ANTI INFLAMMTORY AND CYTOTOXIC ACTIVITY OF METHANOLIC EXTRACT OF *Plectranthus hadiensis* STEM

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Summary

The aim of the present study was to explore the probable *in vitro* anti inflammatory and cytotoxic activity of methanolic extract of the stem of *Plectranthus hadiensis*. The *in vitro* anti inflammatory assays included BSA denaturation inhibition, Platelet aggregation inhibition and HRBC membrane stabilization, and the MTT assay was carried out in cervical cancer (HeLa) cell line. The extract at 1mg/mL concentration showed 86.10% BSA denaturation inhibition, 87.49% Platelet aggregation inhibition and 87.26% HRBC membrane stabilization, which was similar to the standard non-steroidal drug used, viz., Diclofenac. An IC_{50} value of 141.3μg/mL was observed for the cytotoxicity assay on HeLa cells. The results suggest that the methanolic extract of *P. hadiensis* has promising therapeutic potential.

Keywords: BSA denaturation, HRBC membrane stabilization, MTT assay

Introduction

Chronic inflammation represents a major pathologic basis for the majority of human malignancies. The role of inflammation in carcinogenesis has first been proposed by Rudolf Virchow in 1863, when he noticed the presence of leukocytes in neoplastic tissues (1). Since Virchow's early observation that linked inflammation and cancer, accumulating data have supported that tumors can originate at the sites of infection or chronic inflammation (2). Although inflammation acts as an adaptive host defense against infection or injury and is primarily a self limiting process, inadequate resolution of inflammatory responses often leads to various chronic ailments including cancer (3, 4). Although the present treatment regimes for both inflammation related ailments and cancer are effective, their adverse side effects are far more detrimental. Hence, there is a need to develop new drugs with novel modes of action that do not produce considerable side effects. Plants used in Ayurvedic formulations are a source of compounds which have high potential as therapeutic molecules. *Plectranthus hadiensis* (Lamiaceae) is a common constituent of Ayurvedic formulations given for treating patients with any type of carcinoma, ailments related to chronic inflammation, and possess significant anti-fungal and anti-bacterial activity (5). In trade, this plant is substituted with other plants of the same species either due to improper identification or due to insufficient availability of raw materials. Thus, *Plectranthus hadiensis* was selected for the study as no comprehensive and systematic study has been performed using the plant, although it is used in many formulations. In the present study, the anti inflammatory activity of the methanolic extract was evaluated by BSA denaturation assay, Platelet aggregation inhibition and HRBC membrane stabilization assays and the cytotoxic properties of the extract was assessed by MTT assay with HeLa cell line.

The methanolic extract of *Plectranthus hadiensis* was used for the anti inflammatory and cytotoxicity studies, since it had shown comparatively good antioxidant activity and high content of phenolic- acids compared to other solvent extracts.

Materials and methods

Anti inflammatory assays:

BSA denaturation inhibition assay (6): Each extract (5mL) was dried in vacuum oven and redissolved in 5 mL of isosaline. Different concentrations of the methanolic extract of *P. hadiensis* (50, 100, 250, 500, 1000 μg/mL) were from the above stock solution were prepared and added to 1.8 mL of 1 % of BSA solution. The pH was adjusted to 6.5 using 1N HCl and the solution was incubated at 37^0C for 20 minutes and heated to 57^0C for 10 to 15 minutes. After cooling, absorbance was measured at 660 nm. Diclofenac sodium was used as the standard and the control was taken without the extract.

The activity was calculated using the formula: (Control – Test)/Control x 100.

Platelet aggregation inhibition (7-9): The platelet rich plasma with 1.2 x 10^7 platelet cells for each assay was re-suspended in pH 7.4 Tris buffer. The platelet aggregation was recorded as absorbance values of spectrophotometer measurement. To determine the *in vitro* inhibition of platelet aggregation, different concentrations of methanolic extract of *P. hadiensis* (50, 100, 250, 500, 1000 μg/mL) in isosaline were used. The platelet aggregation was induced with ADP at a concentration of 1mM. Diclofenac sodium was used as the standard. The absorbance was recorded after 5minutes at 660nm. Control was taken without the extract.

The activity was calculated using the formula: (Control – Test)/Control x 100.

HRBC membrane stabilization assay (10): Blood was collected freshly and mixed with equal volume of Elsevier's solution. It was then centrifuged at 3000 rpm for 15 minutes. The packed cells were washed with isosaline and a 10 % suspension was made with isosaline. Different concentrations of methanolic extract of *P. hadiensis* (50, 100, 250, 500, 1000 μg/mL) were prepared in isosaline. To 0.5 mL of the extract, 1 mL phosphate buffer, 2 mL hyposaline and 0.5 mL HRBC suspension was added and incubated for 30 minutes at 37^0C and then centrifuged at 3000 rpm for 20 minutes. Absorbance was measured at 560 nm. Diclofenac sodium was used as the standard and control was taken without the extract served as negative control.

The activity was calculated using the formula: (Control – Test)/Control x 100.

Cytotoxicity assay

MTT assay (11, 12): The cytotoxic effect of methanolic extract of *P. hadiensis* was assessed in human cervical cancer HeLa cells by MTT assay. Cells were grown at 37°C in humidified 5% CO_2 and 95% air atmosphere in Dulbecco's Modified Eagle Media (DMEM) (2mM L-glutamine, 45 g/L glucose, 1 mM sodium pyruvate, 2g /L sodium bicarbonate and 10% fetal bovine serum). From the stock of 1x10^5 cells/mL, 100μL of cell suspension were seeded into 96-well plates at plating density of 10000 cells/mL allowed to grow and attach for 24h and subsequently exposed to different concentrations of methanolic extract of *P. hadiensis* dissolved in DMSO and diluted in media, for 48h. The final volume in each well was 200 μl and the plates

were incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 48h. The medium without samples served as control. Triplicate was maintained for all concentrations. After 48h, 15µL of MTT was added to each well and incubated at 37^0C for 4h. 100µL DMSO was added to solubilize the formazan crystals formed and the absorbance was measured at 570nm using microplate reader. The % cell inhibition was determined using the formula:

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC_{50} was determined using GraphPad Prism software.

Statistical analysis
Data were subjected to one-way analysis of variance (ANOVA). GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA) was used for all the statistical analyses as well as IC_{50} value calculation.
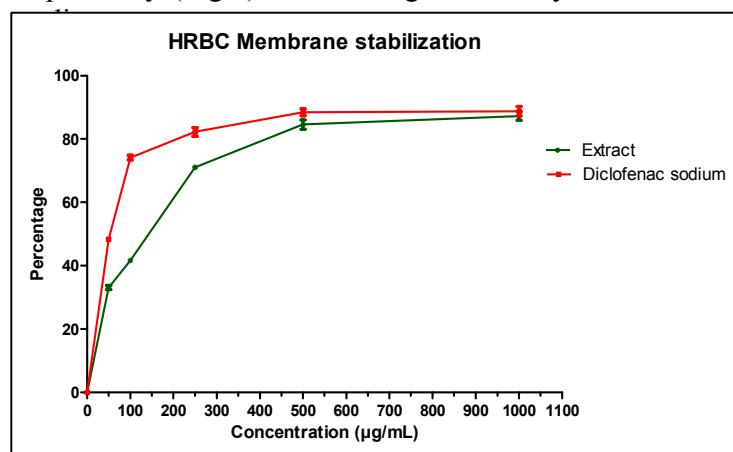
Results

Anti inflammatory assays
BSA denaturation inhibition assay: The inhibitory effect on protein (BSA) denaturation by the methanolic extract is shown in Fig 1. The percentage inhibition of extract and standard, Diclofenac, at 1mg/mL concentration, was 86.10% and 92.93% respectively.
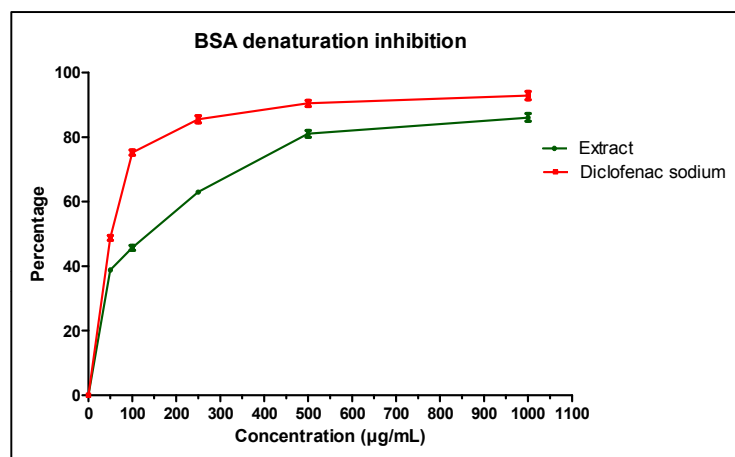
Platelet aggregation inhibition*:* Platelets are essential for normal haemostasis. Activation of the clotting cascade by trauma, results in platelet activation, which is followed by aggregation. The percentage aggregation inhibition of extract and standard, Diclofenac, at 1mg/mL concentration, were 87.49% and 92.31% respectively (Fig. 2).
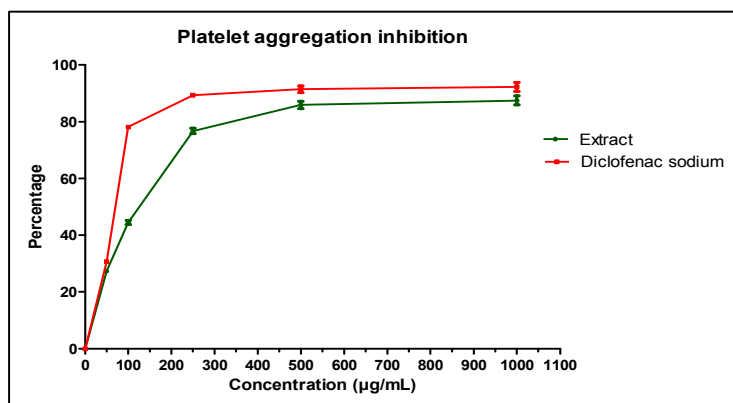
HRBC membrane stabilization assay: Various concentrations of the methanolic extract in iso-saline was assessed for its membrane stabilization properties and it was observed that, at 1mg/mL concentration, both extract and Diclofenac showed similar effects, 87.26% and 88.78% respectively (Fig.3). The analogous activity makes the extract a potential candidate for further

Cytotoxicity assay

MTT assay: The result of cytotoxicity activity of the methanolic extract is shown in Table 1. Increase in extract concentration up to 500µg/mL reduced the cell viability significantly in a dose dependent manner (Fig. 4). 100% growth inhibition was observed at a concentration of 500µg/mL. The IC_{50} value of the extract was found to be 141.3µg/mL.

Concentration (µg/mL)	% growth inhibition
31.25	15.46
62.50	26.57
125	37.08
250	67.93
500	100

Table. 1. Effect of the methanolic extract of *P. hadiensis* on HeLa cells (MTT assay).
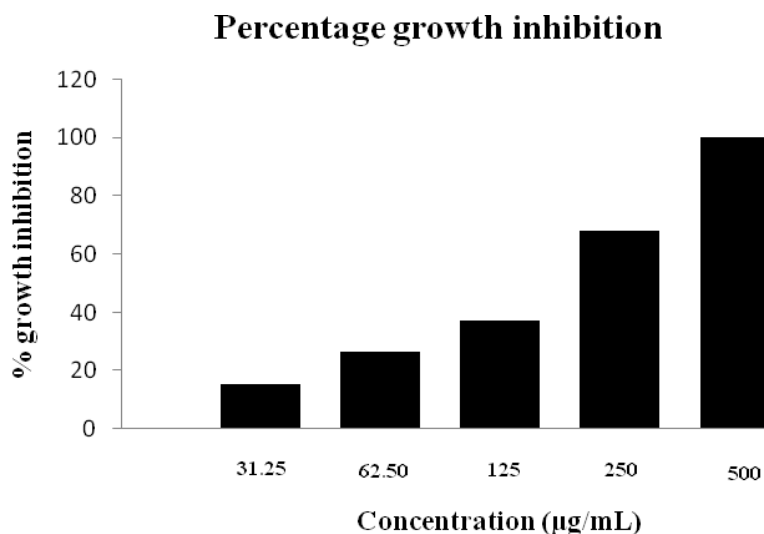
Fig. 4. Effect of the methanolic extract of *P. hadiensis* on HeLa cells (MTT assay).

Discussion

Inflammation covers a series of reparative and protective responses in tissue injury, either caused by infection, auto-immune stimuli, mechanical injury or chemical mediators. In the present study, the anti inflammatory and anti-proliferative activity of the methanolic extract of the stem of *Plectranthus hadiensis* was assessed using *in vitro* assays induced by chemical mediators.

The prevention of hypotonicity induced HRBC membrane lysis was taken as a measure to evaluate the potency of the solvent extracts and further evaluation at different concentrations was carried out in the most potent solvent extract. It was observed that among all the solvent extracts, methanolic extracts showed a dose-dependent HRBC activity compared to hexane and ethyl acetate that was effective only at concentrations above 1 mg/mL. Chloroform extract showed negligible HRBC membrane stabilization activity.

The denaturation of proteins is a well-documented cause of inflammation and rheumatoid arthritis and hence, this assay was adopted in assessing the properties of the extract in stabilizing the protein from the denaturation process. Several anti-inflammatory drugs have been reported to show dose dependent ability to inhibit thermally induced protein denaturation (13). BSA on denaturation expresses antigens associated with type III hypersensitive reaction, related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus. Several nonsteroidal anti-inflammatory drugs such as Indomethacin, Ibufenac, Diclofenac sodium, salicylic acid and flufenamic acid have been reported to prevent denaturation of BSA at pathological pH (6.2-6.5) (14). The effective concentration of the extract in preventing denaturation of the protein was observed to be 134.52 µg/mL compared to the standard drug that showed an inhibitory concentration of 52.66 µg/mL.

Since platelet aggregation is a vital pathogenic marker of inflammation, the activity of the extracts to inhibit platelet aggregation was assessed and compared with the standard NSAID. It is reported that platelets, essential for normal haemostasis on activation leads to aggregation and is

initiated by thromboxane A2 (TXA2). During the formation of the primary haemostatic plug ADP is released from the platelet and induces further platelet aggregation (15, 16). In addition, many other activation pathways and chemical mediators (PGs and 5-HT) are considered to cause platelet aggregation (17, 18). The antiplatelet activity was tested in the concentration ranges of 50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL for the study. There was 27 % aggregation of platelet against ADP with a dose of 50 µg/mL extracts which gradually decreased (87.49 % for 1000 µg/mL) with an increase in the dose. There was no appreciable change on further increase of the dose. The results on platelet aggregation reflects that the extract could either inhibit PGs synthesis pathway or 5-HT release. The development of inflammation is bi-phasic, the first phase is attributed to the release of histamine, 5-HT and kinins, while, the second phase is related to the release of prostaglandins. Diclofenac sodium that is widely used and selected in this study as positive control, is reported to exert its effect on the prostaglandins biosynthetic pathway and the cyclooxygenase activity of the enzyme (19, 20).

The methanolic extract exhibited relatively higher stabilization effect of HRBC membrane by inhibiting the hypotonicity-induced lyses of erythrocyte membrane at a concentration of 137.64 µg/mL. Though the standard drug and the extract differed in their stabilization effect at low concentrations, it was similar at a concentration of 1mg/mL. The inflammatory responses get limited by preventing the release of lysosomal constituents of activated neutrophils thereby the damage to the tissue is reduced. Some of the non-steroidal anti-inflammatory drugs are known to possess membrane stabilization properties, which may contribute to the potency of their anti-inflammatory effect. The exact mechanism of stabilization of the membrane by the extract is not known, but it can be observed that the osmotic loss of intracellular electrolytes and fluid components was inhibited under induced hemolysis (21).

Although MTT assay is considered a preliminary assay to test the cytotoxicity of the extract, it is usually used to correlate to the anticancer activity of the extract. Increased formation of free radicals, during inflammatory conditions tends to be cytocidal and is generally detected by testing the extent of reduction of the tetrazolium dye by the superoxide generated in the cells. The reduction of MTT by the intracellularly generated superoxide in the presence of succinate in HeLa cells was completely inhibited by the extracts at 500 µg/mL concentration and 50 % inhibition was observed at 141.3 µg/mL. Since the formazan crystals lysed in to the solution correlates to the growth of the cells, the amount of formazan produced is directly proportional to the number of viable cells. Madesh and Subramanian (1997) reported that the inhibition of MTT reduction was linear at low concentrations of Super oxide dismutase with 96% inhibition at high concentration of 192 ng/mL (22).

Conclusion

The methanolic extract of *Plectranthus hadiensis* stem, showed considerable anti-inflammatory and cytotoxic activity compared to the standard NSAID. The data on the IC_{50} values of the extract in the assays studied i.e., BSA denaturation inhibition (134.52 µg/mL), Plate aggregation inhibition (121.84 µg/mL) and HRBC membrane stabilization (137.62 µg/mL) shows that it is a good lead to carry out extensive *in vivo* studies on its anti-inflammatory properties. The activity of the methanolic extract could be attributed to the presence of high content of phenolic acids and further work is in progress in isolating and characterizing the constituents by bioactive guided assays and to understand its effect on the exudative phase of inflammation by *in vivo* studies. Studies on lipid-derived eicosanoids, enzyme expression (COX-2, lipoxygenase) and

cytokines are underway to understand the mechanism of action in relation to the observed anti-inflammatory and cytotoxic activity.

Acknowledgements

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