In vivo Antiinflammatory Potential of Leaf Extracts of *Acacia auriculiformis* Benth.

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Rangra *et al.*: Antiinflammatory activity of leaves extract of *Acacia auriculiformis*

The prime objective of the present study was to evaluate the probable *in vivo* antiinflammatory potential of various extracts of the leaves of *Acacia auriculiformis* Benth. using carrageenan and formalin-induced inflammation in the rats. Thereafter gas chromatography-mass spectrometry analysis of the bioactive extract was performed to identify the compounds responsible for the antiinflammatory activity. The *in vitro* antioxidant potential was determined through 2,2-diphenyl-1-picrylhydrazyl scavenging assay, hydrogen peroxide scavenging assay and reducing power assay. Overall butanol and methanol leaf extracts showed significant antioxidant free radical scavenging activity. The methanol and petroleum ether leaf extracts at a dose of 400 mg/kg showed the highest percent inhibition of 84.88 and 82.12, respectively in the carrageenan-induced rat paw edema model, whereas, in formalin-induced rat paw edema model the chloroform and methanol leaf extracts showed the highest percent inhibition of 65.68 and 63.34 at a dose of 400 mg/kg, respectively. In both the antiinflammatory models, indomethacin (standard, 40 mg/kg) was used for comparison with test extracts. The gas chromatography-mass spectrometry analysis of bioactive butanol and methanol extracts showed the presence of compounds like sterols (stigmasterol, β-sitosterol and γ-sitosterol) and phenolic compounds (phenol, 2,4-bis(1,1-dimethylethyl),2,4-ditert-butylphenol), which could be responsible for the pharmacological activities observed. These *in vitro* and *in vivo* studies indicated that the antiinflammatory and antioxidant potential of the methanol leaf extract of *Acacia auriculiformis* Benth. could due to the presence of high phenolic and sterol content.

Key words: Antioxidant, antiinflammatory, GC-MS, *Acacia auriculiformis* leaf extracts

Over decades, medicinal plants have been reported to contribute raw materials for traditional (Ayurveda, Homeopathy, Unani, Siddha, and Chinese) as well as modern medicine system. The rural population of many developing countries still depend on herbal drugs for primary health care. The remedial prospective of herbal drugs rely on its form used i.e. whether whole plant, or parts of the plant, or extracts, or isolated bioactive phytoconstituents. Herbal medicines usually consist of parts of medicinal plants or crude plant extracts composed of several phytoconstituents, which frequently show a synergistic effect.

Inflammation is an immunobiological response of body towards irritation, infection, harmful stimuli, and injury of body cells or tissues. Main symptoms of inflammation are redness, heat, swelling, pain and even loss of function[1]. Inflammation may last for a shorter (acute) or a longer (chronic) duration. In the acute phase, the inflammation is distinguished by raised blood flow and vascular permeability with fluid, leukocytes and cytokine (inflammatory mediators) accumulation. Whereas in the chronic or subacute phase, inflammation is distinguished by the initiation of specific cellular and humoral immune response to pathogens at the place of tissue injury. At present steroids, and non-steroidal drugs and even some herbal drugs available are used to counteract inflammation by inhibiting the release of cyclooxygenase (COX) enzymes i.e. COX-1 and COX-2, which produce prostaglandins, thromboxane, and other inflammatory mediators[2,3].

Reactive oxygen species (ROS) like hydroxyl, peroxynitrite radicals and superoxide anion are earlier reported to be involved in the inflammation process. The excessive generation of ROS leads to cellular

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biomolecular injury to carbohydrate, lipids, proteins, and nucleic acids, leading to cell and tissue damage, which in succession initiates inflammation process[4,5]. Therefore, compounds with scavenging properties towards ROS might have therapeutic efficacy towards inflammation. But to discover and develop more effective and less toxic drugs have become a challenge for the pharmaceutical chemist. Medicinal plants appear to be potential source of new chemical entities with high efficacy.

Acacia auriculiformis A. Cunn. ex Benth. is an evergreen tree, which is distributed throughout India and other parts of the world. It is commonly known as "Australian acacia" a member of Mimosaceae or Fabaceae, usually found as ornamental shade tree having greenish-white shade clustered flowers; seen in roadsides and in parks. Traditionally it has been used in the treatment of rheumatism, sore eyes, aches, itching, and allergy[6]. Recently several reports showed various pharmacological activities like CNS depressant[7,8], antimalarial[9], antifilarial[10], cestocidal[11], antimicrobial[12], antimutagenic[7], antioxidant[13], chemopreventive[7], spermicidal[14], wound healing[15], antidiabetic and hepatoprotective[16]. In addition, various phytochemical investigations revealed the presence of flavonoids, tannins and triterpenoidal saponin glycosides in different parts of this plant[17].

Phenolic group-bearing compounds with one or more hydroxyl groups in an aromatic ring impart a broad range of pharmacological activities like antimutagenic, anticarcinogenic, antiinflammatory and antioxidant activities[18,19]. Although, to the best of our knowledge, the leaves of *Acacia auriculiformis* Benth. have not been previously explored for *in vivo* antiinflammatory activity. Therefore, an attempt was made to mainly investigate and highlight the *in vivo* antiinflammatory potential of various extracts of *A. auriculiformis* leaf.

MATERIALS AND METHODS

Plant materials:

Leaves of *A. auriculiformis* (Mimosaceae) were collected from Jharkhand (India) in September 2017. The plant was identified a voucher specimen was deposited in the Botanical Survey of India, Kolkata India (CNH/Tech.II/2015/42/32111).

Preparation of the plant extracts:

The maceration process was used in the preparation of plant extract. Clean leaves were air-dried at room temperature and made in to a coarse powder. The dried powdered leaves of *A. auriculiformis* were defatted using petroleum ether and subsequently extracted using chloroform, ethyl acetate, acetone, butanol, and methanol. All the extracts were concentrated under vacuum and lyophilized separately for further use. The extraction yield was calculated for all the extracts which were, methanol (7.4 g), petroleum ether (4.1 g), chloroform (3.5 g), ethyl acetate (4.6 g), n-butanol (4.8 g) and acetone (3.2 g).

Preliminary phytochemical screening:

The phytochemical analysis of all the leaf extracts was performed to detect the presence of various secondary metabolites like alkaloids (Dragendorff reagent, Mayer reagent test, Wagner reagent test, and Hager reagent test), steroids (Salkowski test, Libermann-Burchard test) flavonoids (Shinoda and alkaline reagent test), glycosides (sodium hydroxide reagent, Keller-Kiliani test), saponins (foam test), and tannins (chlorogenic test, catechin test and vanillin-hydrochloric acid test)[20]. The detailed procedure of the above-performed tests is shown in Table 1.

DPPH free radical scavenging assay:

The antioxidant free radical scavenging activity for various leaf extracts towards stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) was carried out using the DPPH assay. The mechanism behind the antioxidant effect is hydrogen donating potential of the compound to the unstable radical form of DPPH. In this way, the radical form of DPPH is scavenged by an antioxidant leading to change in colour from purple to yellow. A gradual decrease in absorbance was measured at 517 nm. With the modest change in procedure depicted by Kant *et al.*, free radical scavenging potential of all the leaf test extracts was performed[21]. Stock solutions (1 mg/ml) of butylated hydroxytoluene (BHT), the standard and the test leaf extracts (methanol, petroleum ether, chloroform, ethyl acetate, butanol, and acetone) were prepared in ethanol. Test solutions were prepared by pipetting out 10, 20, 30, 40 and 50 μg/ml of BHT or the leaf extract aliquots from the stock solutions in test tube and the volumes were made up with ethanol. Thereafter the pipetted out aliquots were transferred to test tubes containing 2 ml of the 0.1 mM DPPH solution prepared in ethanol. The mixture was allowed to stand for 30 min at 25° after vigorous shaking. Gradual decrease in absorbance was observed on a UV spectrophotometer (Shimadzu 2450, Japan) at 517 nm, when compared to blank, which was composed of

TABLE 1: QUALITATIVE PHYTOHEMICAL TEST PROCEDURES EMPLOYED

Test	Procedure	Inference
Dragendorff's reagent test	Extract (2 mg)+distilled water (5 ml)+HCl (1 ml)+Dragendorff's reagent (1 ml)	Orange/orange red precipitates
Mayer reagent test	Extract (2 mg)+Mayer's reagent (few drops)	White/pale yellow precipitates
Wagner reagent test	Extract (2 mg)+HCl (1.5 % vv)+Wagner's reagent (few drops)	Yellow/brown precipitates
Hager reagent test	Extract (2 mg)+Hager's reagent (few drops)	Yellow precipitates
Salkowski test	Dry extract (2 mg) shaken with chloroform+H_2SO_4 added to chloroform layer	Formation of red colour
Libermann-Burchard test	Dry extract (2 mg) dissolved in acetic anhydride+heated to boiling+cooled+concentrated H_2SO_4 (1 ml)	Formation of green colour
Shinoda test	Extract (2 mg) dissolved in 5 ml of ethanol+10 drops of dilute HCl+small piece of magnesium	Formation of pink, reddish or brown colour
Alkaline reagent test	Test solution+NaOH solution (few drops)	Formation of intense yellow colour turns to colourless with dilute acetic acid addition
Sodium hydroxide reagent test	Extract (5 mg) dissolved in 0.5 ml of 20 % H_2SO_4 solution+NaOH solution (few drops)	Formation of blue colour
Keller-Kiliani test	Extract (1 ml) boiled with 10 ml of 70 % alcohol for 2-3 min and filtered filtrate+water (5 ml)+lead acetate (0.5 ml)+shaken well filtrate (clear)+chloroform evaporated to extractives extractives+glacial acetic acid+$FeCl_3$ solution (2 drops)+H_2SO_4 solution (2 ml)	Formation of bluish green colour from reddish brown layer on standing
Foam test	Extract solution (1 ml)+distilled water (20 ml)+shaken for 15 min	Formation of stable foam
Catechin test (Match stick test)	Match stick dipped in aqueous extract and dried near burner and moistened with HCl	Matchstick wood turns pink or red due to formation of phloroglucinol
Chlorogenic test	Extract solution+aqueous ammonia	Green colour on exposure to air
Vanillin-hydrochloric acid test	Extract solution+vanillin-hydrochloric acid reagent (1 g vanillin+10 ml alcohol+10 ml concentrated HCl)	Pink or red colour formed due to formation of phloroglucinol

300 µl/ml of ethanol in 2 ml of DPPH solution. Triplicate measurements were performed. Percent inhibition of free radical DPPH was calculated using the Eqn., I % = (Ab–As/Ab)×100, where Ab is the absorbance of the control reaction and As is the absorbance of the test compound.

Hydrogen peroxide scavenging assay:

The hydrogen peroxide scavenging assay for various leaf extracts was performed according to the procedure stated by Dey *et al.* with little modifications[22]. The standard (BHT) and test leaf extracts of *A. auriculiformis* having concentrations 10, 20, 30, 40, 50 µg/ml were prepared in Mili-Q water. Hydrogen peroxide (2 M, 2.5 ml) in phosphate buffer (0.2 M, pH 7.4) was blended with 1.5 ml test leaf extract and standard (BHT). The mixture was shaken and incubated for 10 min away from light and absorbance was recorded at 230 nm. The blank solution used in the above study was phosphate buffer without hydrogen peroxide. Triplicate measurements were performed. Percent inhibition of hydrogen peroxide scavenging activity was calculated using the Eqn., I % = (Ab–As/Ab)×100, where Ab is the absorbance of the control reaction and As is the absorbance of the test compound.

Reducing power assay:

The reducing power assay for different leaf test extracts of *A. auriculiformis* was carried out according to the procedure stated by Dey *et al.* with slight changes[22]. The standard (BHT) and test leaf extracts of *A. auriculiformis* having concentrations 10, 20, 30, 40, 50 µg/ml were prepared in Mili-Q water. One milliliter of test extract solution was mixed with 2.5 ml of 0.2 M KH_2PO_4 buffer solution having pH 6.6 and 2.5 ml of $K_3[Fe(CN)_6]$. This reaction mixture was allowed to stand for 20 min and subjected to treatment for the temperature of 50°. The reaction blend was cooled and trichloroacetic acid (2.5 ml of 10 %) was added followed by the centrifugation for 10 min at 2000 rpm. Thereafter 2.5 ml of the upper centrifuged layer was considered and $FeCl_3$ solution (0.5 ml of 0.1 %) and Mili-Q water (2.5 ml) was added to it. Absorbance was measured at 700 nm using UV

spectrophotometer. The control solution was prepared in a likely way (as described above) without considering the test leaf extracts. Triplicate measurements were performed. Percent inhibition of reducing power assay was calculated using the Eqn., $I\% = (Ab-As/Ab)\times100$, where Ab is the absorbance of the control reaction and As is the absorbance of the test compound.

Experimental animals:

Wistar rats weighing between 150-220 g were used in these studies. Rats were maintained in the standard experimental laboratory animal house (conditions at $25\pm2°$, 50 ± 15 % RH and normal photoperiod 12 h dark/12 h light) in the Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, India. To all animals, water and commercial pellet diet were provided *ad libitum*. The animal experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) with the protocol number PROV/BIT/PH/IAEC/08/2016.

Acute toxicity study:

The acute toxicity study was carried out according to the Organisation for Economic Co-operation and Development (OECD) guidelines 425 (acute oral toxicity: Up-and-Down Procedure)[23]. Wistar rats (150-200 g) of either sex were used for this study. Five rats were chosen for animal studies. Animals were fasted overnight and extracts were given orally at a single dose of 2000 mg/kg. Mortality, abnormal behavior and any physical lesions were studied for 14 d.

Carrageenan-induced rat paw edema assay:

Leaf extract samples were screened for *in vivo* antiinflammatory studies using a slightly modified carrageenan-induced rat paw edema model as described earlier[24]. In the right hind paw of ether anesthetized rat, edema was induced by subplantar injection of 0.1 ml carrageenan (1 % w/v) in 0.9 % saline. One hour before carrageenan injection, test samples (prepared in 1 % w/v gum acacia) were administred orally (po) at a dose of 200 and 400 mg/kg. Indomethacin (40 mg/kg, po) was used as the standard while the control group was administered vehicle only. Using a plethysmometer (Samitek Instrument) thickness of the paw was measured immediately before injecting the carrageenan at 0 h and at 2, 4, and 6 h after carrageenan injection. From 0 to 6 h the increase in paw thickness measurement was observed and expressed as a difference in paw thickness. Percent inhibition in volume was shown as a value with respect to the control group at various time

spans. % Inhibition = (1–difference in paw volume of treatment/difference in paw volume of control)×100.

Formalin-induced paw edema in the rat:

Antiinflammatory activity of leaf extracts was tested using slightly modified and earlier described formalin-induced paw edema model[25]. The 0.05 ml of 2.5 % formalin was injected into the sub-plantar region of the right hind paw of ether anesthetized rat. All test leaf extracts (200 and 400 mg/kg), standard indomethacin (40 mg/kg) and saline 2 ml/kg were administered orally (po) 1 h before formalin injection and at next 6 consecutive h using an oral gavage needle. The elevated paw edema was measured using a plethysmometer. The paw thickness was measured just prior to formalin injection i.e. 0 h and at 2, 4, and 6 h after formalin injection, using a plethysmometer. From 0 to 6 h, rise in paw thickness measurement was observed as a difference in paw thickness. Percent inhibition in volume was expressed as a value with respect to the control group at various time intervals. % Inhibition = (1–difference in paw volume of treatment/difference in paw volume of control)×100.

Statistical evaluation of data:

Results of *in vivo* antiinflammatory activity were expressed as the mean %±SEM (standard error of mean) of n=6 animals. The statistical differences between the test or treated and control groups were analyzed through one way ANOVA (SPSS version 9.05) followed by Dunnett's test application. In statistical analysis, the values having p<0.05 were regarded as significant.

Gas chromatography-mass spectroscopy (GC-MS) analysis of the bioactive extracts:

The GC-MS analysis of butanol and methanol leaf extracts was performed using GC-MS instrument (Thermo Scientific Co., Thermo GC-TRACE ultra ver.:5.0, Thermo MS DSQ II). The experimental conditions maintained during analysis in the instrument were as follows, HP 5-MS capillary standard non-polar column was used having dimensions 30 m length, 0.25 mm diameter and the film thickness of 0.25 μm. The mobile phase used was pure helium (He, carrier gas) having 99.995 % purity and flow rate fixed at 1 ml/min. The oven temperature in GC was elevated from 80° to 280° at the rate of 5° and the injection volume was set at 1 μl. The butanol and methanol test extract samples were dissolved in chloroform and run at a range of 50-650 *m/z*. The Wiley spectral library search programme was used to compare the results.

RESULTS AND DISCUSSION

The qualitative preliminary phytochemical screening test results of leaf extracts (Table 2) revealed the presence of secondary metabolic constituents like phenolic content, flavonoids, saponins, tannins, and glycosides.

The *in vitro* DPPH scavenging assay results (fig. 1A) revealed significant activity in butanol, ethyl acetate, acetone, and methanol leaf extracts. The butanol, ethyl acetate, acetone, and methanol leaf extracts showed 74.27, 63.66, 60.05, and 53.05 % inhibition of paw volume, respectively at 20 µg/ml as compared to standard (BHT), which showed an inhibition of 97.29 % at 40 µg/ml. The highest percent inhibition of 98.42 was shown by the butanol leaf extract at 50 µg/ml as compared to BHT, which showed 97.52 % inhibition at 50 µg/ml concentration. The petroleum ether and chloroform leaf extracts showed relatively lower percent inhibition of 82.17 and 62.11 at 40 µg/ml as compared to other leaf extracts.

The measurable aspect of the DPPH free radical scavenging assay is a change in color from purple to yellow when the antioxidant reacted with DPPH. The prominence of the antioxidant action lies in the intense color change from purple to yellow. The reason behind this phenomenon is the formation of a stable complex due to the reaction between the antioxidant and DPPH. Polyphenolic compounds are reported to possess potent antioxidant activity[26]. Among the tested leaf extracts, butanol leaf extract showed the highest significant DPPH scavenging activity followed by ethyl acetate, acetone, and methanol leaf extracts at a concentration of 20 µg/ml. The order of activity of other extracts tested was found to be in the order: butanol<ethyl acetate<acetone<methanol<chloroform<pet   ether. This could be due to the presence of high flavonoid content in these extracts. The high amount of phenolics

and tannins is also responsible for imparting significant reducing activity of the above extracts.

The *in vitro* H_2O_2 scavenging assay results shown in fig. 1B revealed significant activity in the methanol, acetone, butanol and ethyl acetate extracts of the leaves as compared to BHT, the standard used. Percent inhibition shown by methanol and acetone extracts at 20 µg/ml were 61.25 and 50.05, respectively when compared to BHT, which showed a percent inhibition of 51.53 at 20 µg/ml. The butanol and ethyl acetate extracts at 30 µg/ml showed percent inhibition of 65.09 and 60.03, respectively as compared to the standard, which showed 66.48 % inhibition at 30 µg/ml. The highest inhibition of 93.94 % was shown by the methanol extract at 50 µg/ml dose, while the standard showed 98.43 % inhibition at the same concentration. The petroleum ether (72.07 %) and chloroform (52.01 %) extracts showed relatively lower activity at 40 µg/ml as compared to other leaf extracts.

Hydrogen peroxide is known as a weak oxidizing agent, which denatures enzymes directly by oxidizing the important thiol groups. It is highly light sensitive and quickly enters the cell through cell membranes and reacts with Fe^{2+} and Cu^{2+} ions to form hydroxyl radicals[22]. The methanol extracts showed the highest activity among all the tested extracts at a concentration of 20 µg/ml as compared to BHT. The activity of other extracts in a concentration-dependent manner was found to be in the order, methanol<acetone<butanol<ethyl acetate<chloroform<petroleum ether. The hydrogen donating potential of phenolic constituents present in the extracts appeared to be responsible for scavenging of hydroxyl radicals.

The *in vitro* reducing power assay results (fig. 1C) disclosed significant activity of the ethyl acetate, acetone, methanol, and chloroform extracts as compared to BHT. Percent inhibition shown by ethyl acetate and acetone extracts at 20 µg/ml dose were,

TABLE 2: QUALITATIVE PHYTOCHEMICAL SCREENING OF DIFFERENT LEAF EXTRACTS OF *ACACIA AURICULIFORMIS*

Phytochemical tests	Leaf extracts					
	Pet. ether	Chloroform	Ethyl acetate	n-Butanol	Acetone	Methanol
Alkaloids	++	+	-	+++	+	+
Flavonoids	-	-	+++	+++	+++	+++
Steroids	+++	++	+	+	+	+
Tannins	-	+	++	++	+++	++
Glycosides	-	+	+++	+++	++	+++
Saponins	-	+	++	+++	+++	++

'+' Slightly present, '++' moderately present, '+++' highly present, '-' absent
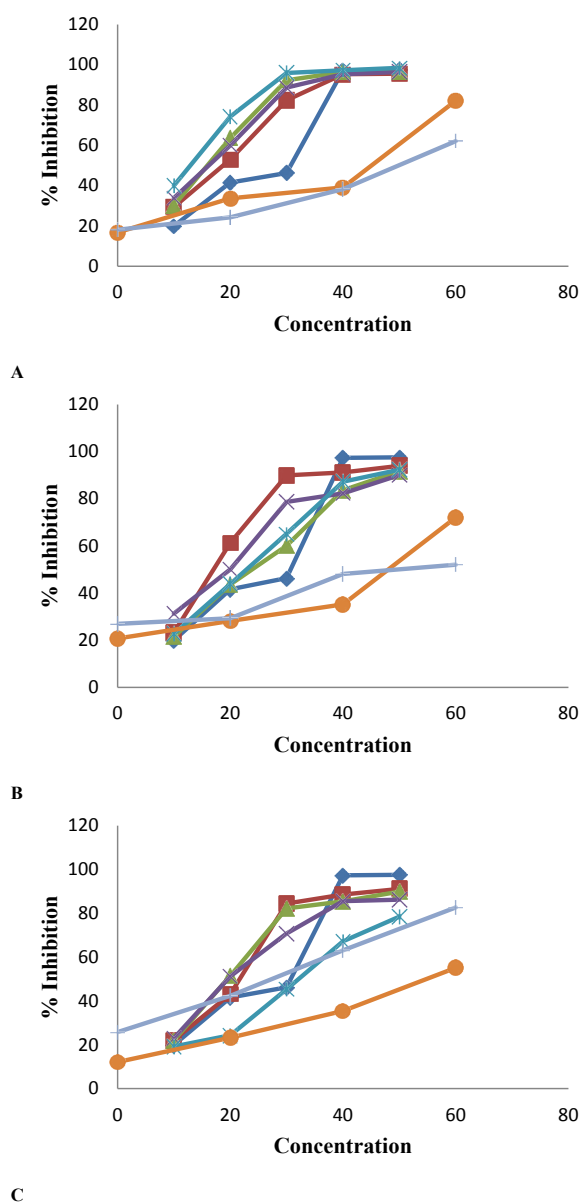
Fig. 1: Antioxidant effects of leaf extracts of *Acacia auriculiformis*

(A) DPPH free radical scavenging assay, (B) hydrogen peroxide (H$_2$O$_2$) scavenging assay and (C) reducing power assay. —◆— **% inhibition BHT;** —■— **% inhibition methanol extract;** —▲— **% inhibition ethyl acetate extract;** —×— **% inhibition acetone extract;** —✳— **% inhibition butanol extract;** —●— **% inhibition petroleum ether extract;** —|— **% inhibition chloroform extract**

51.44 and 51.25, respectively, whereas BHT showed of 56.68 % inhibition at 30 μg/ml. The highest percent inhibition of 91.09 was shown by methanol extract at 50 μg/ml while the standard showed 97.92 % inhibition at the same concentration. The methanol and chloroform extracts showed inhibition of 84.41 and 63.12 %, respectively at 30 μg/ml. The petroleum ether (55.12 %) and butanol (67.21 %) leaf extracts showed low activity at 40 μg/ml as compared to other leaf extracts.

The basic principle behind the reducing power assay is the conversion of Fe^{3+} ion in FeCl$_3$ to Fe^{2+} in presence of reducer antioxidant agents. The elevation in the absorbance of the reaction blend increases the antioxidant activity[22]. The ethyl acetate leaves extract showed the highest significant activity among all the tested leaf extracts at a concentration of 20 μg/ml as compared to standard (BHT). The order of activity of other leaf extracts in concentration-dependent manner was found to be in the order: ethyl acetate<acetone<methanol<chloroform<pet ether<butanol. This may be due to the presence of reductones or antioxidants in the test leaf extracts, which cause reduction of ferric ion or ferric cyanide complex to ferrous ion form.

The acute toxicity study demonstrated that the extracts tested up to a dose of 2000 mg/kg (po, single dose) in rats and observed for 14 d showed no mortality, no abnormal behavior and physical lesions. So, the tested leaf extract of plant *A. auriculiformis* were found to be safe up to the dose of 2000 mg/kg. On the basis of this toxicity study as well as reported studies, the doses of 200 and 400 mg/kg were selected.

After an exhaustive literature survey, it is possible to conclude that for the very first time, the *in vivo* antiinflammatory evaluation of the various leaf extracts of *A. auriculiformis* is being reported. The antiinflammatory activity of different *A. auriculiformis* leaf extracts were studied in carrageenan-induced rat paw edema model. The methanol and petroleum ether extracts administered orally at a dose of 400 mg/kg showed the highest percent inhibition (fig. 2A) of 84.88 and 82.12, respectively. Comparison was made with the standard drug, indomethacin (40 mg/kg), which showed a percent inhibition of 94.01 at a dose of 40 mg/kg. At 0 h just before the injection of carrageenan, the range of basal values was observed to be 1.22± 0.86 ml in control and in the inflammation control 1.21±0.47 ml. Whereas the paw volumes of rats treated with 400 mg/kg of the methanol extract were 1.15± 0.88 ml at the 0 h, 2.02±0.42 ml at 2 h, 1.96±0.23 ml 4 h and 1.30±0.12 ml at 6 h. The difference in paw volume between 0 and 6 h was found to be 0.15±0.001 ml and the percent inhibition was 84.88. The other extracts i.e. methanol, acetone, ethyl acetate, chloroform, butanol, and petroleum ether at a dose of 200 mg/kg showed percent inhibitions of 76.90, 62.01, 60.99, 66.81, 43.40, and 70.12, respectively. Whereas the acetone, ethyl acetate, chloroform, and butanol extracts at a

dose of 400 mg/kg showed percent inhibition of 77.65, 60.02, 67.12, and 64.86, respectively.

The carrageenan-induced rat paw edema model revealed the efficacy of methanol leaves extract at 400 mg/kg when compared to the standard drug indomethacin (40 mg/kg). The decrease in paw volume or decrease in paw edema was considered an effective antiinflammatory action. All the values were represented as mean %±SEM and n=6; whereas the values with p<0.05 were considered to be significant (fig. 2B). The different extracts were found effective against carrageenan-induced paw edema in rats after 6 h as compared to inflammation control treated group. Methanol and petroleum ether extracts at the dose of 400 mg/kg were found to be the most effective (p<0.05).

The antiinflammatory effect of different leaf extracts of *A. auriculiformis* on the paw edema induced by the formalin method was also studied. The chloroform and methanol extracts showed the highest percent inhibition (fig. 3A) of 65.68 and 63.34 at a dose of 400 mg/kg, po, respectively when compared to the standard drug indomethacin, which showed a percent inhibition of 76.81 at a dose of 40 mg/kg. At 0 h just before the injection of carrageenan, the range of basal values was observed to be 1.22±0.86 ml in normal control and in inflammation control 1.14±0.30 ml. In the 400 mg/kg chloroform extract treated group the paw volume values were 1.07±0.23 ml at 0 h, 1.69±0.35 ml at 2 h, 1.96±0.28 ml at 4 h and 1.43±0.28 ml at 6 h. The difference in paw volume between 0 and 6 h was found to be 0.36±0.002 ml and percent inhibition was 65.68. The methanol, acetone, ethyl acetate, chloroform, butanol, and petroleum ether extracts at a dose of 200 mg/kg showed percent inhibition of 56.11, 40.73, 39.80, 45.60, 35.02, and 37.12, respectively, while the acetone, ethyl acetate, butanol, and petroleum ether extracts at a dose of 400 mg/kg showed percent inhibition of 52.62, 52.98, 43.71, and 45.43, respectively.

The formalin-induced rat paw edema model used for *in vivo* antiinflammatory evaluation results showed that of all the leaf extracts tested, the methanol extract at a dose of 400 mg/kg was found to be significant when compared to the standard indomethacin (40 mg/kg). All results for statistical evaluation were presented as mean %±SEM of n=6 and the probability level with p<0.05 was considered to be significant (fig. 3B). Different extracts were found to be effective
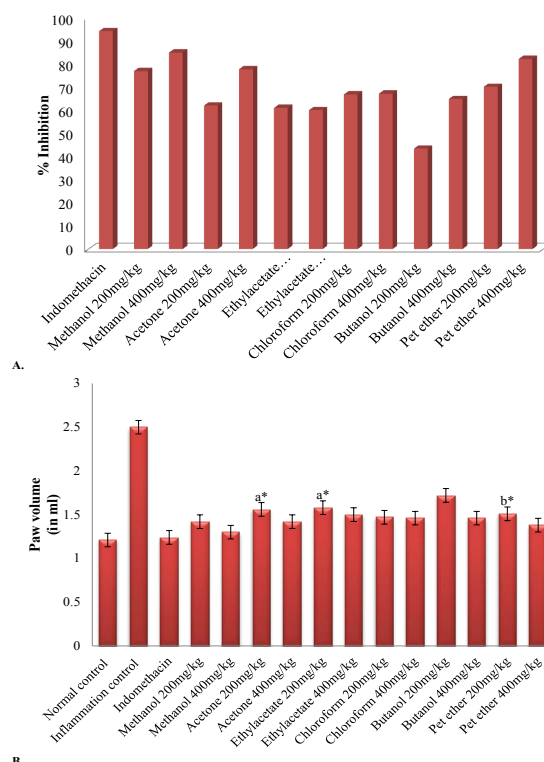
Fig. 2: Antiinflammatory effects of leaf extract of *Acacia auriculiformis* in rat carrageenan-induced paw edema model
Values are expressed as mean±SEM of n=6. p<0.05 was considered to be significant. a is *vs* inflammation control; *p< 0.05
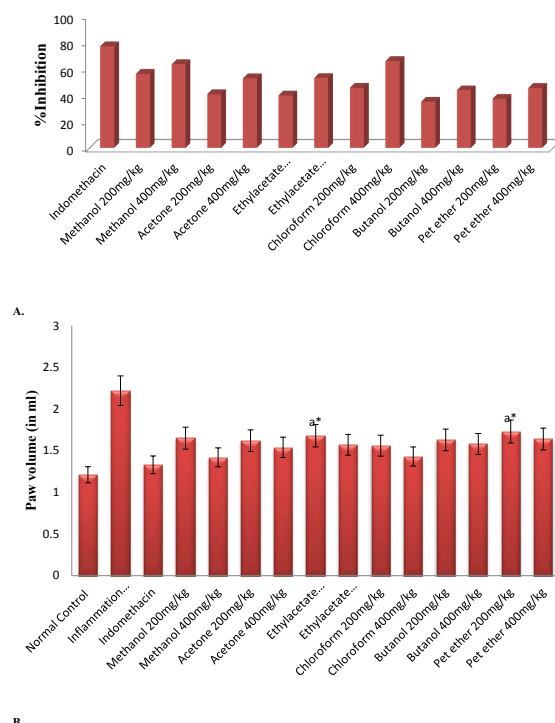
Fig. 3: Antiinflammatory effects of leaf extract of *Acacia auriculiformis* in formalin-induced paw edema in rats
Values are expressed as mean±SEM of n=6. p<0.05 was considered to be significant. a is *vs* inflammation control; *p< 0.05

against formalin-induced paw edema in rats after 6 h as compared to inflammation in control rats. The chloroform extract at 400 mg/kg and methanol extract at 400 mg/kg were found to be most effective. The maximum antiinflammatory activity was observed at 400 mg/kg dose.

The carrageenan-induced rat paw edema and formalin-induced paw edema models were considered to be effective models for the evaluation of antiinflammatory action. The inflammation is generally considered to be biphasic. The first phase lasted up to one hour and included secretion of histamine and serotonin whereas after one hour the second phase was initiated with the release of kinins, prostaglandins and other COX products. Bradykinin maintained the continuity between the two phases[27]. The standard drug indomethacin inhibited COX enzyme. The methanol extract exhibited a dose-dependent inhibitory effect similar to indomethacin in both the antiinflammatory models employed. The methanol extract at 400 mg/kg was found to be effective in both the models. This could be due to the presence of high levels of phenolic, flavonoid and sterol content in this extract, which led to antioxidant action[28]. Therefore, it could be noted that the antiinflammatory action of *A. auriculiformis* leaf extracts during the first and second phase of inflammation was due to the inhibition of COX, an enzyme necessary for the release of inflammatory mediators like prostaglandins, histamine, serotonin, bradykinin, and metabolism of arachidonic acid[29]. Selvam and Jachak reported that the carrageenan and formalin-induced paw edema models were more effective in evaluating the COX enzyme inhibition activity[30]. The *Acacia* genus has already been explored extensively for antiinflammatory activity[31-33]. On the basis of the above-reported evidence, it could be concluded that the antiinflammatory action of *A. auriculiformis* extract on the carrageenan and formalin-induced paw edema models could be mediated by the inhibition of COX enzyme.

The phytoconstituents present in methanol and butanol extract of *A. auriculiformis* are shown in Tables 3 and 4. The elution order and retention time were the basis of identifying the bioactive compounds. The peak retention time, peak area, percent peak area, peak height and percent peak height were presented. Based on the retention time, the major phytoconstituents present

TABLE 3: COMPOUNDS IDENTIFIED THROUGH GC-MS ANALYSIS OF BUTANOL EXTRACT OF *ACACIA AURICULIFORMIS*

| Peak	Name	Retention time	Area	Area (%)	Height	Height (%)	Base (*m/z*)
1	Phenol, 2,4-bis(1,1-imethylethyl)	15.597	5650693	5.96	1585685	9.58	191.15
2	(E)-9-octadecene	17.564	339115	0.36	141000	0.85	55.05
3	3-Hydroxy-ß-damascone	18.023	921425	0.76	207644	1.25	69.05
4	Megastigmatrienone	18.333	652090	0.69	228290	1.38	133.10
5	2-(3-Isopropyl-4-methyl-pent-3-en-1-ynyl)-2-methyl-cyclobutanone	22.642	1185064	1.25	348995	2.11	147.10
6	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	24.272	395711	0.42	155479	0.94	57.05
7	Hexadecanoic acid methyl ester	24.676	1192974	1.26	485099	2.93	74.05
8	1-Heptadecene	26.030	311486	0.33	137699	0.83	57.05
9	(E,E)-9,12-octadecadienoic acid, methyl ester	27.866	592188	0.62	241052	1.46	67.05
10	10-Octadecenoic acid, methyl ester	28.008	530171	0.56	140420	0.85	55.00
11	Methyl stearate	28.493	289267	0.31	130754	0.79	74.05
12	Campesterol	34.368	7179548	7.58	611633	3.70	130.10
13	2-monopalmitoylglycerol	34.974	4214578	4.45	1442175	8.72	98.10
14	Docosanoic acid, methyl ester	35.241	274692	0.29	121450	0.73	74.05
15	Stigmasterol	36.111	21637780	22.83	1578532	9.54	55.00
16	α-Monostearin	38.076	745937	0.79	261504	1.58	98.10
17	ß-Sitosterol	38.833	12188664	12.86	1919681	11.60	55.05
18	Delta-8(14)-stigmastenol	38.867	4444179	4.69	1865368	11.27	414.40
19	γ-Sitosterol	38.974	18213558	19.22	2852026	17.23	55.05
20	3-ß-Cholest-5-en-3-ol, 24-propylidene	39.380	3760112	3.97	697422	4.21	55.05
21	Cholest-4-en-3-one, 26-acetyloxy	40.035	3666170	3.87	446784	2.70	124.10
22	Cycloartenol acetate	40.718	3374761	3.56	448027	2.71	69.05
23	Delta-4-sitosterol-3-one	43.340	3204726	3.38	501155	3.03	124.10

TABLE 4: COMPOUNDS IDENTIFIED THROUGH GC-MS ANALYSIS OF METHANOL EXTRACT OF *ACACIA AURICULIFORMIS*

Peak	Name	Retention time	Area	Area (%)	Height	Height (%)	Base (*m/z*)
1	2,4-Ditert-butylphenol	15.577	5080233	8.88	1432098	8.98	191.2
2	3-Heptadecanol	22.235	600188	1.05	234753	1.47	59.05
3	2-Methyl-2-(4H-1,2,4-triazol-4-ylamino) propanenitrile	22.492	1692219	2.96	473617	2.97	124.1
4	Isopropyl myristate	22.619	643946	1.13	206490	1.29	60
5	Neophytadiene	22.899	3726755	6.51	1476272	9.26	68.05
6	Phytol acetate	23.77	1251973	2.19	523506	3.28	82.1
7	(Z)-7-Hexadecenoic acid, methyl ester	24.56	297630	0.52	134349	0.84	57.05
8	Hexadecanoic acid methyl ester	24.68	2628617	4.59	1107727	6.95	74.05
9	E-15-Heptadecenal	26.037	488789	0.85	214456	1.34	57.05
10	9,12-Octadecadienoic acid methyl ester	27.87	845084	1.48	328977	2.06	67.05
11	(Z,Z,Z)-9,12,15-Octadecatrienoic acid methyl ester	27.981	1425963	2.49	416132	2.61	79.1
12	Phytol	28.199	4248618	7.43	1659589	10.41	71.05
13	Eicosane	28.403	411563	0.72	158432	0.99	57.05
14	Methyl stearate	28.498	824697	1.44	359533	2.25	74.05
15	1-Heneicosanol	29.729	263909	0.46	123012	0.77	57.05
16	Formamide, N-{4-[2-(1,1-dimethylethyl)-5-oxo-1,3-dioxolan-4-yl]butyl}	34.363	1411792	2.47	545830	3.42	130.1
17	Hexanoic acid pentadecyl ester	34.667	1359889	2.38	153897	0.96	117.1
18	2-Monopalmitoylglycerol	34.983	11311798	2.38	3336569	20.92	98.1
19	Stigmasterol	35.86	5789844	10.12	620446	3.89	55.05
20	β-monolinolein	37.613	784256	1.37	249656	1.57	67.05
21	α-Glyceryl linolenate	37.732	2227656	3.89	354042	2.22	55.05
22	Octadecanoic acid 2,3-dihydroxypropyl ester	38.073	2561676	4.48	950686	5.96	98.1
23	γ-sitosterol	38.697	7333765	12.82	889072	5.57	55.05

in butanol extract were considered to be phenol-2,4-bis(1,1-dimethylethyl), (e)-9-octadecene, 3-hydroxy-beta-damascone. Whereas in the methanol extract, the major compounds identified were, 2,4-ditert-butylphenol, 3-heptadecanol and 2-methyl-2-(4h-1,2,4-triazol-4-ylamino) propanenitrile. The GC-MS chromatograms of both extracts are shown in figs. 4A and B.

GC-MS analysis of butanol and methanol extracts revealed the existence of various compounds like phenolic compounds (phenol-2,4-bis(1,1-dimethylethyl) and 2,4-ditert-butylphenol), sterols (campesterol, stigmasterol, β-sitosterol, delta-8(14)-stigmasterol, γ-sitosterol, delta-4-sitosterol-3-one, stigmasterol, β-monolinolein and γ-sitosterol), alcoholic compounds (2-monopalmitoylglycerol, 3-heptadecanol, phytol, 1-heneicosanol and 2-monopalmitoylglycerol) and easters (hexadecanoic acid methyl ester, (e,e)-9, 12-octadecadienoic acid methyl ester, 10-octadecenoic acid methyl ester, docosanoic acid methyl ester, 7-hexadecenoic acid, (Z)-methyl ester, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid methyl ester, (Z,Z,Z)-9,12,15-octadecatrienoic acid methyl ester, hexanoic acid pentadecyl ester, octadecanoic acid-2,3-dihydroxypropyl ester). Stigmasterol, sitosterol, phytol, and hexadecanoic acid were the major compounds present in different quantities both in butanol and methanol extracts. Phytol is a precursor of vitamin K and E, which exhibits potent antioxidant, cytotoxic and antinociceptive activity[34-37]. Phytosterols such as stigmasterol, β-sitosterol and γ-sitosterol showed antiinflammatory action by blocking the inflammatory cytokines and inducing apoptosis. Sterols even possessed anticancer and antioxidant properties. Sterols prevented angiogenesis by enhancing antioxidant enzymes and blocked the release of ROS and prevented oxidative stress[38,39]. Phenolic compounds exhibited antiinflammatory properties and their mechanism of action was reported to be similar to that of nonsteroidal antiinflammatory drugs. Besides COX enzyme inhibition, phenolic compounds also inhibited other pro-inflammatory mediators and their activity through gene suppression. Some phenolic compounds in inflammatory and antioxidant pathways can up/downregulate the transcriptional factors like
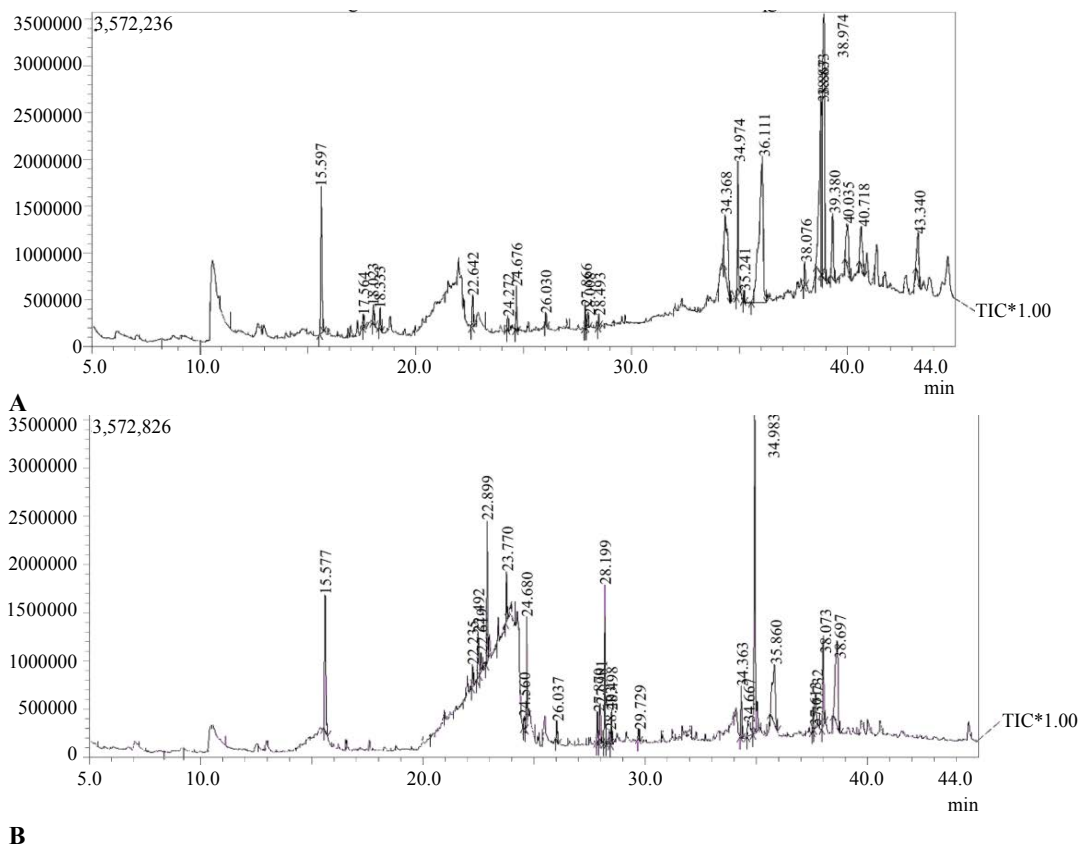
Fig. 4: GC-MS chromatogram of butanol and methanol extract of *Acacia auriculiformis*
GC-MS chromatogram of (A) butanol and (B) methanol extract of leaves of *Acacia auriculiformis*

nuclear factor-kB or Nrf-2[32]. On the basis of the above-reported evidence, it could be summarised that the compounds identified through GC-MS analysis possessed pharmacological significance indicating the antioxidant and antiinflammatory potential of the plant *A. auriculiformis*.

The methanol and butanol extracts were able to significantly scavenge the free radicals and reduce inflammation. This confirmed the justification of the traditional use and efficacy of this plant to treat many free radical-born and inflammatory-related major diseases. The GC-MS analysis of butanol and methanol extracts revealed the presence of various sterols and phenolic compounds, which might be responsible for imparting the antiinflammatory as well as antioxidant action. The separation and characterization of these bioactive phytoconstituents from the methanol and butanol extracts responsible for the *in vitro* antioxidant scavenging and *in vivo* antiinflammatory activities are currently under progress.

Acknowledgments:

Conflict of interest:

The authors declare no conflict of interest.

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