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

P. Lalitha[1]* and P. Gayathiri[2]

[1]Department of Biotechnology, Pondicherry University, Pondicherry, India.
[2]Department of Agricultural Biotechnology, MetaHelix Life Science, Bangalore, India.

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Baliospermum montanum **(Muell – Arg) which belong to Euphorbiaceae family is a well known perennial herb in Indian medicine used to treat various disorders like asthma, bronchitis, purgative, anthelmintic, diuretic, diaphoretic, rubefacient and tonic. The anti-inflammatory activity of four different solvent extracts of *B. montanum* leaf was investigated in Phytohaemagglutinin (PHA) induce peripheral blood mononuclear cells (PBMC) cells using MTT assay. Among the samples, ethyl acetate extract revealed good anti-inflammatory response comparatively with other extracts such as hexane, acetone, methanol and the preliminary screening of phytochemical test was investigated. The results of phytochemical screening revealed the presence of flavonoids, tannin, steroids, glycosides, amino acid and carbohydrates. Our study demonstrates that the ethyl acetate extracts of *B. montanum* leaves contains effective anti-inflammatory agents, which could ultimately be used as functional material and traditional remedy against inflammation.**

Key words: *Baliospermum montanum*, anti-inflammatory, MTT assay, phytochemical.

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, Malaya 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 - hydroxyphorbol - 13 myristate. Leaves contain 8-sitosterol, and 8-D-glucoside and hexacosamol was observed from roots and 11, 13-dihydroxytetracos-trans-9-enoic acid was reported from seeds of *B. montanum* (Johnson et al., 2010; Husain et al., 1980). The prelimnary 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 ethanobotanical 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

*Corresponding author. E-mail: lallubio19@gmail.com. Tel: +091-8122195038.

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 Ayuvedic 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, amphotericine B, 3-(4,5-Dimethylthiazlo-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% CO_2.

Isolation of PBMC

PBMC were isolated from heparinized venous blood by Histopaque-1077 (Sigma) gradient centrifugation. The cells were suspended in RMPI-1640 medium containing 1% penicillin, streptomycin and amphotericin B, supplemented with 10% fetal bovine serum. Ten milliliter (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 10^6 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 10^6 /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% CO_2 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% CO_2 and 90% humidity. Medium from the wells were removed and 10 µl of MTT (5 mg/ml re-supended 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 lyses 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 Borntrager test was used for the detection of anthraquinones. Two milliliter (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 *Baliospermum montanum* leaf against PBMC cells.

Concentration (µg/ml)	Anti-inflammatory activity (% Inhibition) (24 h)			
	Hexane	Ethyl acetate	Acetone	Methanol
0.1	6.277±0.18	8.5319±0.25	11.27338±0.33	5.2451±0.157
1	7.658±0.22	15.295±0.45	20.85575±0.62	10.381±0.311
10	9.838±0.29	55.012±1.65	37.12529±1.11	15.368±0.461
50	15.06±0.45	64.452±1.93	45.17038±1.35	17.140±0.514
100	18.08±0.54	80.245±2.40	49.01358±1.47	21.265±0.637

Test for flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample (Sofowora, 1993; Harbrone, 1998). Five milliliter (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 H_2SO_4. 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 flavornoids (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 H_2SO_4. 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 H_2SO_4 (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 $p \leq 0.05$.

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 leafs 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

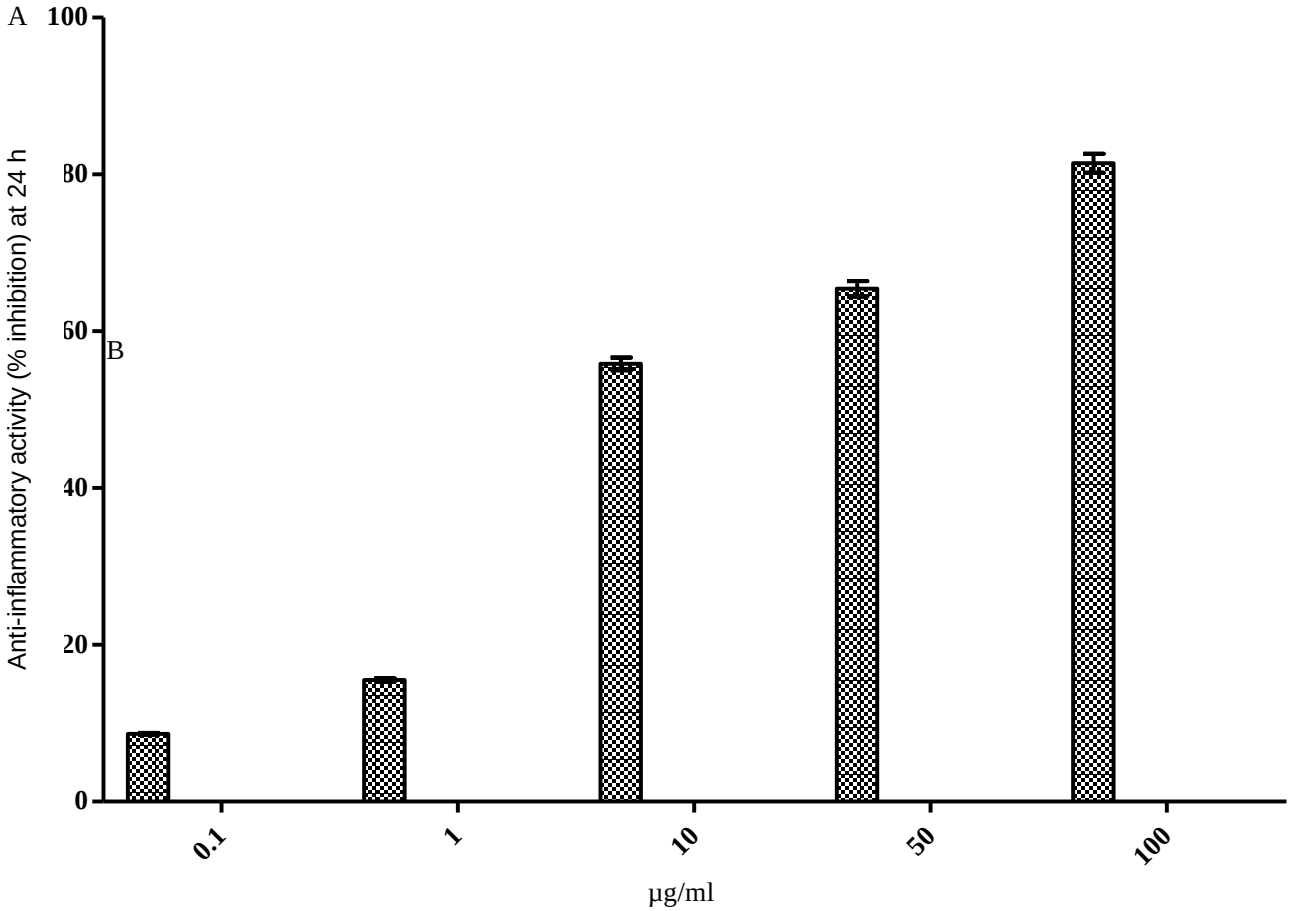

Figure 1. (A) Inhibitory effect of crude ethyl acetate leaf extracts from *Baliospermum montanum* on mitogen induced PBMCs with different concentrations (0.1, 1, 10, 50 and 100 µg/ml). The IC50 value was found to be 9.08 µg/ml.

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 constitutents 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 flavornoids 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 *Morindatinctoria roxb,* and *Vernonia amygdalina* (Sivaraman and Muralidharan, 2010; Udeme et al., 2009). Inspite of 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 *Pafaffia 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-

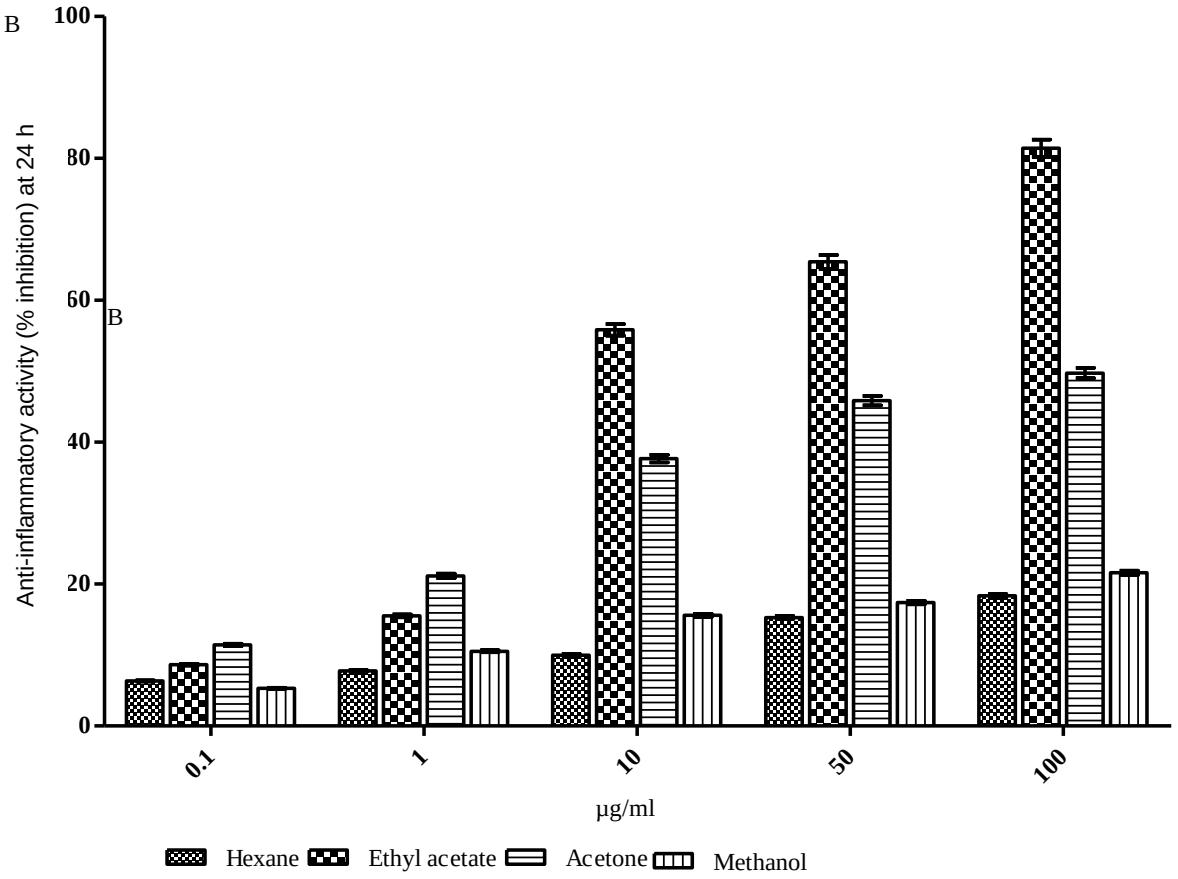

Figure 1. (B) Inhibitory effect of crude leaf extracts from *Baliospermum montanum* on mitogen induced PBMC with different concentrations (0.1, 1, 10, 50 and 100 µg/ml): increasing polarity viz Hexane, Ethyl acetate, Acetone, Methanol.

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

Phytochemical	Hexane	Ethyl acetate	Acetone	Methanol
Alkaloids	–	–	–	–
Anthraquinones	–	–	–	–
Flavonoids	–	+	+	–
Glycosides	–	–	+	–
Phlobatannins	–	–	–	–
Phenolic compound	–	–	–	–
Saponins	–	–	–	–
Steroids	–	+	+	–
Tannins	–	+	+	–
Terpenoids	–	–	–	–
Amino acid	–	+	+	–
Protein	–	–	–	–
Carbohydrates	–	+	+	–

+ = Present; – = absent.

tions (Johnson et al., 2010). 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

Ashalatha K, Venkateswarlu Y, Moushum Priya A, Lalitha P, Krishnaveni M, Jayachandran S (2010). Anti-inflammatory potential of Decalepis hamiltonii (Wight and Arn) as evidenced by down regulation of pro-inflammatory cytokines-TNF-α and IL-2. J. Ethnopharmacol. 130: 167-170.

Akinpelu DA, Aiyegoro OA, Okoh AI (2008). In vitro antimicrobial and phytochemical properties of crude extract of stem bark of Afzelia africana (smith). Afr. J. Biotechnol. 7(20): 3665-3670.

Bhakuni DS, Dhar ML, Dhar MM, Dhawan BN, Gupta B, Srimal RC (1971). Screening of Indian medicinal plants for biological activity Part III. Ind J. Experi Biol. 9: 91.

Bignold LP, Ferrante A (1987). Mechanism of separation of polymorphonuclear leukocytes from whole blood by the one-step Hypaque-Ficoll method. J. Immunol. Method. 96: 29-33.

Edeoga HO, Okmu DE, Mbaebie BO (2005). Phytochemical constituents of some Nigerian medicinal plants. Afr. J. Biotechnol. 4(7): 685-688.

Ekhaise FO, Soroh AE, Falodum A (2010). Antibacterial properties and preliminary phytochemical analysis of methanolic extract of Ocimum Gratissium (scent leaves). Bayer. J. Pure Appl. Sci. 3(2): 81-83.

Husain S, Ahmed MU, Osman SM (1980). New hydroxyl fatty acid from seed oil of Baliospermum montanum. Phytoche. 19: 75-77.

Harborne JB (1998). Phytochemical Methods London Chapman and Halls. 91.

Johnson M, Manickam VS (2003). *In vitro* micropropagation of Baliospermum montanum (Willd.) Muell.Arg. a medicinal plant. Indi. J. Exper. Biol. 41: 1349-1351.

Johnson M, Wesely EG, Zahair Hussain MI, Selvan N (2010). *In vivo* and *In vitro* phytochemical and antibacterial efficacy of Baliospermum montanum (Willd). Muell. Arg. Asian pacif. J. Tropi. Medi. 894-897.

Karin M, Greten FR (2005). NF-κB: Linking inflammation and immunity to cancer development and progression. Nat. Rev. Immunol. 5: 749-759.

Kubmarawa D, Ajoku GA, Enwerem NM, Okorie DA (2007). Preliminary phytochemical and antimicrobial screening of 50 medicinal plants from Nigeria. Afr. J. Biotechnol. 6(14): 1690-1696.

Libby P, Ridker P, Maseri A (2002). Inflammation and atherosclerosis. Circulation 105: 1135-1143.

Mali RG, Wadekar RR (2008). In vitor Anthelmintic activity of Baliospermum montanum Muell-Arg roots. Indi. J. Pharma. Scien. 70: 131-3.

Mali RG, Mahajan SG, Mehta AA (2006). Antimicrobial activity of Baliospermum montanum Muell-Arg leaves. Plant. Med. 2: 13-14.

Mahesh G, Ramkanth S, Mohamed Saleem TS (2011). Anti-inflammatory drugs from medicinal plants – Comprehensive a review. Int. J. Rev. Life Sic. 1(1): 1-10.

Neto AG, Costa JMLC, Belati CC, Vinhalis AHC, Possebom LS, Da Silva Filho AA, Cunha WR, Carvalho JCT, Bastos JK, Silva E MLA (2005). Analgesi and anti- inflammatory activity of a crude root extract of pfaffia glomerata (Spring) Pedersen. J. Ethnopharmacol. 96(2-1): 87-91.

Ogura M, Koike K, Cordell GA, Farnsworth NR (1978). Potential anticancer agents VIII. Constituents of Baliospermum montanum (Euphorbiaceae). Plant. Med. 33: 128-43.

Obianime AW, Uche FI (2007). The phytochemical screening and the effects of methanolic extract of phyllanthus amarus leaf on the biochemical parameters of male guinea pigs. J. Appl. Sci. Environ. Manage. 12(4): 73-77.

Raju I, Moni M, Subramanian V (2005). Anti-inflammatory and antioxidant activities of Cassia fistula linn bark extract. Afr. J. Tra. Comple. Alter. Med. 2(1): 70-85.

Schett G (2006). Rheumatoid arthritis: Inflammation and bone loss. Wien. Med. Wochenschr. 156: 34-41.

Sivaraman D, Muralidharan P (2010). Anti-Ulcerogenic evaluation of root extract of ficus hispida linn, in aspirin ulcerated rats. Afr. J. Phar. Pharmaco. 4(2): 079-082.

Sofowora A (1993). Medicinal plants and traditional medicine in Africa Chichester John. Wiley & Sons, New York. 97-145.

Selvakkumar C, Gayathri B, Vinay Kumar KS, Lakshmi BS, Balakrishnan A (2007). Potential anti inflammatory properties of crude alcoholic extract of Ocimum basilicum L in human peripheral blood mononuclear cells. J. Heal. Scien. 53: 500-505.

Souza-Fagundes EM, Queiroz ABR, Filho OAM, Giovanni G, Corrêa-Oliveira R, Alves TMA, Zani CL (2002). Screening and fractionation of plant extracts with anti proliferative activity on human peripheral blood mononuclear cells. Int. J. Biol. Biomed. Res. 97: 1207-1212.

Trease GE, Evans WC (1983). Textbook of Pharmacognasy. 12th edition, Balliere, Tindall, London. 57-59: 343-383.

Udeme OG, Owunari AG (2009). Evaluation of anti-inflammatory activity of extract of Vernonia amygdalina. East. J. Med. 14: 20-22.

Wadekar RR, Supale RS, Tewari KM, Patil KS, Jalalpure SS (2008). Screening of roots of Baliospermum montanum for heptoprotective activity against paracetamol induced liver damage in albino rats. Interna. J. Green Phar. 2: 220-223.