-inflammatory potential of *Cassia fistula* revealed the responsibility of flavonoid and alkaloids in anti-inflammatory reactions. Similarly, flavonoid with anti-inflammatory potential are reported from *Morinda citrifolia* and *Vernonia amygdalina* (Sivaraman and Muralidharan, 2010; Udeme et al., 2009). Despite flavonoids, steroids were noticed in both the extracts (ethyl acetate and methanol) and studies of Neto et al. (2005) reported the presence of steroids with anti-inflammatory potential in *Paaffia glomerata*.

The ethyl acetate extract showed good anti-inflammatory response comparatively with other extracts. The preliminary phytochemical test suggested the presence of flavonoids, steroids, tannins, glycosides, amino acids and carbohydrates in the ethyl acetate and acetone extracts. Hexane and methanol crude extracts did not show remarkable phytochemical activity. Results of the present investigation are directly correlated with previous observa-
Figure 1. (B) Inhibitory effect of crude leaf extracts from *Baliospermum montanum* on mitogen induced PBMC with different concentrations (0.1, 1, 10, 50 and 100 µg/ml): increasing polarity viz Hexane, Ethyl acetate, Acetone, Methanol.

Table 2. Preliminary phytochemical screening of various extracts of *Baliospermum montanum* leaf.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Hexane</th>
<th>Ethyl acetate</th>
<th>Acetone</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Glycosides</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Phenolic compound</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Saponins</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Steroids</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tannins</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Amino acid</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Protein</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

+= Present; − = absent.

So, this study first reported the anti-inflammatory potential of leaf ethyl acetate extract from *B. montanum*. Results from our study demonstrated that the ethyl acetate extract of *B. montanum*
leaves contains effective anti-inflammatory agents, which could ultimately be used as functional material and traditional remedy against inflammation. Future studies are required for isolation of bioactive compounds for analysis of the molecular mechanisms responsible behind its anti-inflammatory potential.

REFERENCES


Halls. 91.


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