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Research Article

IN VITRO STUDY OF ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY OF SOME MEDICINAL PLANTS AND THEIR INTERRELATIONSHIP

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ABSTRACT

Objective: The objective of the present work is to study the *in vitro* anti-inflammatory and antioxidant activity of medicinal plants. The extent and correlation between anti-inflammatory and antioxidant activity have been studied.

Method: Methanolic and aqueous extracts of five medicinal plants, namely, *Ficus racemosa*, *Aloe vera*, *Cannabis sativa*, *Datura stramonium,* and *Calotropis gigantean* have been taken for *in vitro* anti-inflammatory and total antioxidant activity.

Result: The study showed that the inhibition of protein (albumin) denaturation was maximum in aqueous extract of *A. vera* with 97.55±1.45%. Proteinase inhibitory action of different plant extracts showed significant action and was found to be maximum in aqueous extract of *D. stramonium* with 87.89±2.58%. Heat-induced hemolysis showed that maximum inhibition was with aqueous extract of *F. racemosa* with 90.72±3.33%. When hypotonicity-induced hemolysis activity was done it was found maximum in methanolic extract of *C. gigantea* with 90.58±3.04%. Anti-lipoxygenase activity was found maximum in methanolic extract of *F. racemosa* with 94.05±4.24%. When total antioxidant activity was done, it was found highest in *F. racemosa* (4.38±0.546 mM equivalent of ascorbic acid/g tissue).

Conclusion: An overall strong positive correlation between anti-inflammatory and antioxidant activity was observed, indicating that antioxidant activity of the plant species studied might be responsible for their anti-inflammatory property. Further work needs to be undertaken to fully elucidate the antioxidants responsible for anti-inflammatory action and to develop better herbal drug formulations.

Keywords: Anti-inflammatory, Antioxidant, Hemolysis, Lipoxygenase, Medicinal plants.

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INTRODUCTION

A free radical is a molecule or atom that carries one or more unpaired electrons and is able to exist independently [1]. Free radicals are short-lived, highly reactive, and unstable as they possess odd number of electrons. Subsequently, to obtain stability, free radicals can react quickly with other elements trying to catch the required electron. Meanwhile, the confronted molecule can become a free radical by losing its electron and start a chain reaction cascade causing damage to the living cell [2]. It was observed that most of the reactive oxygen species are produced in cells through the mitochondrial respiratory chain [1,2]. Lipids and proteins are essential targets for oxidative attack and alteration of these molecules can enhance the mutagenesis process [3,4]. In the inflammatory response, leukocytes and mast cells are present in the damaged areas which are responsible for "respiratory burst" as a result of further uptake of oxygen and therefore boost the release of reactive oxygen species at the damaged area [3,4]. Although inflammatory cells produce more soluble inflammatory mediators such as arachidonic acid, cytokines, and chemokines, that act through end to end at active inflammatory cells in the area of infection and discharge more reactive species. These indispensable markers can rouse signal transduction flows in addition to modifications in transcription factors, such as nuclear factor of activated T cells, signal transducer and activator of transcription 3, nuclear factor kappa B, activator protein-1, NF-E2 related factor-2, and hypoxia-inducible factor-1α, that mediates cellular stress reactions. Initiation of cyclooxygenase-2, inducibility of nitric oxide synthase, and high expression of inflammatory cytokines, including tumor necrosis factor-β, interleukin-1 β, (IL-6), and chemokines, in addition to changes in the expression of specific microRNAs have also been revealed to possess oxidative stress-induced inflammation [5,6]. This inflammatory/oxidative environment activate harmful sphere, that can lead to damaging of healthy stromal cells and adjoining epithelial cells, which may cause carcinogenesis, if delayed [2,5]. The present study is aimed to explain the mode of action and relationship between antioxidant and anti-inflammatory activity of various medicinal plants to develop herbal anti-inflammatory drugs.

METHODS

Plant samples of *Ficus racemosa*, *Aloe vera*, *Cannabis sativa*, *Datura stramonium,* and *Calotropis gigantea* were collected in fresh condition from the herbal garden at Amity University, Uttar Pradesh, Lucknow, India. The plant material was thoroughly washed with tap water followed by distilled water. For drying washed tissues were dried on blotting paper and spread out at room temperature in the shade. Shade-dried materials were ground to fine powder with tissue blender. The extracts were prepared according to Tiwari *et al*. [7] with few modifications. Two types of solvents, namely, methanol and water were used to prepare a variety of extracts from the plant samples. The extracts were filtered through Whatman filter paper and evaporated till dryness. The dried extracts were dissolved in 1mg/ml stock solution with methanol and water, respectively, filtered with 0.45 µm syringe filter for analysis. The stock solutions were diluted to different concentrations of 500 µg/ml, 400 µg/ml, 300 µg/ml, 200 µg/ml, and 100 µg/ml for further analysis.

Assessment of *in vitro* anti-inflammatory activity

Inhibition of albumin denaturation

The anti-inflammatory activity of methanolic and aqueous extracts of *F. racemosa*, *A. vera*, *C. sativa*, *D. stramonium,* and *C. gigantean* were studied

according to the protocol of Mizushima *et al*. and Sakat *et al*. [8,9] with some modifications. Inhibition of albumin denaturation was done according to the protocol. The reaction mixture consists of an equal volume of test extracts of different concentrations (100–500 µg/ml) and 1% aqueous solution of bovine albumin (Fraction V). The pH of the reaction mixture was adjusted using a small amount of 1N HCl. The sample extracts were incubated at 37°C for 20 min and then heated to 51°C for 20 min. The absorbance was measured after cooling the samples at room temperature. The turbidity formed was measured at 660 nm using ultraviolet (UV)-visible spectrophotometer (Model: Shimadzu UV-1800). The percentage inhibition of protein denaturation was calculated as follows:

$$\%\text{inhibition} = \frac{\text{Abs control -Abs sample}}{\text{Abs control}} \times 100$$

Antiproteinase action

The activity was performed by the protocol of Sakat *et al*. and Oyedepo *et al*. [9,10] with some modifications. The reaction mixture (2 ml) containing 0.001% trypsin, 1 ml of 1mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100–500 µg/ml). The mixture was incubated at 37°C for 5 min and then 1 ml of 0.02% (w/v) casein was added. The mixture was incubated for an additional 20 min at 37°C. 2 ml of 2% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The percentage inhibition of proteinase inhibitory activity was calculated.

$$\%\text{inhibition} = \frac{\text{Abs control -Abs sample}}{\text{Abs control}} \times 100$$

Membrane stabilization

Preparation of red blood cells (RBCs) suspension [9,11]. The blood was obtained from a healthy human volunteer who had not taken any nonsteroidal anti-inflammatory drugs for 2 weeks before the experiment. The blood sample was centrifuged at 3000 rpm for 10 min and was washed 3 times with equal volume of normal saline. The amount of blood was measured and re-constituted as 10% v/v in normal saline suspension.

Heat-induced hemolysis

The activity was done according to the protocol of Sakat *et al*. and Shinde *et al*. [9,12] with some modifications. The reaction mixture (2 ml) comprised 1 ml test sample at different concentrations (100–500 µg/ml) and 10% RBCs suspension (1 ml). For control, only saline was added to the test tube. Aspirin as a reference was used. The reaction mixture was incubated in water bath for 30 min, at 56°C. The reaction was stopped by cooling the reaction mixture under running tap water. The reaction mixture was then centrifuged at 2500 rpm for 5 min. The supernatant collected was used to take absorbance at 560 nm. The experiment was performed in triplicates. The percentage inhibition of hemolysis was calculated as follows:

$$\%\text{inhibition} = \frac{\text{Abs control -Abs sample}}{\text{Abs control}} \times 100$$

Hypotonicity-induced hemolysis

Hypotonicity-induced hemolysis was done according to the protocol of Azeem *et al*. [13] with some modifications. Extracts of different concentration (100–500 µg/ml), reference sample (diclofenac sodium 100 µg/ml), and control were individually mixed with phosphate buffer pH 7.0 (1 ml), hyposaline (2 ml), and RBC suspension (0.5 ml). The reaction mixtures were incubated at 37°C for 30 min. It was then centrifuged at 3000 rpm. The supernatant was transferred, and absorbance was taken at 560 nm. The percentage hemolysis was calculated by assuming control as 100%.

$$\%\text{protection} = 100 - \frac{\text{OD sample}}{\text{OD control}} \times 100$$

Anti-lipoxygenase activity

Anti-lipoxygenase activity was studied with minor modifications, using linoleic acid as substrate and lipoxidase as an enzyme [12]. Test samples were dissolved in 2M borate buffer pH 9.0 (0.25 ml) and (0.25 ml) lipoxidase enzyme solution (20,000 U/ml). The reaction mixture was incubated for 5 min at 25°C. Then, 0.6 mM lenoleic acid solution (1.0 ml) was added. The reaction mixture was vortexed, and absorbance was measured at 234 nm. Indomethacin as a reference was used. The percent inhibition was calculated from the following equation:

$$\%\text{inhibition} = \frac{\text{Abs control -Abs sample}}{\text{Abs control}} \times 100$$

Total antioxidant activity

Total antioxidant activity was assayed by the protocol of Cacig *et al*. and Iqbal *et al*. [14,15] with some modifications. 1.0 g of tissue was homogenized in 4 ml of double distilled water and incubated for 24 h at 4°C. It was then filtered twice with Whatman No. 1 filter paper and the filtrate collected was stored at 4°C. Suitable amount of sample was taken in a 3 ml glass cuvette containing the oxidative mixture of 0.18 ml potassium permanganate (0.01 M); 0.42 ml sulfuric acid (2M) and distilled water to make up volume up to 3 ml. The decrease in absorbance was measured at 535 nm. Ascorbic acid was taken as standard. Readings were taken in triplicaste and the mean activity (± standard deviation) was calculated and used for plotting the graph.

To quantitatively compare the antioxidant activities, the following formula was used:

$$A50 = t\,\text{standard} \cdot \frac{C\,\text{standard}}{t\,\text{plant sample}} \cdot \frac{V\,\text{standard}}{m\,\text{plant}} \cdot \frac{V\,\text{extract}}{V\,\text{plant sample}}$$

Where:

A50 - antioxidant activity expressed, reproduced in the time until the sample makes a decrease of the oxidizing agent (potassium permanganate) concentration up to one half, compared against a standard (ascorbic acid) (mM equivalent standard/gram plant).

t plant sample - the time until the sample induces a decrease of the potassium permanganate concentration up to one half (min).

t standard - the time till the standard (ascorbic acid) induces a decline of the potassium permanganate concentration up to one half (min) (0.66 min as seen in standard curve).

C standard - standard (ascorbic acid) concentration (mM/mL) (0.01 mM/mL).

m plant - weight (gram) of the plant sample subjected to extraction (1 g).

V plant sample - volume of the plant extract subjected to the analysis (0.1 ml).

V standard - volume of the standard subjected to the analysis (1 ml).

V extract - volume (mL) of the obtained extract (4 ml).

Statistical analysis

Results are expressed as mean ± standard deviation. The difference among experimental sets was compared by two-way analysis of variance (ANOVA). Correlation was established between total antioxidant and different anti-inflammatory activities.

RESULTS AND DISCUSSION

There are many methods to estimate the anti-inflammatory action of drugs. At present, a comparative study has been done on a methanolic and aqueous extract of five different medicinal plants species for anti-

inflammatory activity. Primarily the inhibition of protein (albumin) denaturation was studied and was found the maximum in aqueous extract of *A. vera* with 97.55±1.45%, while minimum in aqueous extract of *C. gigantea* with 47.80±1.75%. Results are represented in Fig. 1. In a similar study by Reshma *et al*. [16] plant extracts of *Aegle marmelos* and *Ocimum sanctum* were effective in inhibiting heat-induced albumin denaturation and were observed 95.64 µg/mL and 42.17 µg/mL, respectively. In a comparative study, *A. vera* was found to be marginally more active in inhibition of protein (albumin) denaturation than *Citrus sinensis*. It may be due to the higher flavonoids contents of *A. vera* [17].

The proteinase inhibitory action of different plant extract showed significant action. It was found the maximum in aqueous extract of *D. stramonium* with 87.89±2.58%, and minimum in aqueous extract of *F. racemosa* with 2.95±0.57% (Fig. 2). Similarly, Duraid [18] found *D. stramonium* as a potent anti-inflammatory plant. Studies on other plants also revealed significant proteinase inhibitory action. The aqueous extract of leaves of *A. marmelos* and *O. sanctum* exhibited significant antiproteinase activity. Maximum inhibition was observed in *A. marmelos* leaf extract. *A. marmelos* and *O. sanctum* were observed as 74.45 µg/mL and 49.70 µg/mL, respectively [16]. It was earlier investigated that leukocyte proteinase plays a significant part in the enhancement of tissue damage during inflammatory reactions and substantial level of defense was delivered by proteinase inhibitors [19].

The extract was also effective in reducing the heat-induced hemolysis at various concentrations. The results showed that maximum inhibition was with aqueous extract of *F. racemosa* with 90.72±3.33% while minimum in methanolic extract of *C. sativa* with 2.94±0.48% (Fig. 3). All the extracts were effectively inhibiting the heat-induced hemolysis involving stabilization of RBC membrane. Heat-induced hemolysis of HRBC by methanolic extract of *Ficus virens* is effective with maximum inhibition of 64% at 200 µg/ml [20]. A similar study by Govindappa *et al*. [21] also showed effective inhibition of heat-induced hemolysis by ethanolic extract of *Wedelia trilobata*. The maximum inhibitions were 78.11% from leaf extract followed by the stem (74.17%) and flower (58.74%).

The hypotonicity-induced hemolysis activity was found to be maximum in methanolic extract of *C. gigantea* with 90.58±3.04%, while minimum in aqueous extract of *C. sativa* 8.29±0.88% (Fig. 4). Saumya *et al*. [22] reported *C. gigantea* to possess anti-inflammatory activity on Wistar

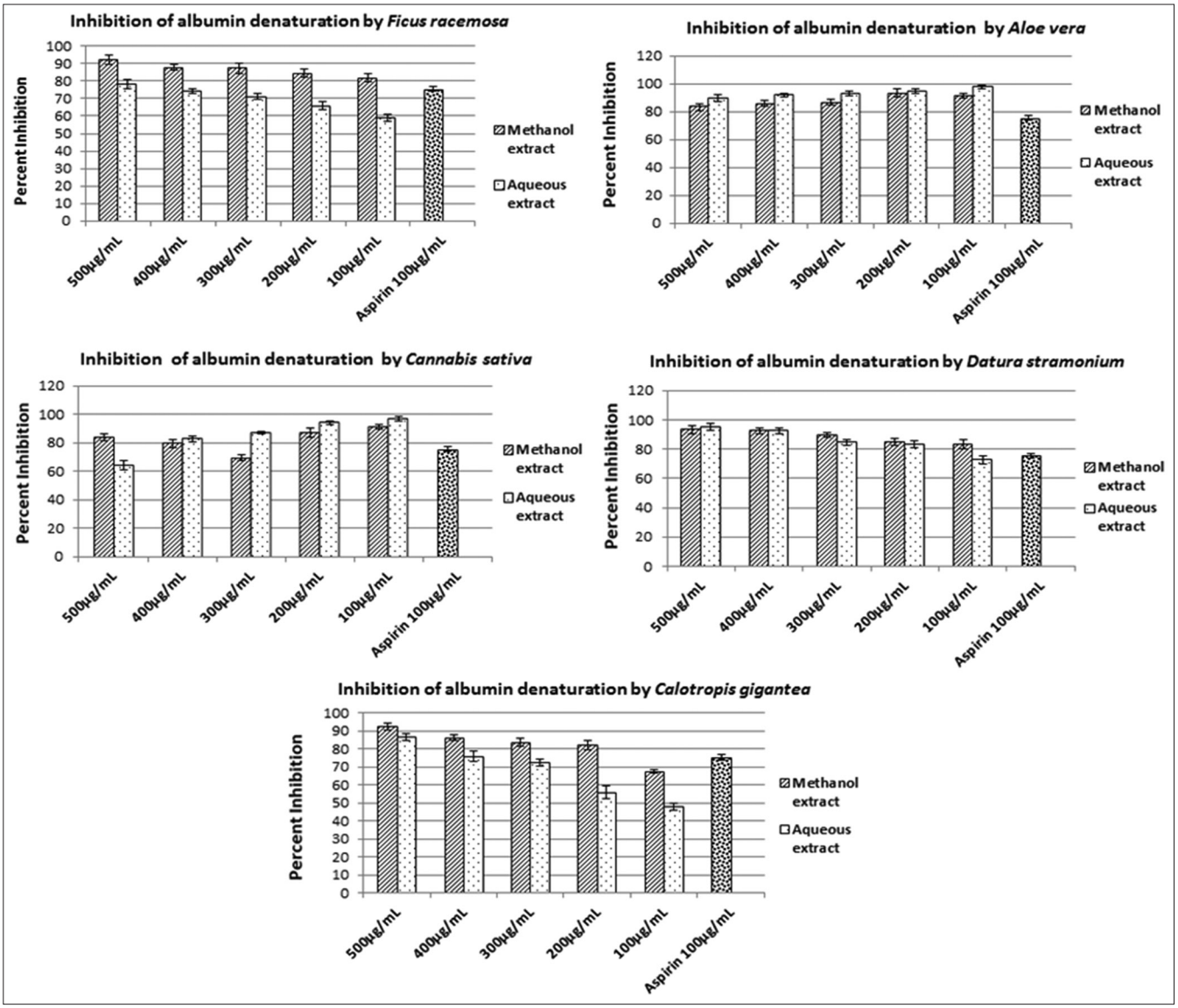

Fig. 1: Inhibition of protein (albumin) denaturation by different methanolic and aqueous extracts of medicinal plants. Two-way analysis of variance between methanolic and aqueous extracts confirms significant variation with p>0.05 (*Ficus racemosa* - 0.005, *Aloe vera* - 0.0009 *Cannabis sativa* - 0.2459, *Datura stramonium* - 0.2356, and *Calotropis gigantean* - 0.0149)

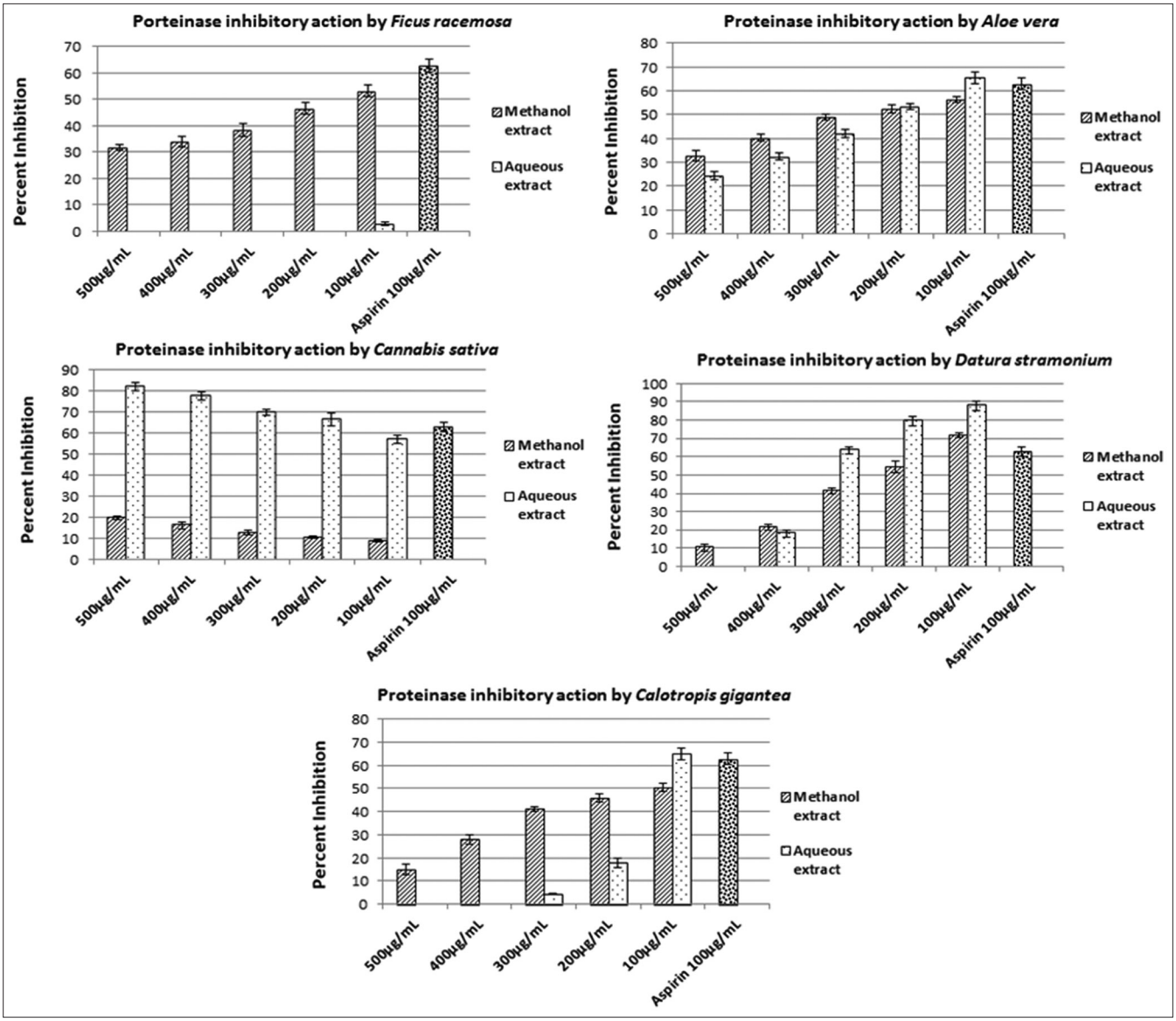

Fig. 2: Proteinase inhibitory action by different methanolic and aqueous extracts of medicinal plants. Two-way analysis of variance between methanolic and aqueous extracts confirms there is no significant variation with p<0.05 (*Ficus racemosa* - 0.0003, *Aloe vera* - 0.4931, *Cannabis sativa* - 2.26, *Datura stramonium* - 0.2311, and *Calotropis gigantean* - 0.1052)

albino rats. Antioxidant potential of *C. gigantea* was also reported by Iqbal *et al*. [23], affirming it to be a virtuous source of antioxidant. Leelaprakash and Mohan [24], also showed that *Enicostemma axillare* at a concentration range of 200–500 µg/ml protect hypotonicity-induced hemolysis of the erythrocyte membrane significantly against lysis induced by hypotonic solution. At the concentration of 500 µg/ml, *E. axillare* exhibited maximum 75% of protection.

Anti-lipoxygenase activity was found to be maximum in methanolic extract of *F. racemosa* with 94.05±4.24%. On the other hand, minimum activity was observed in aqueous extract of *A. vera* with 8.95±0.92% of inhibition, Fig. 5. *F. racemosa* is reported as an effective anti-inflammatory plant. The extract at 200 and 400 mg/kg doses has been found to have noteworthy anti-inflammatory activity on the experimental models [25]. Anoop and Bindu [26], who studied the effects of *Syzygium zeylanicum* leaf extracts on production, of leukotrienes, were estimated by the inhibition of lipoxygenase activity. The anti-inflammatory activity of *F. racemosa* extract was estimated on rat hind paw edema models; the results were found satisfactory with 79.26% in ethyl acetate extract and 77.65% in aqueous extract. In another study anti-lipoxygenase activity using *Momordica charantia* extract and protein free *M. charantia* extract at different time intervals, pH, temperature and different extract concentration was observed and found to be same for both the cases, confirming phytochemicals present in the extract are responsible for the activity, rather than protein [27].

Total antioxidant activity (established on the redox reactions between the antioxidant sample and the $KMnO_4$ in acidic (H_2SO_4) media, resulting in discoloration of $KMnO_4$) was recorded highest in *F. racemosa* (4.38±0.546 mM equivalent of ascorbic acid/g tissue) at 500 µL of sample used, while lowest in *A. vera* (0.620±0.030 mM equivalent of ascorbic acid/g tissue) at 500 µL of sample used (Fig. 6). In a study by Johora *et al.* [28], the antioxidant activity of methanolic extract of *F. racemosa* was studied. It was found that the scavenging activity of *F. racemosa* from leaves and bark extracts was 48.54701% and 73.46154%, respectively. A number of studies have been done on antioxidant properties from different plant part (mainly fruits) of *F. racemosa* are also reported [29,30].

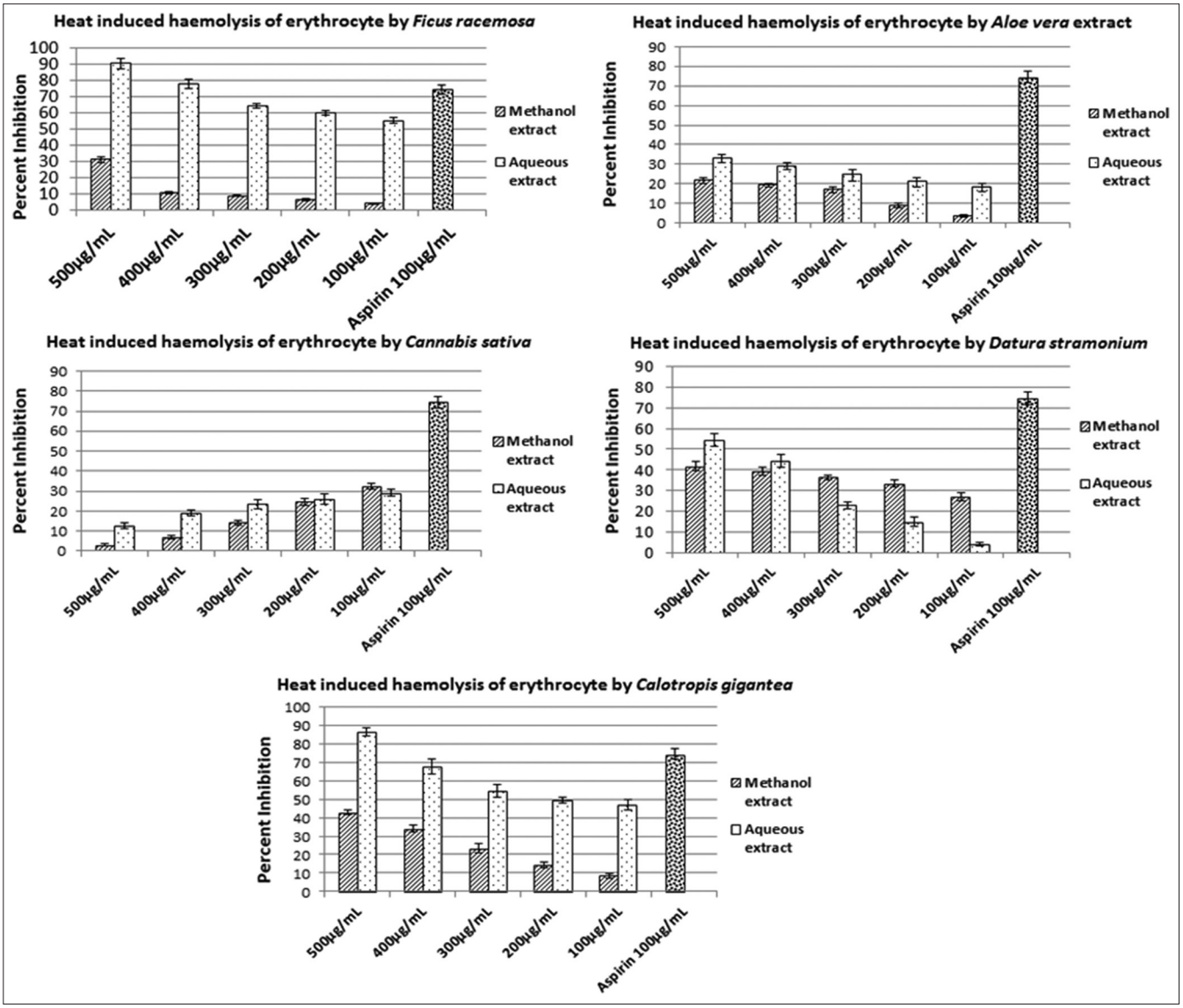

Fig. 3: Heat-induced hemolysis of erythrocyte by different methanolic and aqueous extracts of medicinal plants. Two-way analysis of variance between methanolic and aqueous extracts confirms there is no significant variation with p<0.05 (*Ficus racemosa* - 3.44, *Aloe vera* - 0.0007, *Cannabis sativa* - 0.1102, *Datura stramonium* - 0.3445, and *Calotropis gigantean* - 7.30)

Antioxidant compounds originating from plants can reduce the formation of free radicals [31,32], and can lessen the diseases initiated by oxidative stress [33,34]. The flavonoids and phenolics compounds obtained from remedial herbs account for the antioxidative property of plants [31,35,36] and thus act as an anti-inflammatory mediator [37]. Production of pro-inflammatory biomolecules such as nitric oxide and tumor necrosis factor (TNF-α) can lessen inflammation. These inflammatory biomolecules respond with free radicals, which can be a cause of irreparable mutilation to cell membranes, causing tissue damage and cell death [38].

Furthermore, a strong positive correlation was observed between various anti-inflammatory activities and antioxidant activity (Table 1), which shows the relationship among the two factors. In a study by Chohan *et al*. [39] on culinary herbs, the relationship was found between their anti-inflammatory activity, polyphenol content, and antioxidant capacity. In the study, anti-inflammatory activity of cooked, uncooked, cooked and *in vitro* digested, and standardized (30mg/mL) culinary herbs was evaluated by determining their influence on IL-8 release from Caco-2 cells and stimulated human peripheral blood lymphocytes. The Trolox equivalent capacity and estimated total phenolic content of the herbs were also determined. The substantial correlations between Trolox equivalent capacity and phenolic content and anti-inflammatory activity suggest a possible contributory role of polyphenols to the anti-inflammatory activity of the culinary herbs scrutinized, indicating a relationship between anti-inflammatory activity and antioxidant level.

CONCLUSION

Many of the analgesics drugs are known for their side-effects such as gastrointestinal and severe gastric disorders. Anti-inflammatory drugs comprise "biologicals" such as anticytokine therapies, which resist the activities of kinases and express a considerable decline in host resistance for infections. Due to health problems and side effects of existing anti-inflammatory drugs, naturally occurring anti-inflammatory supplements (plants) are now becoming popular. In recent years, various experimental techniques are employed to verify natural antioxidant and anti-inflammatory drugs from natural product resources. The present work is an attempt in the direction of anti-inflammatory drug development for several chronic inflammatory and allied diseases. It can be concluded that the plant species studied

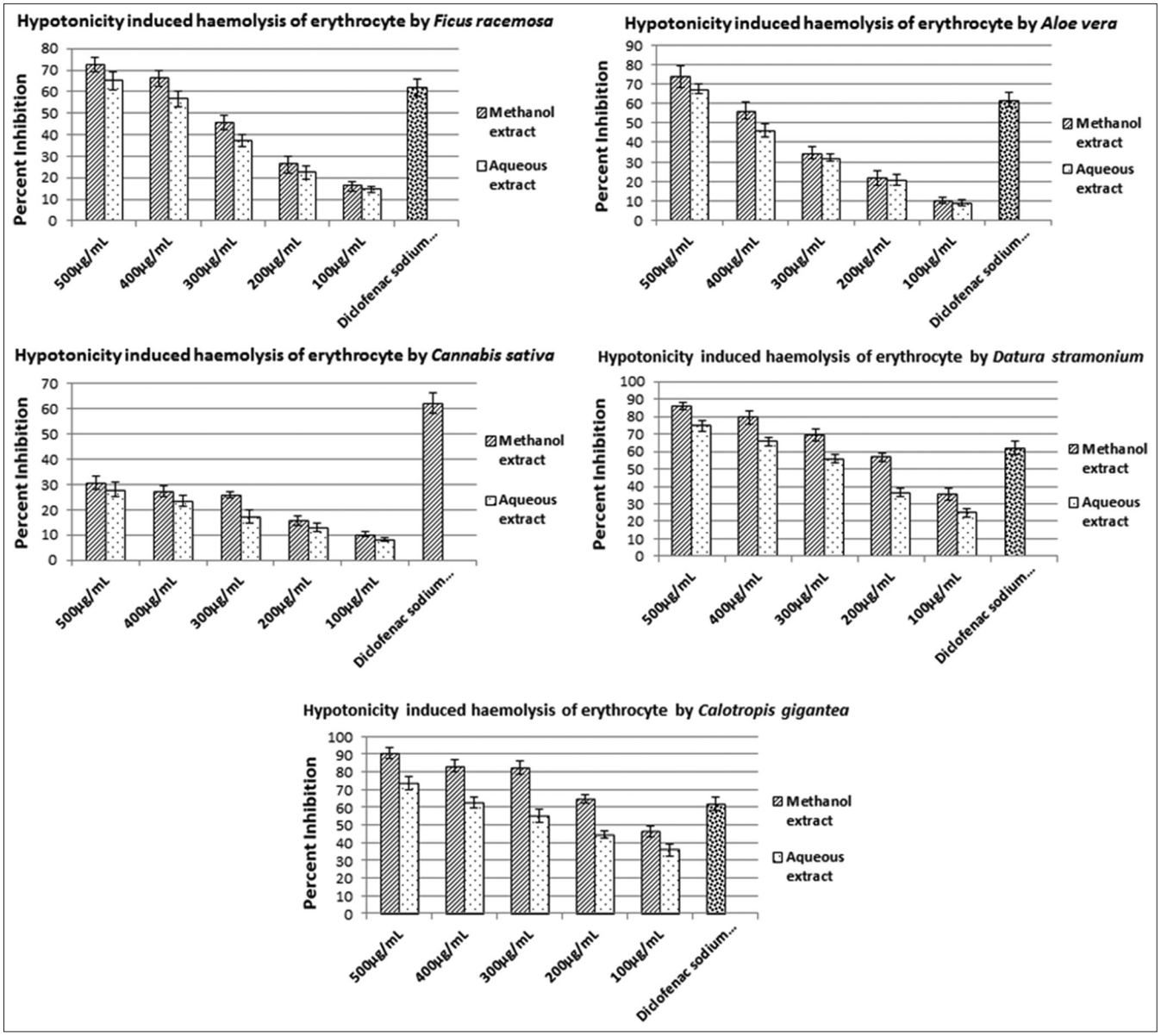

Fig. 4: Hypotonicity-induced hemolysis of erythrocytes by different methanolic and aqueous extracts of medicinal plants. Heat-induced hemolysis of erythrocyte by different methanolic and aqueous extracts of medicinal plants. Two-way analysis of variance between methanolic and aqueous extracts confirms significant variation with p>0.05 (*Ficus racemosa* - 0.0143, *Aloe vera* - 0.0716, *Cannabis sativa* - 0.0267, *Datura stramonium* - 0.0012, and *Calotropis gigantean* - 0.0022)

Table 1: Correlation matrix between *in vitro* anti-inflammatory activity and antioxidant activity of plants used in the study

Assessment of *in vitro* anti-inflammatory activity	Antioxidant activity of *F. racemosa*		Antioxidant activity of *A. vera*		Antioxidant activity of *C. sativa*		Antioxidant activity of *D. stramonium*		Antioxidant activity of *C. gigantea*	
	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract
Inhibition of albumin denaturation	0.9709	0.9540	0.9847	0.9912	0.9222	0.8883	0.9561	0.9352	0.8864	0.9642
Antiproteinase action	0.9966	0.7851	0.9432	0.9973	0.9998	0.9660	0.9901	0.9320	0.9253	0.9115
Heat-induced hemolysis	0.9041	0.9939	0.9300	0.9955	0.9970	0.9470	0.9362	0.9885	0.9955	0.9811
Hypotonicity-induced hemolysis	0.9725	0.9853	0.9977	0.9977	0.9263	0.9898	0.9325	0.9659	0.8902	0.9888
Anti-lipoxygenase activity	0.9097	0.9581	0.9930	0.9732	0.9972	0.9924	0.9694	0.9835	0.9054	0.9923

F. racemosa: Ficus racemosa, A. vera: Aloe vera, C. sativa: Cannabis sativa, D. stramonium: Datura stramonium, C. gigantean: Calotropis gigantean

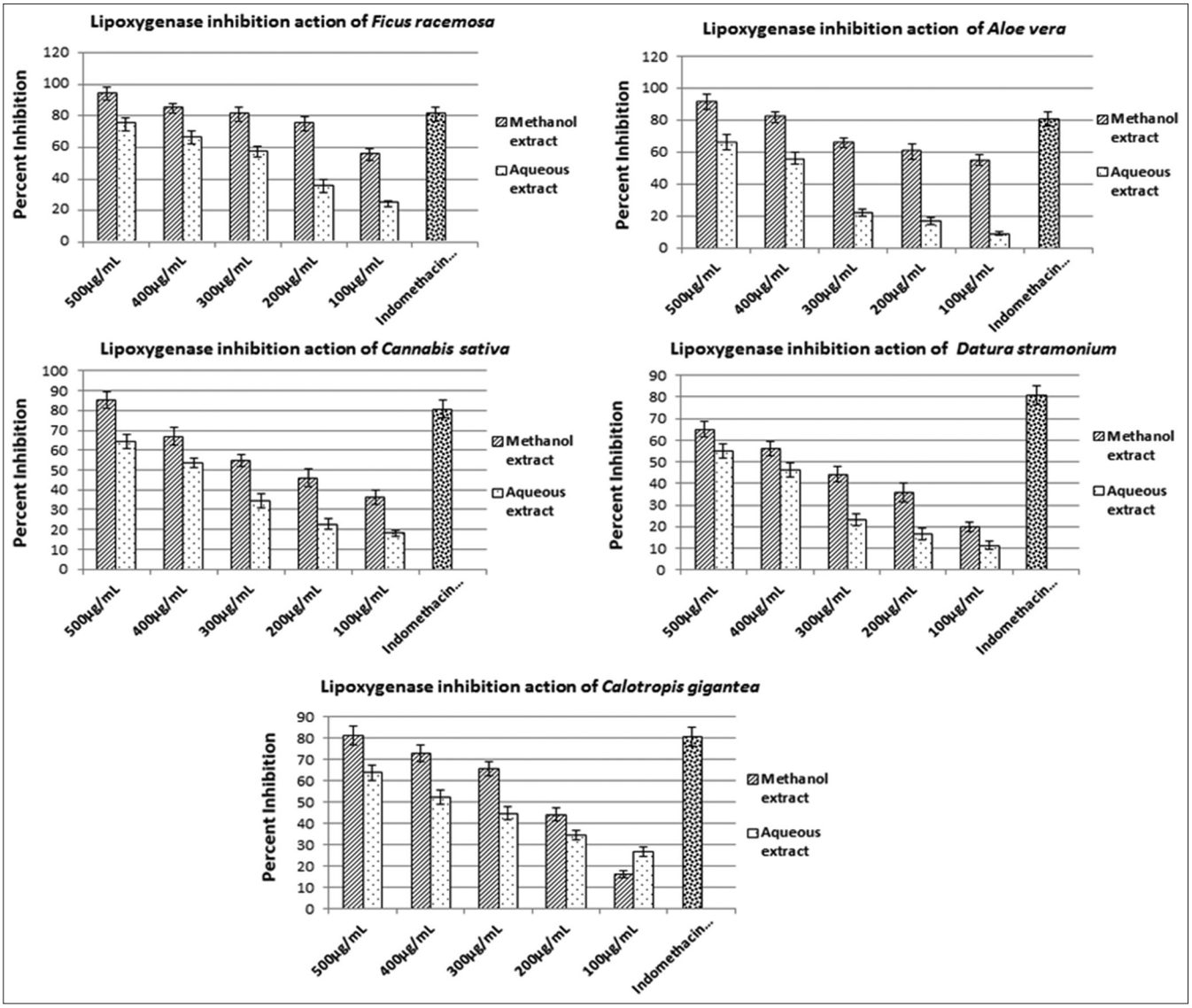

Fig. 5: Lipoxygenase inhibition action by different methanolic and aqueous extracts of medicinal plants. Two-way analysis of variance between methanolic and aqueous extracts confirms significant variation with p>0.05 (*Ficus racemosa* - 0.0025, *Aloe vera* - 0.0013, *Cannabis sativa* - 0.0003, *Datura stramonium* - 0.0065, and *Calotropis gigantean* - 0.1244)

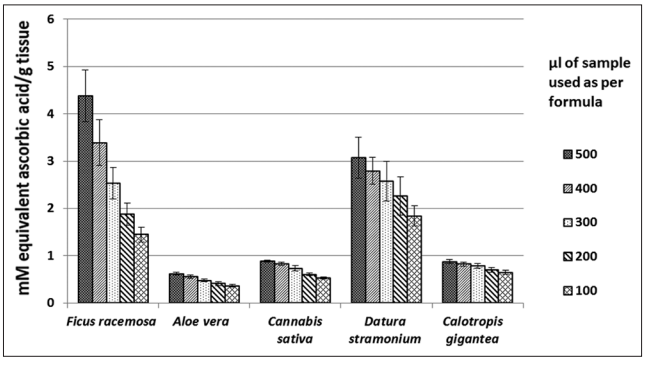

Fig. 6: Total antioxidant activity of different medicinal plants

possess significant anti-inflammatory and antioxidant activity. Their anti-inflammatory activity may be due to the antioxidants present in the plants. The correlation established between both the activities in the study establishes their relationship. Further work is required to fully elucidate the antioxidants responsible for this action and their mechanisms of action, for future herbal drug formulations.

AUTHOR'S CONTRIBUTION

All the authors contributed equally in planning, conductance of study, interpretation of results and writing.

CONFLICTS OF INTERESTS

Authors do not have any conflicts of interests to declare.

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