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ANALGESIC & ANTI INFLAMMATORY SCREENING OF CRUDE ISOLATES OF ANNONA SQUAMOSA LEAF EXTRACT

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ABSTRACT
In the present study we evaluated the anti-inflammatory and analagesic activity of crude fractions of *Annona squamosa* leaf methanolic extract. From the phytochemical analysis of the methanolic extract, it has been inferred that the methanol extract of *Annona squamosa* leaves contain the maximum number of phytoconstituents like alkaloids, glycosides, protein, flavonoids, tannins and polyphenol. By the help of column chromatography the bioactive fractions were isolated which might be responsible for the therapeutic activity. Of the total 8 fractions (F1-8), **F6** and **F7** indicate the presence of phenolic compounds and flavonoids. These two fractions exhibited analgesic and anti-inflammatory effect in tail flick and carrageenan- induced paw oedema models respectively. This study confirmed that flavonoid fraction obtained from *Annona squamosa* leaves extract are responsible for its anti inflammatory and analgesic effects.

KEYWORDS: *Annona squamosa*, carageenan-induced edema, analgesic, antiinflammatory, tail flick.

INTRODUCTION
Medicinal plants are part of human society to combat diseases, from the dawn of civilization. About 80% of the world population relies on plants and their products for primary health care.[1] *Annona squamosa* is a small, semi-deciduous tree widely cultivated for its edible fruit. It is also used medicinally in treating diarrhoea, dysentery, colds, chills, rheumatism, and sleeplessness.[2]

Taxonomical Classification
Kingdom: Plantae
Subkingdom: Tracheobionta
Super division: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Sub class: Magnoliidae
Order: Magnoliales
Family: Annonaceae
Genus: *Annona* L.
Species: *Annona squamosa*

The ethanolic extract of the seeds of *Annona squamosa* are found to contain annonaceous acetogenins: squamocenin, annotemoyin-2, reticulatain-2, squamocin-I, squamocin-B, squamocin, motrilin, squamostatin-D, squamostatin-E, cherimolin-1, and cherimolin-2.[3] The leaves of the plant on extraction with methanol and further sujection to isolation of active principles was found to contain Annonaretin A, kaurenoic acid, taraxerol, β-sitosterol, 16α- hydro-19-al-ent-kauran-17-oic acid, 6β-hydroxystigmast-4-en-3-one, 17-acetoxy-16β-ent- kauran-19-oic acid, 16α-hydro-ent-kauran-17,19-dioic acid, and (2S)-di-O-methylquiritigenin.[4] The various parts of the plant have been traditionally used for management of diabetes. Scientifically the plant has been explored for pharmacological as well as biological actions like insecticidal, antidiabetic, antidysentric, antiovulatory, antiinflammatory, antithyroidic, antilipidemic, antioxidant, antiulcer, vasorelaxant, antitumor, hepatoprotective, genotoxic, and larvicidal.[5]

The literature pointed out several pharmacological and biological actions of *Annona squamosa*. The presence of phenolic compounds paves way for a number of bioactivities in plant extracts. Hence it was envisioned to perform chromatographic separation of various solvent fractions fromthe crude extract of *Annona squamosa* and screen the isolates for analgesic and anti- inflammatory activities using animal models.

MATERIAL AND METHODS
Collection and identification of plant material
Annona squamosa leaves of vouchered herbarium specimen were prepared and preserved along with crude drug sample at the herbarium of RB Science, Bhopal (M.P.), India. The plant material was shade dried, reduced to coarse powder and stored in airtight container till further use.

Preparation of extract[6,7]

500 gram of powdered of leaves of *Annona squamosa* was packed in soxhlet apparatus and extracted with methanol as solvent.

The extraction was carried out with the aid of electrical heating of the solvent until the completion of the extraction which was confirmed by the colour of the siphoned liquid. The extract was filtered while hot, and the solvents were removed by distillation and the last traces of solvent being removed under reduced pressure. The ethanol extracts were stored in refrigerator for further experimental work.

The extract was weighed and the percentage value was recorded and also the physical appearance and color was evaluated and recorded and thereafter, was stored in refrigerator for further experimental work.

Qualitative phytochemical tests[8]

Qualitative chemical tests were performed to determine the presence of alkaloids, carbohydrates, cardiac glycosides, polyphenols, saponins, tannins and terpenoids.

Fractionation of the methanolic crude extract with different solvents

Wet packing method was adopted for packing the column. Slurry of activated silica gel (neutral) was prepared in solvent system and was poured into the column with the help of a hollow glass cylinder.

Prepartion and application of sample

About 10 gm of extract was mixed with 50 gm of silica gel for CC (60-120 mesh) & small quantity of an appropriate solvent was mixed. This mixture was triturated to obtain a free flowing mixture. This mixture was carefully placed on the top of the silica bed and appropriate solvent was fed to the column.

Isolation of Fractions

The *Annona squamosa* extract was subjected to column chromatography using silica gel (60- 120 mesh size), and eluted with the following solvent ratios of Hexane: ethylacetate (EA), 100:0, 75:25, 50:50, 25:75, 0:100, then with 75:25, 50:50, 25:75, 0:100, EA:Ethanol (EtOH). Finally elution was done with 75:25, 50:50, 25:75, 0:100, EtOH:Methanol (MeOH). The fractions (25 ml) were collected from the column. The elute collected were monitored by thin layer chromatography (eluent: EA-MeOH, 9:1 and 3:2) for homogeneity and the similar fraction were pooled together. The eight different fractions were collected and dried. The three fractions were further analyzed for phytochemical screening to determine the nature of isolated compound. The phytochemical analysis of each fraction was carried out.

Pharmacological activitySelection of animals

Male Wistar rats (150-200 gm) were used for the study after acclimattization to the laboratory conditions. All experiments were approved by the institutional ethical committee and were carried out according to the animal ethics committee guidelines.

Anti-inflammatory activity[9]

Animal were divided into various groups and six animals in each group as follows: Group I (control group) – Treated with distilled water; Group II – Treated with standard drug Aceclofenac at 10 mg/kg body weight; Group III – Treated with F6 at 50 mg/kg body weight; Group IV – Treated with F7 at 50 mg/kg body weight

Acute inflammation was produced by injecting 0.1ml of 1% carrageenan suspension in normal saline into the subplantar region of right hind paw after 60 minutes of drug administration. The control group was administered only distilled water. The isolated fractions and standard drugs administered intraperitoneally 1h before carrageenansuspension administration. A mark was made on the leg at the malleolus to facilitate uniform dipping at subsequent readings. The volume of paw oedema volume was measured with the help of plethysmograph by mercury displacement method immediately before and five hours after the drug administration. The inhibition of oedema in various treated groups was then calculated by using statistical analysis.

Analgesic activity[10]

The analgesic activity was evaluated using tail flick method. Animals were divided into four groups of six animal each as follows: Group I - Control - treated with vehicle (normal saline); Group II - Standard drug – Ibuprofen; Group III– F6 was administered in dose of 50 mg/kg; Group IV – F7 was administered in dose of 50 mg/kg.

About 5 cm from the distal end, tail of each rat was immersed in warm water maintained at 50°C. The reaction time (in seconds) was the time taken by the rat to flick its tail due to pain. The first reading was omitted and reaction time was taken as the average of the next two readings. The reaction time was recorded before (0 min) and at 15, 30, 45, and 60 min after the administration of the treatments. The maximum reaction time was fixed at 15 sec to prevent any tail tissue injury. If the reading exceeds 15 sec, it would be considered as maximum analgesia. The maximum possible analgesia (MPA) was calculated as follows:

$$MPA = \frac{\text{Reaction time for treatment} - \text{reaction time for saline}}{15 \text{ sec} - \text{reaction time for saline}} X\ 100$$

RESULTS AND DISCUSSION

The hot continous extraction method was used for extracting the phytoconsituents from the leaf of the plant. The yield of methanolic extracts of leaves of *Annona squamosa* was 21.7%. Preliminary phytochemical investigations of the extracts of leaves of *Annona squamosa* revealed the presence of flavonoids, tannins, phenolic compounds, alkaloids, glycosides, and proteins

(table 1).

Table 1: Phytochemicals present in leaves of *Annona squamosa* extract.

Chemical Tests	Observation checked for	Observation	Inference
Mayer's reagent	cream colour precipitate	+	AlkaloidPresent
Hager's reagent	yellow colour precipitate	+	
Wagner's reagent	reddish brown precipitate	+	
Dragendorff's reagent	reddish brown precipitate	+	
Froth test	Frothing is seen	+	Glycosidepresent
Kedde's Test	No color	-	
Bontrager's Test	Rose pink or red color in theammonical layer not found	+	
Keller-Kiliani	No color in acetic acid layer	-	
Ferric chloride	Blue green color	+	Phenolics/Tannins Present
Gelatin Solution	White precipitate	+	
Alkaline reagent test	Yellow to red precipitate	+	
Vanillin HCl test	Purplish red color	+	
Shinoda test	red color	+	Flavonoids
Alkaline reagent test	Yellow color that turns red onacidification	+	Present
Zinc HCl reductinotest	red color	+	
Millon's Test	white precipitate, turns red onheating	+	Protein Present
Ninhydrin Test	Voilet color	+	
Liberman-BurchardTest	Brown ring at junctionUpper layer turns green	-	Sterols Absent
Salkowski Test	Yellow color in lower layer	-	

Isolation of compound from ethanol extracts

The methanolic extract of *Annona squamosa* leaf was subjected to column chromatography. The fraction F1, F2 and F3 were containing waxy material; the fractions F4 and F8 were powder but quantity was very little. The yield of fraction F5, F6 and F7 were 285 mg, 322 mg and 337 mg, respectively. These three fractions were further analyzed for phytochemical screening to determine the nature of isolated compound.

The phytochemical investigation of **F5** of *Annona squamosa* leaves revealed the presence of alkaloids and glycosides. The **F6** and **F7** indicate the presence of glycoside, flavonoids, tannins & phenolic compound.

Antiinflammatory and analgesic action

Many investigations have proven that varieties of flavonoid molecules possess anti- inflammatory activity on various animal models of inflammation. Fractions **F6** and **F7** containing polyphenol and flavonoids compound were evaluated for antiinflammatory and analgesic action.

The effect of the **F6** and **F7** isolated from *Annona squamosa* on carrageenan- induced paw oedema is presented in (table 2, figure 1).

Table 2: Effect of isolated fraction from *Annona squamosa* leaves extract oncarrageenan induced paw oedema.

Group	Paw volume after induction				
	1 hr	2 hr	3 hr	4 hr	5 hr
Control	0.323 ± 0.008	0.745 ± 0.010	0.935 ± 0.015	1.22 ± 0.010	1.06 ± 0.017
Aceclofenac(10 mg/kg)	0.26 ± 0.021	0.42 ± 0.026*	0.358 ±0.007*	0.261 ±0.011*	0.233 ±0.010*
F6 (50 mg/kg)	0.283 ± 0.012	0.566 ± 0.010	0.633 ±0.010*	0.523 ±0.012*	0.428 ±0.007*
F7 (50 mg/kg)	0.273 ± 0.010	0.635 ± 0.008	0.626 ±0.026*	0.538 ±0.016*	0.468 ±0.018*

Values are expressed as mean □ SD, n = 6 in each group. *P<0.05 compared to controlgroup

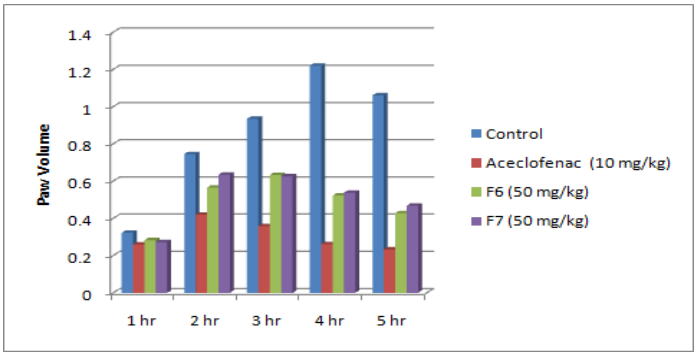

Figure 1: Graphical representation of effect of *Annona squamosa* leaves extract oncarrageenan induced paw oedema.

The results of analgesic activity of fractions of *Annona squamosa* extract by tail flick method areshown in table 3.

Table 3: Effect of *Annona squamosa* fractions on tail flick response.

Group	Response Time in seconds				
	0 min	15 min	30 min	45 min	60 min
Vehicle Control	3.07 ±0.055	3.21 ±0.021	3.47 ±0.044	3.39 ±0.027	3.51 ±0.049
Ibuprofen	4.15 ±0.128	5.69 ±0.332	6.22 ±0.171	6.79 ±0.142	7.98 ±0.324
F6 (50 mg/kg)	3.47 ±0.136	4.26 ±0.120	6.06 ±0.057	6.09 ±0.052	6.08 ±0.119
F7 (50 mg/kg)	3.53 ±0.170	4.37 ±0.358	6.15 ±0.242	6.21 ±0.064	6.11 ±0.074

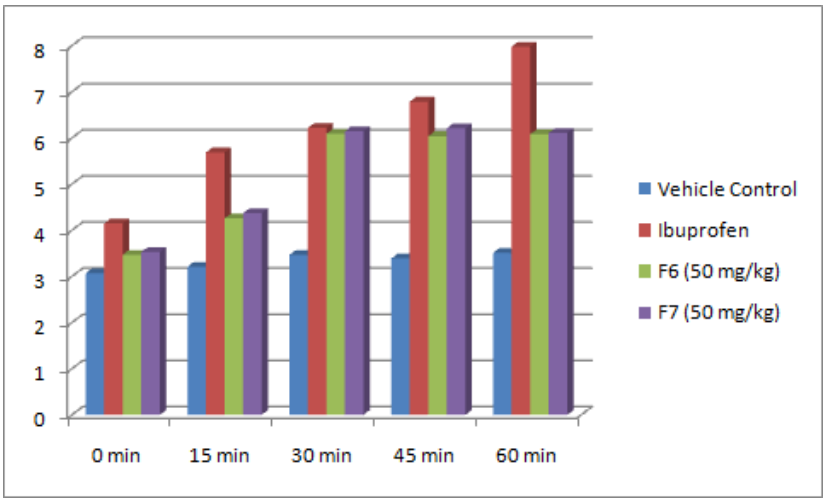

Figure 2: Comparison of analgesic effect of ibuprofen and F6 & F7.

The duration of response time in Ibuprofen and *Annona squamosa* was significantly higher ascompared to the saline treated animals. The highest reaction time for *Annona squamosa* was 6.21 ± 0.064 sec (**F7**) at 45 min post administration while it was 3.51 ± 0.049 sec and 7.98 ± 0.324 sec for vehicle treated group and Ibuprofen respectively at the 60 min post administration.

CONCLUSION

Annona squamosa is rich in secondary metabolite such as alkaloid, glycoside, flavonoids, polyphenol etc. In the present study an attempt was made to isolate the various crude fraction and evaluate their anti-inflammatory and analgesic activity. It was concluded that the fractions **F6** and **F7** isolated from *Annona squamosa* leaves extract exhibited moderate to high anti-inflammatory and analgesic activity.

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