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COX and LOX inhibitory potential of *Abroma augusta* and *Desmodium gangeticum*

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Abstract

The present study was aimed to evaluate the cyclooxygenase (COX) and lipoxygenase (LOX) inhibitory activity of *Abroma augusta* (AA) and *Desmodium gangeticum* (DG). Initially In-vivo anti-inflammatory activity of aqueous extract (AqE) of aerial parts of DG (100 mg/ml) and petroleum ether extract (PEE) of roots of AA (250 mg/ml) was assessed in carrageenan induced paw oedema model in albino wistar rats. In addition, each plant extract was evaluated for COX-1, COX-2 and LOX inhibitory activity to determine the possible mechanism of their anti-inflammatory activity. The results of the study demonstrated that each plant extract significantly ($p < 0.0001$) reduces the paw volume compared with standard drug Ibuprofen (100 mg/kg b.w.). The percentage inhibitory activity of AqE of DG against COX-2 ($IC_{50} = 39.5 \mu\text{g/ml}$) were generally higher to that of COX-1 ($IC_{50} = 49.5 \mu\text{g/ml}$), however, at the same concentrations PEE of AA demonstrated inhibitory activity against COX-1 ($IC_{50} = 36.5 \mu\text{g/ml}$) to a greater extent than COX-2 ($IC_{50} = 59 \mu\text{g/ml}$). Moderate inhibition of LOX activity was demonstrated by DG ($IC_{50} = 57.0 \mu\text{g/ml}$). AA exhibited weak inhibitory action on LOX activity at the same concentrations ($IC_{50} = 75.5 \mu\text{g/ml}$). The results of the study concluded that anti-inflammatory activity of plant extracts could be due to inhibition of COX and LOX enzymes and thus supports the traditional use of the above mentioned plants in inflammatory disorders.

Keywords: *Desmodium gangeticum*, *Abroma augusta*, Inflammation, Cyclooxygenase, Lipoxygenase.

Introduction

Inflammation is an orchestrated biological process, induced by microbial infection or tissue injury.¹ Inflammatory diseases include different types of rheumatic disorders such as rheumatic fever, rheumatoid arthritis, ankylosing spondylitis, polyarthritis nodosa, systemic lupus erythematosus and osteoarthritis.² It is a body defense reaction in order to eliminate or limit the spread of injurious agent. There are various components of an inflammatory reaction that can contribute to the associated symptoms and tissue injury. Oedema, leukocyte infiltration, and granuloma formation represent such components of inflammation.³

The mechanisms of inflammation involve a series of events in which the metabolism of arachidonic acid plays an important role.⁴ Two cyclooxygenases (constitutive COX-1 and inducible COX-2) and lipoxygenase (5-LOX) enzymes are responsible for the transformation of arachidonic acid into the potent biologically active lipid mediators that are intimately involved in inflammation.⁵ COX catalyzes the first key steps in the synthesis of all the prostaglandins (PGs) by converting arachidonic acid into PGH₂.

Thus, COX is a bifunctional enzyme exhibiting both cyclooxygenase (from arachidonic acid to PGG₂) and peroxidase (from PGG₂ to PGH₂) activities. COX-1 is constitutively expressed as a housekeeping enzyme in nearly all the tissues and mediates physiological responses (e.g., cytoprotection of the stomach, platelet aggregation). On the other hand, COX-2 is expressed by cells that are involved in inflammation and has emerged as the isoform primarily responsible for the synthesis of prostanoids involved in acute and chronic inflammatory states of pathological processes. Inhibition of COX-2-dependent PG synthesis accounts for the anti-inflammatory and analgesic effects of NSAIDs, whereas suppression of COX-1 can lead to many unwanted side effects (e.g., gastrointestinal ulceration and bleeding, platelet dysfunctions).^{6,7}

Lipoxygenases (LOX) enzymes are correlated with inflammatory and allergic reactions because of the formation of the leukotrienes (LTs). High levels of LTs could be observed in the case of asthma, psoriasis, allergic rhinitis, rheumatoid arthritis and colitis ulcerosa. The production of LTs can be prevented via inhibition of the lipoxygenase pathway. Lipoxygenase inhibitors may lead to the design of biologically and pharmacologically targeted therapeutic strategies inhibiting L isoforms and/or their biologically active metabolites which may be useful in cancer treatment.⁸

An array of drugs is available in the market to treat inflammatory disorders, but only very few are free from toxicity. Gastrointestinal problems associated with the use of anti-inflammatory drugs are still an enduring dilemma of the medical world. It is very important that profound research with ethnobotanical plants possessing anti-inflammatory and analgesic properties can definitely open up new vistas in inflammatory disorders. Purified natural compounds from plants can serve as a template for the synthesis of new generation anti-inflammatory drugs with low toxicity and higher therapeutic values.² Various herbal medicines derived from plant extracts are being used in the treatment of a wide variety of clinical diseases, though relatively little knowledge about their mechanisms of action is known. Many herbal preparations are being prescribed widely for the treatment of inflammatory conditions. There is a need for research and developmental work in herbal medicine because apart from the social and economic benefits, it has become a persistent aspect of present day healthcare in developing countries.⁹

Desmodium gangeticum (L.) DC (Family Fabaceae) is a perennial non-climbing herb or shrub widely distributed in tropical and sub-tropical habitats and particularly abundant in India. It is known as Sarivan in Hindi and Shalaparni in Sanskrit. It has been extensively used in Indian system of medicine as a bitter tonic, febrifuge, digestive, anticatarrhal, antiemetic, in inflammatory conditions of the chest and other inflammatory conditions due to 'vata' disorders.^{10, 11} *Abroma augusta* Linn (Sterculiaceae), commonly known as Ulatkambal (Bengali and Hindi) is a large spreading bushy shrub with fibrous bark and irritant hairs, widely distributed (native or cultivated) throughout the hotter part of India- U.P., Sikkim (3000 ft.), Khasia hills (4000 ft.) and Assam. The fresh viscid sap of the root bark is considered to be a valuable emmenagogue and uterine tonic. It is also used in dysmenorrhoea.¹²

The present study was designed to investigate the anti-inflammatory activity of AqE and PEE of DG and AA respectively, and to evaluate the COX and LOX inhibitory potential of both the extract to contribute to an understanding of their possible mechanism in treatment of inflammatory disorders.

Materials and Methods

Chemicals

All the chemicals used in the present study were of analytical grade. Carrageenan was supplied by High Media laboratories Pvt., Ltd., Mumbai, India. Mercury was purchased from the Central Drug Home Company, New Delhi. Ibuprofen (API) was gifted by Ranbaxy lab, Poanta Sahib, (HP). COX inhibition was measured by using a colorimetric human COX inhibitor screening assay kit (Cayman Ann Arbor, MI, USA). The contents of the kit (for COX) are Prostaglandin screening EIA antiserum, Prostaglandin Screening AChE Trace, Prostaglandin Screening EIA Standard, EIA Buffer, Wash Buffer, Mouse Anti-rabbit IgG Coated Plate, Plate Cover, Elliman's Reagent, Reaction Buffer, COX-1 (ovine), COX-2 (human recombinant), Heme, Arachidonic Acid (AA), Potassium Hydroxide, Hydrochloric Acid, Stannous Chloride. For LOX assay, Soybean lipoxygenase (1.13.11.12) type I-B and linoleic acid were purchased from Sigma (St. Louis, MO, USA).

Experimental Animals

Albino Wistar rats of either sex weighing about 150-200 gm., Obtained from Shri Guru Ram Rai Institute of Technology and Science, Patel Nagar, Dehradun (Uttarakhand), were selected for the experiment. The animals were kept under standard conditions of 12:12 h light and dark cycle in polypropylene cages and fed with standard laboratory diet and water ad libitum. The animals were acclimatized to laboratory condition for seven days before commencement of the experiment. The Institutional Animal Ethical Committee (IAEC), SGRRITS, Dehradun reviewed the entire animal protocols prior to conducting the experiments (Regd. No. 264/CPCSEA).

Plant Material

The crude drugs of DG (aerial parts) and AA (roots) were collected from the local herbal garden of Dehradun, Uttarakhand. These crude drugs were authenticated at Forest Research Institute of India (FRI), Dehradun. The voucher specimens (No.157028 and 157029 respectively) of the plants were deposited in the Forest Research Institute herbarium. Soon after authentication collected parts of the plants were shade dried until they were free from moisture and were ground to coarse powder.

Acute Toxicity Study

Acute oral toxicity study for the test extracts of the plants were carried out using OECD/OCED guideline 425.¹³ The test procedure minimizes the number of animals required to estimate the oral acute toxicity. The test also allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

Healthy albino Wistar rats (200–250 g) were used for this study. Animals were fasted (food but not water was withheld overnight) prior to dosing. The fasted body weight of each animal was determined, and the dose was calculated according to the body weight.

Limit test at 2000 mg/kg

The drug (each plant extract) was administered in the dose of 2000 mg/kg body weight orally to one animal. A total of six animals were tested. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter, for a total of 14 days. No animal died. Therefore, the LD₅₀ is greater than 2000 mg/kg.

An investigation with 1/20th, 1/10th, and 1/5th of 2000 mg/kg, i.e. 100, 200, and 400 mg was done in pre-screening. 100 mg/kg of AqE of DG and 250 mg/kg of PEE of AA (slightly more than 200 mg/kg) were found to be effective against inflammation.

Effect of Plant Extract in Carrageenan Induced Inflammation

Induction of Inflammation

Anti-inflammatory activity of the plant extracts was determined by the method of Winter, C.A.¹⁴ The rats weighing 150-200 g were divided into five groups, and each group consisting of six animals. Group-1: Normal control, Group-2: Inflammatory control, Group-3: Inflammatory control + Ibuprofen, Group-4: Inflammatory control + PEE of AA, Group-5: Inflammatory control + AqE of DG. Paw edema was induced by s.c. injection of 0.1 ml of freshly prepared 1% carrageenan (in 0.3% CMC) suspension into the subplantar region of left hind paw of each rat after one hour administration of standard drug (Ibuprofen) and test drugs (AA and DG). After administration of carrageenan, animals were deprived of water during experiments to ensure uniform hydration and to minimize variability in edematous response. The measurement of the paw volume was done on the principle of volume displacement using plethysmometer. The readings were taken before and at 0, 30, 60, 120 and 180 min intervals after the administration of carrageenan injection for a period of 3 hours. The oedema at each time was calculated in relation to the paw volume before the injection of the carrageenan. The anti-inflammatory activity of AA and DG was determined as the percentage of inhibition of inflammation after it was induced by carrageenan by taking volume of inflammation in inflammatory control groups.

The percentage inhibition was calculated by using the formula:

$$\% \text{ Inhibition} = \frac{\text{Mean paw inflammation of inflammatory control} - \text{Mean paw inflammation of test}}{\text{Mean paw inflammation of inflammatory control}} \times 100$$

COX inhibitory activity of plant extracts

The ability of the test compound to inhibit COX-1 (ovine) and COX-2 (human recombinant) was determined by using enzymes immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA) according the manufacturer's

instructions. AqE of DG and PEE of AA (10, 50 and 100 µg/ml) were used for inhibition studies as per manufacturer's protocol. The absorbance at 415 nm was read by using a microtitre plate reader.

LOX inhibitory activity of plant extracts

Inhibition studies in presence of various concentrations of both the plant extracts (10, 50 and 100 µg/ml) and reference compound viz., quercetin (10, 50 and 100 µg/ml) was recorded at 234 nm using UV-Vis spectrophotometer.⁸ In assay protocol, 160ml of 100 mM sodium phosphate buffer (pH 8.0), 10 µl of test samples and 20 µl of soybean lipoxygenase solution (167 U/ml) were mixed and incubated at 25°C for 10 min. The reaction was then initiated by the addition of 10 µl of the substrate in the form of sodium linoleic acid solution. The enzymatic conversion of sodium linoleic acid to form (9Z, 11E)-(13S)-13-hydroperoxyoctadeca-9,11-dienoate was measured by monitoring the change of absorbance at 234 nm over a period of six min using UV-vis spectrophotometer. Another reaction mixture (a negative control) was prepared by replacing 10 µl samples with 2.47 ml mixture of sodium phosphate buffer (5 ml) and DMSO (25 µl) into the quartz. All the reactions were performed in triplicates. The percentage of inhibition was calculated as:

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

The IC₅₀ value was determined by graphical method.

Statistical Analysis

Results of anti-inflammatory activity were expressed as the mean ± Standard error mean (SEM). Data was analyzed by

using two way ANOVA followed by tukey's multiple comparison as post-hoc test. The limit of statistical significance was set at P<0.05.

Results

Acute toxicity study

Acute toxicity studies conducted revealed that the administration of each plant extract (up to a dose of 2000 mg/kg) did not produce significant changes in behavior of the animals. No death was observed up to the dose of 2000 mg/kg b.w. The rats were physically active. These effects were observed during the experimental period (14 days). The results showed that in single dose the plant extracts had no adverse effect, indicating that the medium lethal dose (LD₅₀) could be greater than 2000 mg/kg body weight in rats. In acute toxicity study, no toxic symptoms were observed up to dose of 2 g/kg body weight. All animals behaved normally. No neurological or behavioral effects could be noted. No mortality was found up to 14 days study.

Anti-inflammatory activity of *D. gangeticum* and *A. augusta*

The AqE of DG and PEE of AA were evaluated for anti-inflammatory activity in carrageenan induced paw oedema model and the results are summarized in Table 1 and 2. The result obtained indicates that the extracts found to have significant (P< 0.0001) anti-inflammatory activity in rats. AqE of DG and PEE of AA reduced the carrageenan induced oedema by 39.06 % and 35.17% respectively at 3 h, whereas the standard drug showed 40.39% of inhibition as compared to the control group.

Table 1: Percentage (%) paw oedema inhibition in carrageenan induced inflammation

Treatment	% Paw oedema inhibition in rats				
	After 0 min	After 30 min	After 60 min	After 120 min	After 180 min
Normal Control	---	---	---	---	---
Inflammatory control	---	---	---	---	---
Inflammatory control+ Standard	6.25	32.45	37.68	46.63	40.39
Inflammatory control+ AqE of DG	1.25	30.00	34.68	43.38	39.06
Inflammatory control + PEE of AA	2.5	26.48	26.91	33.17	35.178

Table 2: Anti-inflammatory activity of plant extracts in carrageenan induced paw oedema

Treatment	PAW VOLUME (Mean in ml. ± SEM)				
	After 0 min	After 30 min	After 60 min	After 120 min	After 180 min
Normal Control	1.52±0.040	1.47±0.030	1.43±0.028	1.50±0.020	1.42±0.037
Inflammatory control	1.60±0.040	3.183±0.030 ^{a*}	3.503±0.028 ^{a*}	4.16±0.020 ^{a*}	3.75±0.158 ^{a*}
Inflammatory control+ Standard	1.50±0.114	2.15±0.116 ^{b*}	2.183±0.121 ^{b*}	2.22±0.108 ^{b*}	2.24±0.158 ^{b*}
Inflammatory control+ AqE of DG	1.58±0.046	2.228±0.053 ^{b*}	2.288±0.055 ^{b*}	2.355±0.041 ^{b*}	2.29±0.121 ^{b*}
Inflammatory control + PEE of AA	1.56±0.176	2.34±0.073 ^{b†}	2.56±0.123 ^{b‡}	2.78±0.175 ^{b*}	2.43±0.056 ^{b*}

COX and LOX inhibitory activity of *D. gangeticum* and *A. augusta*

COX-1 inhibitory activity of indomethacin (standard drug) and plant extracts

At various concentrations viz. 2.5, 5, and 10 µg/ml, the percentage inhibition exhibited by indomethacin were found to be 23.57%, 55.10% and 77.39% for COX-1 enzyme with an IC₅₀ value of 4.6 µg/ml. The percentage inhibition of COX-1 exhibited by different concentrations (10, 50 and 100 µg/ml) of AqE of DG and PEE of AA were found to be 15.49%, 24.33%, 32.45% (IC₅₀=49.5 µg/ml) and 18.35%, 34.81% and 68.79% (IC₅₀=36.5 µg/ml) respectively (Figure 1, 2 and 3).

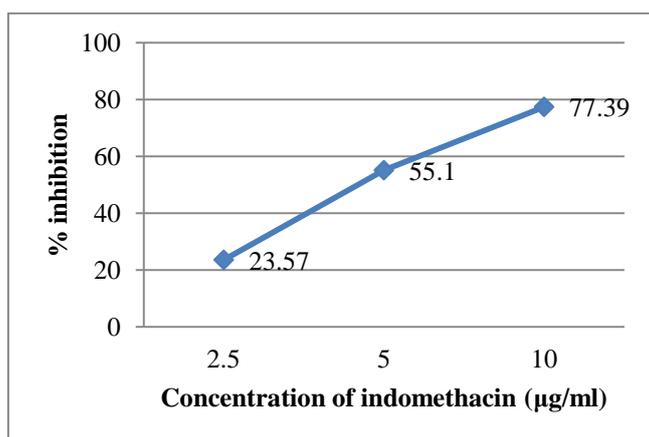


Figure 1: COX-1 inhibitory activity of Indomethacin

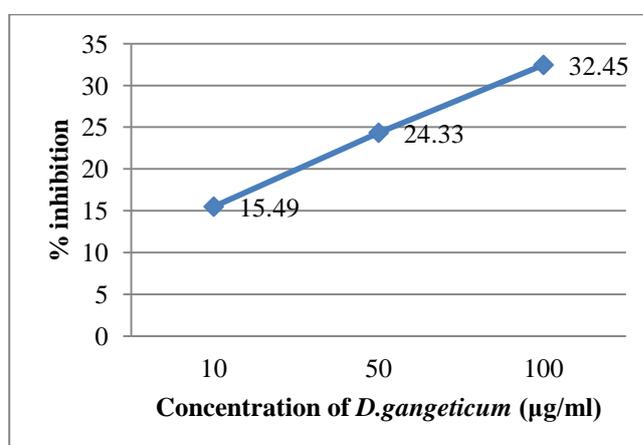


Figure 2: COX-1 inhibitory activity of AqE of *D.gangeticum*

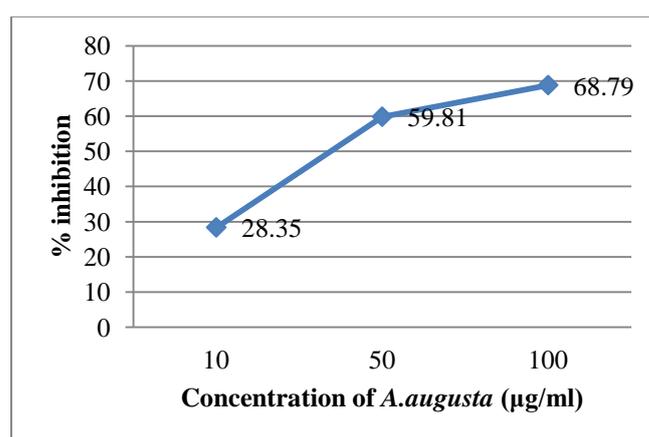


Figure 3: COX-1 inhibitory activity of PEE of *A.augusta*

COX-2 inhibitory activity of indomethacin (standard drug) and plant extracts

Dose dependent inhibition of COX-2 was exhibited by standard drug and both the plant extracts. Indomethacin showed maximum inhibition at 10 µg/ml (80.36%) with an

IC₅₀ value of 4.2 µg/ml. The percentage inhibition of COX-2 exhibited by various concentrations (10, 50 and 100 µg/ml) of AqE of DG and PEE of AA were found to be 29.56%, 52.63%, 72.35% (IC₅₀=39.5 µg/ml) and 13.79%, 23.46% and 37.51% (IC₅₀=59.0 µg/ml) respectively (Figure 4, 5 and 6).

Maximum LOX inhibitory effect of quercetin was found to be 59.89% at 100 µg/ml with an IC₅₀ value of 39.5 µg/ml. Dose dependent inhibition of LOX was demonstrated by both the extracts. At different concentration (10, 50 and 100 µg/ml), percentage inhibition exhibited by AqE of DG and PEE of AA were found to be 24.95%, 38.73%, 69.53%; and 10.38%, 17.63% and 32.10 % µg/ml with an IC₅₀ value of 57.0 µg/ml and 75.5 µg/ml respectively (Figure 7, 8 and 9).

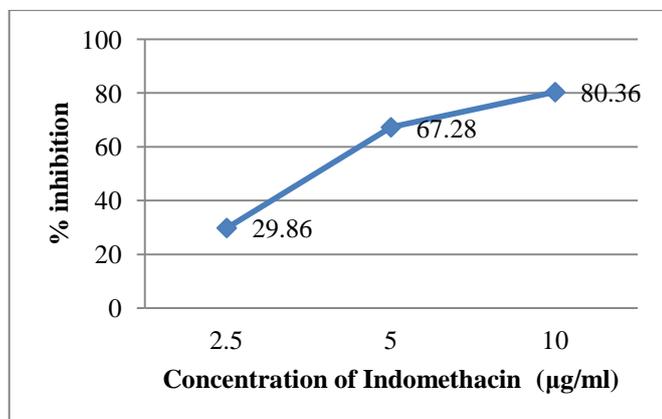


Figure 4: COX-2 inhibitory activity of Indomethacin

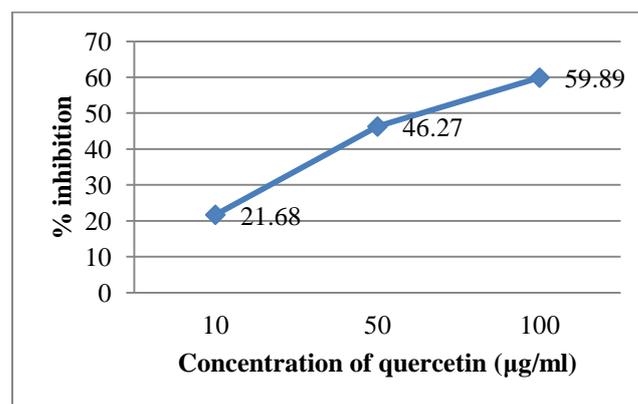


Figure 7: LOX inhibitory activity of quercetin

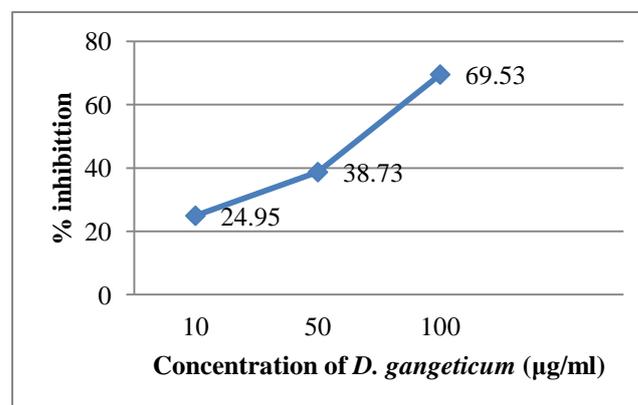
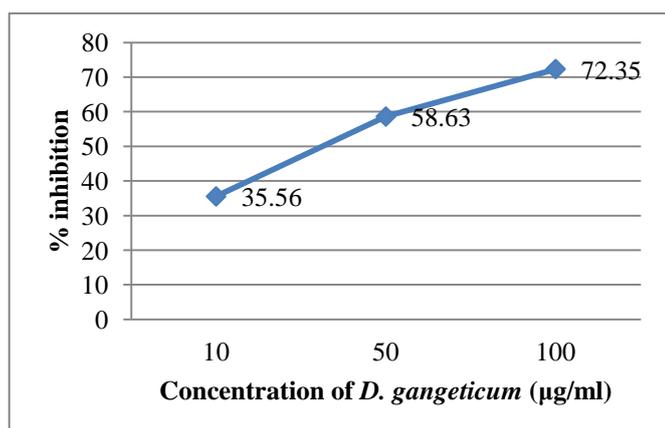


Figure 8: LOX inhibitory activity of AqE of *D. gangeticum*

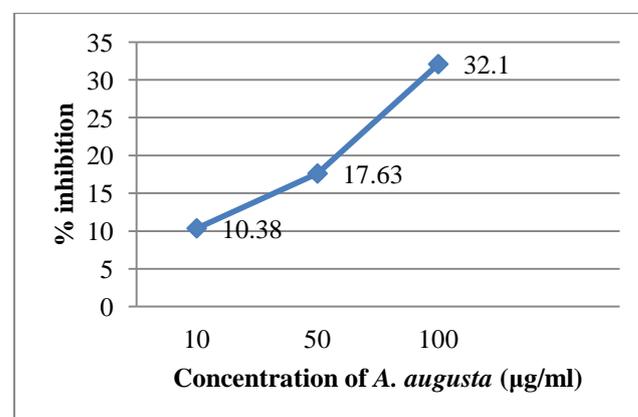
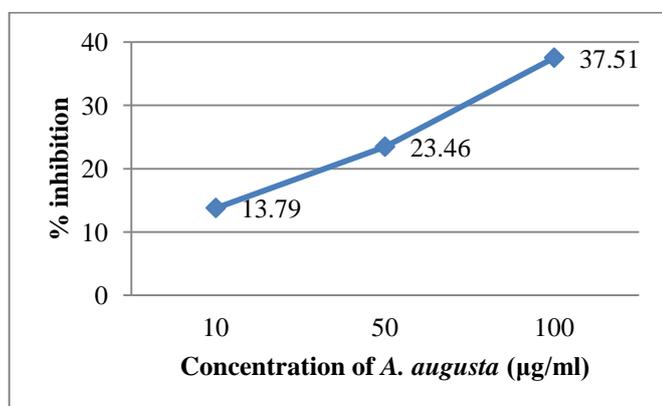


Figure 9: LOX inhibitory activity of PEE of *A. augusta*

LOX inhibitory activity of quercetin (standard drug) and plant extracts

Discussion

The inhibition of carrageenan-induced inflammation in rats is an established model for evaluating anti-inflammatory drugs, which has been used frequently to assess anti-edematous effect of natural products. Carrageenan induced acute inflammation is believed to be biphasic. The early phase occurs within one hour of carrageenan inflammation and is mainly mediated by cytoplasmic enzymes, histamine, serotonin, from the mast cell. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells, and prostaglandins produced by tissue macrophages. Since the extracts significantly inhibited paw edema induced by carrageenan in the second phase, this finding suggests a possible inhibition of cyclooxygenase synthesis by the extract and this effect is similar to that produced by nonsteroidal anti-inflammatory drugs such as indomethacin, whose mechanism of action is inhibition of the cyclooxygenase enzyme.^{9, 15}

The results of study showed that AqE of DG and PEE of AA possessed significant ($p < 0.0001$) anti-oedematogenic effect on paw oedema induced by carrageenan. Since carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation¹⁶, the results of this study are an indication that extracts of DG and AA can be effective in acute inflammatory disorders. Further attempts were made to investigate whether the anti-inflammatory effect exhibited by DG and AA extracts in the carrageenan-induced rat paw edema is caused by inhibition of COX enzyme (one of the mechanisms for anti-inflammatory activity), both the extracts were studied in COX-1 and COX-2 catalysed prostaglandin biosynthesis assay (In-vitro). Effect of plant extracts on LOX enzyme was also examined. Lipoxygenases (LOXs) (LOX; EC 1.13.11.12) are a family of non-heme iron-containing dioxygenases catalyzing the biosynthesis of leukotrienes. Leukotrienes function as initiators of inflammation and their inhibition is considered to be partly responsible for the anti-inflammatory activity.¹⁷ The percent inhibitory activity against COX-1, COX-2 and LOX by the plant extracts at 10, 50 and 100 $\mu\text{g/ml}$ was reported as percent inhibition of prostaglandins and leukotrienes biosynthesis. The percentage inhibitory activity of AqE of DG against COX-2 enzyme ($\text{IC}_{50} = 39.5 \mu\text{g/ml}$) were generally higher to that of COX-1 ($\text{IC}_{50} = 49.5 \mu\text{g/ml}$), at the same concentration however PEE of AA demonstrated inhibitory activity against COX-1 ($\text{IC}_{50} = 36.5 \mu\text{g/ml}$) to a greater extent than COX-2 ($\text{IC}_{50} = 59 \mu\text{g/ml}$). Moderate

inhibition of LOX activity was demonstrated by DG ($\text{IC}_{50} = 57.0 \mu\text{g/ml}$). However AA exhibited weak inhibitory action on LOX activity at the same concentration ($\text{IC}_{50} = 75.5 \mu\text{g/ml}$). The results of the study indicated that inhibitory effect of each plant extract on enzymes were dose dependent.

Conclusion

From the results of the study, it can be concluded that both the plant extracts are endowed with effective anti-inflammatory activity mediated via diverse mechanism of action. Multiple mechanism of anti-inflammatory activity was evident by extracts of DG and AA including reduction in the level of prostaglandins and leukotrienes. The study provided a scientific support for the use of DG and AA for the treatment of inflammatory diseases in ethnomedicine/Ayurveda.

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