EVALUATION OF ANTIINFLAMMATORY ACTIVITY OF FLAVONOIDE FRACTIONS FROM EUPHORBIAEAE MEMBERS ON RAW 264.7 CELL LINES

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SUMMARY

The flavonoid fractions I, II and III isolated from the leaf extract of Baliospermum montanum, Drypetes roxburghii and Codiaeum variegatum possess significant antiinflammatory and cytotoxic activity against mouse leukaemic monocyte macrophage cell line RAW 264.7. In B. montanum, the cytotoxic activity of LPS-stimulated mouse macrophages (RAW 264.7 cells) was found to be 82, 80 and 77% in fractions I, II and III respectively. In D. roxburghii, 76, 79 and 81% in fractions I, II and III respectively whereas in C. variegatum, 76, 71 and 69% in fractions I, II and III respectively. The antiinflammatory activity in B. montanum was in the range of 31–41%, whereas in D. roxburghii and C. variegatum 35–42% and 41–54% respectively. The cytotoxic and antiinflammatory activity against RAW 264.7 cell lines was found to be the highest in B. montanum as compared to the other 2 selected medicinal plants, D. roxburghii and C. variegatum. The present finding indicates the importance of isolation and characterization of the flavonoids and its future need to investigate its capability as antiinflammatory drug.

Keywords: Baliospermum montanum, Drypetes roxburghii, Codiaeum variegatum, flavonoids, antiinflammatory.

INTRODUCTION

Euphorbiaceae is a complex heterogeneous family consisting of about 322 genera and 8900 species. In India, it is represented by 73 genera and 410 species. The family is essentially tropical and occurs in diverse habitats from arid regions to humid tropics. As a result, the plants of this family have developed various life forms from herbs, shrubs, stunted succulents to tall canopy trees. (Balakrishnan & Chakrabarty 2007). Euphorbiaceae is considered as one of the top 25 economically important plant families (Benett 2007). Bijekar & Gayatari (2014) reviewed ethnomedicinal properties of this family and concluded it as rich source of medicinal components and have mentioned the need of doing extensive research on it.
The mystery of the medicinal properties of all plants lies in the phytochemicals i.e., secondary metabolites viz., alkaloids, carbohydrates, glycosides, steroids, flavonoids, coumarins, saponins, fatty acids, tannins, protein and amino acids, gum and mucilage, terpenoids, anthroquinones and phenols. These phytochemicals differ from each other at functional groups and chemical structure and therefore they have different chemical and medicinal properties. Among these, flavonoids have proved to have broad spectrum of medicinal properties such as antioxidative activity, free radical scavenging capacity, coronary heart disease prevention, hepatoprotective, antiinflammatory, anticancer and antiviral activities (Shashank et al. 2013).

The antiinflammatory actions of flavonoids in vitro or in cellular models involve the inhibition of the synthesis and activities of different pro-inflammatory mediators. Prostaglandins and nitric oxide biosynthesis are involved in inflammation, and isoforms of inducible nitric oxide synthase (iNOS) and of cyclooxygenase (COX-2) are responsible for the production of a great amount of these pro-inflammatory mediators. It has been demonstrated that flavonoids are able to inhibit enzymes, as well as other mediators of the inflammatory process such as reactive C protein or adhesion molecules (González-Gallego et al. 2007). The chronic inflammation might lead to a host of diseases, such as hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, and even cancer.

The present investigation aims to isolate and find the potential medicinal constituents responsible for the antiinflammatory property in Baliospermum montanum, Drypetes roxburghii and Codiaeum variegatum.

MATERIALS AND METHODS

*B. montanum* (Wild.) Muell-Ag., *D. roxburghii* (Wall.) Huresawa were collected from Sirsi, Western Ghats of Karnataka and *C. variegatum* (L.) Bl. was collected from Lal Bagh botanical garden, Bengaluru. Plants are being maintained in the Department of Molecular Biology, Bangalore University, Bengaluru.

Leaves of *B. montanum, D. roxburghii* and *C. variegatum* were collected, dried for one wk at room temperature (in shade) and grounded in a blender to fine powder. Crude plant extract was prepared by Soxhlet extraction method. About 750 g of the powdered leaves of *B. montanum, D. roxburghii* and *C. variegatum* using a Soxhlet apparatus with methanol for 8 h. The extracted solution was filtered and concentrated in a rotary evaporator under reduced pressure (rotary vacuum flash evaporator).

The crude methanol extract (28 g) obtained was subjected to chromatography (Silica gel 120 mesh, 500 g) eluted with ethyl acetate–n-hexane (7:3) solvent system. A total of 60 fractions were eluted. A Shinoda test was carried out to confirm the presence of flavonoids. The fractions in which flavonoids are present gave positive for flavonoids producing pink colour with Shinoda test. Fractions 1–25 were negative. Small fractions 26–30, 35–40, 50–55 gave positive test. These fractions were pooled to form fractions I, II and III.
The cytotoxic activity of isolated flavonoids was tested on Mouse leukaemic monocyte macrophage cell line RAW 264.7. The cell line RAW 264.7 cells were seeded in 96-well plates (4×10^4 cells/well) with 200 µl Dulbecco's modified eagle media (DMEM) for 24 h. RAW 264.7 cells were treated with lipopolysaccharide (LPS, 2 µg/ml), following exposure to 50 µg/ml of samples (different pooled fractions- I, II and III) for 30 min. After incubation for 4 h, the supernatant was removed, and the purple crystals were dissolved in 200 µl dimethyl sulfoxide (DMSO). After incubation for 24 h, cell viability was estimated by measuring the absorbance at 550 nm using an ELISA plate reader. Fresh culture medium was used as a blank and RAW 264.7 cells with fresh medium as control.

\[
\text{Cell viability} \% = \frac{A_c - A_b}{A_c - A_b} \times 100
\]

\[A_b = \text{absorbance of blank}
A_c = \text{absorbance of control.}
\]

The antiinflammatory activity was detected by nitric oxide assay. Mouse leukaemic monocyte macrophage cell line RAW 246.7 was cultured in DMEM (2 mM L-glutamine, 45 g/L glucose, 1 mM sodium pyruvate) with 10% fetal bovine serum (FBS). The cells were cultured at 37°C with 5% CO\textsubscript{2} and were subcultured twice a week. RAW 246.7 cells were seeded in 96-well plates at a density of 1 × 10^6 cells/well and incubated for 24 h at 37°C with 5% CO\textsubscript{2} for adherence. The cells were treated with test samples for 1 h and then incubated for 24 h in fresh DMEM with 1g/ml of \textit{Escherichia coli} lipopolysaccharide (LPS). Fresh culture medium was used as a blank. We used 2 categories of control; first one was RAW 246.7 with LPS only and second, cells without LPS and test sample.

The level of NO production in the pre-incubation of RAW 246.7 (Mouse leukaemic monocyte macrophage cell line) cell supernatants was determined according to the quantity of the nitrite indicator, using a colorimetric assay based on the Griess reaction; 100 µl of cell culture medium was mixed with 100 µl Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl-ethylenediamine dihydrochloride). Subsequently, the absorbance of the mixture was measured at 550 nm using an ELISA plate reader, after incubation at room temperature for 10 min.

Percentage inhibition was calculated using the following equation (Srisopa Ruangnoo et al. 2012):

\[
\text{Inhibition} \% = \frac{(A - B)}{(A - C)} \times 100
\]

\[A: \text{LPS (+), sample (–)}; B: \text{LPS (+), sample (+)}; C: \text{LPS (–), sample (–)}.
\]

**OBSERVATIONS**

**Cytotoxic activity**

The effect of isolated flavonoid from \textit{B. montanum}, \textit{D. roxburghii} and \textit{C. variegatum} methanol leaf extracts were tested in vitro on the RAW 264.7 cells. The latter were incubated with different...
fractions (Fractions I, II and III) for 24 h, the cell viability was then measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay revealed that none of the fractions of *B. montanum*, *D. roxburghii* and *C. variegatum* were cytotoxic to RAW 264.7 cells (Figs 1–3). Data represent the mean ± SDs (n = 3) of 3 independent experiments.

Figs 1–3: Effect of different fractions on the viability of LPS-stimulated mouse macrophages (RAW 264.7 cells).

**Antiinflammatory activity:**

Nitric oxide (NO) production in tissues is an indicator of inflammation under several conditions. The NO assay results showed all fractions (Fractions I, II and III) of *B. montanum*, *D. roxburghii* and *C. variegatum* exhibit antiinflammatory activity against RAW 264.7 cells (Figs 4–6). Data represent the mean ± SDs (n = 3) of 3 independent experiments.

**DISCUSSION**

NO is an endogenous free radical species that is synthesized from L-arginine by nitric oxide synthase (NOS) in various animal cells and tissues. Small amounts of NO are important regulators of physiological homeostasis, whereas large amounts of NO have been closely correlated with the pathophysiology of a variety of diseases and inflammation. After exposure to inducers, such as lipopolysaccharide (LPS) from gram-negative bacteria and various cytokines, NO production is induced in various cells,
such as macrophages, kupffer cells, smooth muscle cells, and hepatocytes, to trigger cytotoxicity, tissue damage, inflammation sepsis, and stroke as reported by Marletta (1993) and Jiang et al. (2006). The regulation of NO production is, therefore, an important target for inflammatory disease.

Although steroidal antiinflammatory drugs and NSAIDs are currently used to treat acute inflammation, these drugs have not been entirely successful in curing chronic inflammatory disorders while such compounds are accompanied by unexpected side effects. Therefore, there is an urgent need to find safer antiinflammatory compounds as opined by Yoon & Baek (2005). The use of natural products with therapeutic properties is as ancient as human civilisation and, for a long time, minerals, plant and animal products were the main sources of drugs (De Pasquale 1984).

Traditional medicine has used extracts of different plants for the treatment of a wide variety of disorders including acute and chronic inflammation. Among the active constituents of these extracts, flavonoids are a family of substances whose members have many interesting biological properties including anticancer, antimicrobial, antiviral, antiinflammatory, immunemodulatory and antithrombotic activities (Robak & Gryglewski 1996, Havsteen 2002).

A number of studies have reported that the phenolic compounds are abundant in ethyl acetate and n-butanol fractions of plant extracts as reported by Puangprongpitag et al. (2008) and Rao et al.
In addition, phenolic compounds are known to be potent for inhibiting NO and peroxynitrite productions (Conforti & Menichini 2011). The antiinflammatory properties of flavonoids have been studied recently, in order to establish and characterize their potential utility as therapeutic agents in the treatment of inflammatory diseases. Several mechanisms of action have been proposed to explain in vivo flavonoid antiinflammatory actions, such as antioxidant activity, inhibition of eicosanoid generating enzymes or the modulation of the production of proinflammatory molecules. Recent studies have also shown that some flavonoids are modulators of proinflammatory gene expression, thus leading to the attenuation of the inflammatory response (Ana Garcia-Lafuente et al. 2009).

In vitro cytotoxic and antiinflammatory activity of 3 flavonoid fractions, I, II and III from B. montanum, D. roxburghii and C. variegatum, were tested against RAW 264.7 Mouse leukaemic monocyte macrophage cell lines. Ethnobotanical study has mentioned the antiinflammatory properties of these plants but the medicinal constituent was unknown.

In B. montanum, fraction I showed the least cytotoxic activity, as compared to fractions II and III. Fraction I was also found to have more effective antiinflammatory property i.e., least NO production when compared to other 2 fractions. Lesser the NO production higher will be the antiinflammatory activity. The present investigation does not agree with the findings of Lalitha & Gayathiri (2013) on B. montanum. They reported that in vitro cytotoxic and antiinflammatory activity of crude leaf hexane, ethyl acetate, acetone, methanol extract against peripheral blood mononuclear cells (PBMC) and found ethyl acetate gives best result. Similar report of Kumar et al. (2001) on antiinflammatory property of petroleum ether and ethanol extract of B. montanum showed that it is effective against acute inflammation (carrageenan paw edema, and Ibuprofen-induced paw edema) in a dose related manner.

In D. roxburghii, fraction III showed maximum cell viability and least cytotoxic activity as compared to others fractions and fraction I showed highest antiinflammatory activity. Reanmongkol et al. (2009) have also studied antinociceptive, antipyretic, and antiinflammatory activities of Putranjiva roxburghii (Drypetes roxburghii) leaf extract in experimental animals and found that, extract exhibits moderate inhibitory activity of inflammation in carrageenin-induced paw edema in rats.

In C. variegatum, fraction I showed highest cell viability and fraction III the least. Fraction II was found to have most effective antiinflammatory activity whereas fraction III was least effective. Even though ethnomedicine study has mentioned antiinflammatory property but in vivo and in vitro antiinflammatory activity of C. variegatum was not estimated before. We are the first to study in vitro antiinflammatory activity of C. variegatum.

Among all 3 selected medicinal plants, B. montanum was found to have least cytotoxic effect as compared to other 2 species and highest antiinflammatory property. This cytotoxic and antiinflammatory study agrees with the findings of Neelesh Sharma et al. (2014) who reported cytotoxic and...
antiinflammatory activity of *Euphorbia hirta* (Euphorbiaceae). Further, the present findings is in agreement with Raju et al. (2005) who reported that flavonoids and alkaloids are responsible for antiinflammatory reactions. Similarly, flavonoids with antiinflammatory potential are reported from *Morinda tinctoria* and *Vernonia amygdalina* (Sivaraman & Muralidharan 2010, Udeme & Owunari et al. 2009).

The present findings indicate the importance of isolation and characterization of the flavonoids from *B. montanum*, *D. roxburghii* and *C. variegatum* and there is a need to investigate their utility in formulation of drugs against inflammation.

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