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Research Article

EVALUATION OF IN VITRO ANTIOXIDANT ACTIVITY & EFFECT OF AQUEOUS EXTRACT OF *ANNONA SQUAMOSA* LEAVES ON INDOMETHACIN INDUCED PEPTIC ULCER IN RATS

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ABSTRACT

The present work was conducted to evaluate the In vitro antioxidant activity & to find out the effect of aqueous extract of *AnnonaSquamosa*leaves on indomethacin induced peptic ulcer in rat. For Invitro antioxidant activity evaluation various models were used (DPPH scavenging, Superoxide radical scavenging, Hydroxyl radical scavenging, Nitric Oxide, Inhibition of Lipid peroxidation.). The treatment was given to different groups of rat i.e. Group I, Group II, Group III & Group IV with vehicle, omeprazole 10 mg/kg, test drug 150 mg/kg, test drug 300mg/kg respectively. Indomethacin was used to induce peptic ulcer and various parameters were estimated. As per the results aqueous extract of *AnnonaSquamosa*leaves shown good antioxidant activity and it is having the potential of protecting the gastric mucosa too. The aqueous extract of *AnnonaSquamosa*leaves shown dose dependent effects in both activities.

Keywords: AnnonaSquamosa, antioxidant, peptic ulcer.

INTRODUCTION

Present world is full of competition, busy life style, mental pressure, etc, which develops lot of stress full conditions. Mental stress leads to formation of free radicals leading number pathological conditions in human beings, which requires proper care and treatment otherwise it can cause serious heath related problems. Free radicals are causing cell injury and are capable of damaging tissues & even organs. Antioxidants are the substances which reduces the dangerous effects of free radicals and useful as protective in case of cell/tissue injury.Numbers of plants are having good antioxidant property & hence they become very useful against stress induced or free radical related abnormalities. Natural antioxidants from plant sources can protect human body organs and cells from damage due to free radicals which can cause serious abnormalities like cancer, cardiovascular disorders, stroke, diabetes etc.[1].Increase in the gastric hydrochloric acid secretion leads to damage to gastric mucosa and erosion to the area exposed to acid pepsin mixture, it is termed as peptic ulcer. Peptic ulcer is characterized by abdominal pain, restlessness, hyper acidity, etc.[2]. Some factors like stress, lack of sleep, starvation, improper food intake, spicy food, smoking,drinking alcohol, etc leads to peptic ulcer due to imbalance the gastric juice secretion and decreased defensemechanism. Most of the people are suffering from peptic ulcer, for the treatment of peptic ulcer various

drugs are available but they are having some drawbacks and limitations. Hence use of natural plants and their parts or extracts are widely used for the treatment of ulcer, plants are also having good antioxidant activity hence they will be useful in the protection of gastric mucosa in case of peptic ulcer.Annona Squamosa is one of the most widely used plants traditionally for the treatment of number of pathological conditions. It has antidiabetic[3], anticancer[4], anthelmintic[5], anti-inflammatory[6], analgesic[6], hepatoprotective[7], antibacterial[8],etc activities. It has great therapeutic potential as it contains many chemical constituents. The aqueous extract of *Annona Squamosa* leaves mainly consist of glycosides, alkaloids, flavonoids, saponins, phenolic compounds, etc. these constituents may be responsible for its various activities[9,10]. As Annona squamosa is having so many chemical constituents & uses and it is easily available hence thought to be worthwhile to investigate its invitro antioxidant and protective effect on gastric mucosa damage/injury induced by indomethacin in rat. The experimental work and results are given below.

MATERIALS AND METHODS

Plant Material: Fresh leaves of Annona Squamosa were collected from the premises of the campus of Andhra University,Visakhapatnam. Authenticated by Department of Botany, Andhra University, Visakhapatnam.

Preparation of Aqueous Extract of Annona Squamosa leaves: The leaves of Annona Squamosa were washed with water and 50 g of leaves dried under shade (in absence of sunlight) at 25^0c for 5 days. Then extraction was done with one liter of boiling for water two hours, and then concentrated it to of the volume by boiling in water bath. The dark brown extract was obtained cooled & filtered with filter paper (no.1), filtrate was centrifuged at 10000 rpm at 25^0c. The supernatant was concentrated up to 100 ml under reduced pressure. The lyophilized concentrated crude extract was used for the study.[11]

Drugs & Chemicals: Indomethacin, Omeprazole, Sodium CMC, potassium chloride, ascorbic acid, ammonium ferrous sulphate, sodium dodecyl sulphate, thiobarbituric acid, acetic acid, trichloroacetic acid, ethylene diamine tetra acetic acid(EDTA), nitro bluetetrazolium (NBT), etc. All chemicals were used are of analytical grade.

Experimental Animals: In present study Albino Wistar strain rats of either sex weighing 150-250 g were used. Animals were obtained from Albino Research centre, Hyderabad, and were maintained under standard condition i.e. 24-27^0c temp. 12 hours light and dark cycle, animals were fed with dry pellets obtained from Albino Research centre, Hyderabad, and water ad libitum. Healthy animals were selected for the study.

Acute toxicity study: Healthy adult Wistar albino rats of either sex starved overnight, were divided into four groups (n=6) and were orally fed with aqueous extract of Annona Squamosa leaves at the increasing doses i.e. 100 mg/kg, 500 mg/kg, 1000 mg/kg, 3000 mg/kg, 5000 mg/kg of body weight where the rats were observed continuously for 2hr for behavioral, neurological and autonomic profiles, and then at 24 hr and 72 hr for any lethality.[12] All animals were live after the treatment in all groups.

In Vitro Antioxidant Activity

DPPH radical scavenging activity: It is measured by spectrophotometric method. To a methanolic solution of DPPH (200µM), 0.05 ml of test compound dissolved in ethanol were added at different concentration i.e. 50µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, & 500 µg/ml. An equal quantity of ethanol was added to control. After 20 min, the decrease in absorbance of test mixture was read at 517nm and the percentage inhibition calculated by formula given below, [13,14]

% Inhibition = (control – test) / control ×100

Scavenging of nitric oxide radical[15]: Nitric oxide was generated from sodium nitropruside and measured by Griess reaction.[16, 17] Sodium nitropruside (5mM) in standard phosphate buffer solution was incubated with different concentrations i.e. 50µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, & 500 µg/ml of test extract dissolved in phosphate buffer (0.025 M, $_P$H 7.4), and the tubes were incubated at 25^0c, for 5 hours. Control samples were prepared in the same manner without extract wit equivalent amount of phosphate buffer. After 5 hours, 0.5 ml of incubation solution was removed & diluted with 0.5 ml of Griess reagent (1 % sulphanilamide, 2 % o-phosphoric acid, 0.1 %

napthylethylene, diamine dihydrochloride). The absorbance was read at 546nm. (The experiment was repeated in triplicate).

Scavenging of hydroxyl radical: Hydroxyl radical scavenging activity was measured by studying the competition between deoxy ribose and test compounds for hydroxyl radical generated by the Fe^{3+}– ascorbate – EDTA – H_2O_2 system (Fenton reaction) according to the method of Kunchandy& Rao.[18] The reaction mixture containing, a final volume of 1.0 ml, 100 µl 2-deoxy-ribose, 500 µl of the various concentrations of the methanol extract as well as fraction A (25, 50 $\mu g.ml^{-1}$) and standard compound (Mannitol 50 mM) in KH_2PO_4- KOH buffer (20mM, pH 7.4), 200 µl 1.04 mM H_2O_2 and 100 µl 1.0mM ascorbic acid was incubated at 37°C for 1 hour. One milliliter 1% trichloroacetic acid was added to each test tube and incubated at 100°C for 20 min. After cooling to room temperature, absorbance was measured at 532nm against a control preparation containing deoxyribose and buffer. Percent inhibition was determined by comparing the results of the test and control samples with the above mentioned Eqn.

Scavenging of Superoxide radical: Superoxide radical (O_2) scavenging was measured in terms of inhibition of generation of O_2[19]Te reaction mixture consisted of phosphate buffer (50mM pH 7.4), riboflavin (20 µg/0.2 ml), EDTA (12mM), NBT (0.1 mg/3ml), sodium cyanide (3 µg/0.2 ml) different concentrations i.e. 50µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, & 500 µg/ml of test extract were added to make a total volume of 3 ml. The absorbance was read at 530 nm before and after illumination under UV lamp for 15 min against a control instead of sample. The percentage inhibition was calculated by formula given above.

In Vitro inhibition of lipidperoxidation: Freshly excised goat liver was processed to get 10% homogenate in cold phosphate buffered saline, pH 7.4 using glass Teflon homogenizer and filtered to get a clear homogenate. Te degree of lipidperoxidation was assayed by estimating TBARS by using standard method[20] with minor modification.[14] different concentrations i.e. 50µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, & 500 µg/ml of test extract were added to liver homogenate. Lipidperoxidation was initiated by adding 100 µl of 15mM ferrous sulphate solution to 3 ml of the tissue homogenate. After 30 min 100 µl of this reaction mixture was taken in the tube containing 1.5 ml of 10% TCA, After 10 min tubes were centrifuged for 10 to 15 min and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated for 30 min in boiling water bath. The intensity of pink coloured complex formed was measured at 535 nm. The results were expressed in percentage inhibition by using same formula given above.

Effect of aqueous extract of *Annona Squamosa* leaves on indomethacin induced gastric mucosa damage: Animals were divided in following groups (6 animals per group);
Group I – Control (Vehicle treated with 1% sodium CMC orally)
Group II – Standard (treated with omeprazole 10mg/kg orally)
Group III – test I (treated with aqueous extract of *Annona Squamosa* leaves 150 mg/kg orally)
Group IV – test II (treated with aqueous extract of *Annona Squamosa* leaves 300 mg/kg orally)
All groups were treated as stated above for 15 days. On 15th day after completion of drug treatment Indomethacin was given in 1% sodium CMC at the dose of 20mg/kg to all the group orally.[21]Animals were starved for 12 hours and then sacrificed by spinal dislocation method. Stomach was isolated and gastric juice was collected and volume was measured. Stomach was cut along the greater curvature and ulcer score were given after microscopical examination and ulcer index was calculated, Ulcer score:[22]
0 = Normal
0.5 = Reddening
1 = Spot ulcer
1.5 = Hemorrhagic streaks
2 = ≥ 3 mm ulcer
3 = ≥ 5 mm ulcer

Ulcer Index = Total ulcer score / number of animals ulcerated

$$\% \text{ Ulcer inhibition} = \frac{\text{(Control mean ulcer index – test mean ulcer index)}}{\text{Control mean ulcer index}} \times 100$$

Total Acidity: Total acid output of gastric juice was estimated by titration 0.1 ml of gastric juice with 0.01 N sodium hydroxide using phenolphthalein as an indicator, acid output was expressed as mEq/L.[23]

RESULTS

Invitro antioxidant activity: Several concentrations of aqueous extract of Annona squamosa leaves i.e. 50µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, & 500 µg/ml were tested by using various models invitro DPPH inhibition, Nitric Oxide scavenging, Hydroxyl radical scavenging, Superoxide radical scavenging & Lipidperoxidation inhibition the extract has shown dose dependent free radical scavenging activity. Themaximum percentage inhibition observed in this study for these models are 90.16%, 63.92%, 75.20%, 68.19% & 56.33% respectively. The highest inhibition observed in DPPH inhibition. All results are shown in observation table I. From this study it was observed that aqueous extract of Annona squamosa leaves scavenged free radicals In vitro.

Effect of aqueous extract of Annona squamosa leaves on Indomethacin induced gastric mucosa damage: The aqueous extract of Annona squamosa was tested in this study for its effect on Indomethacin induced gastric lesions or ulcers, test extract was given orally at the dose 150 mg/kg & 300 mg/kg. Gastric content, gastric pH, total acidity, means ulcer index and % inhibition of ulcer was calculated, results obtained are shown in observation table II. In this study test extract shown dose dependent effect when compare with control group in all parameters. The gastric content of Group I, Group II, Group III & Group IV were found 5.48 ml, 2.68 ml, 4.26 ml & 3.05 ml respectively where Group I was control, Group II was standard, Group III & IV were test extract treated (150 mg/kg & 300mg/kg),here gastric content decreased in all treated groups. Total acidity in Group I, Group II, Group III & Group IV were found 98.66 meq/L, 35.83 meq/L, 77.5 meq/L & 48.66 meq/L respectively.Mean ulcer index in Group I, Group II, Group III & Group IV were found 29.63, 11.66, 17.83 & 9.16 respectively. The percentage inhibition of ulcer formation in Group II, Group III & Group IV was 60.64%, 39.82% & 69.08% respectively.

DISCUSSION

Oxidative stress is major reason for the most of the pathological conditions like diabetes, cancer, cardiovascular diseases, inflammatory conditions, aging, neuronal damage etc.[24]many studies reported that antioxidants are having good protective effect over the damage done by free radicals in many cases, with that interest we done this study and we found that aqueous extract of Annona squamosa leaves is having good antioxidant activity as it was scavenged free radicals in various In vitro models, the results obtained in the present study may be due to several reasons, inhibition of ferryl-perferryl complex formation; scavenging of OH, superoxide radical by changing of Fe^{3+}/Fe^{2+}; reducing the rate of conversions of ferrous to ferric or by chelation of iron itself. A leaf of Annona squamosa contains number of chemical constituents and it is rich in bioflavonoid like rutin, quercetin and hyperoside, etc.[25] Flavonoids are natural products having strong antioxidant activity and good therapeutic potential which can be useful for number of pathological conditions. Thus the antioxidant potential shown by aqueous extract of Annona squamosa leaves may be due to presence of bioflavonoid. Further research requiresexploring the exact mechanism behind this activity.

Peptic ulcer is pathological condition occurs due to many reasons, i.e. may be due to increase in gastric acid secretion or due to decrease in cytoprotection by defensive factor like prostaglandin and mucus or due to free radicals or due to infection from H. pylori bacteria, etc. In the present study the aqueous extract of Annona squamosa was used and it has shown very good effect as per the results the control Group I was having more gastric content, total acidity as well as mean ulcer index but in test extract treated groups Group III & Group IV these three parameters decreased significantly in dose dependent manner, i.e. ulcer inhibition in Group III & Group IV were given 39.82% & 69.08% respectively. The higher dose of test extract has shown more inhibition than standard drug i.e. Omeprazole (60.64%). It may be due to antisecretory, antiulcerogenic & cytoprotective action these effects are having lot of importance in treatment and prevention of peptic ulcer. The Annona squamosa contains mainly alkaloids, bioflavonoid etc. and it has also shown very good in vitro antioxidant activity also. As per the results shown we can say that test extract is having good antisecretory, antiulcerogenic and cytoprotective effects, as gastric content

reduced, total acidity reduced & there were significant inhibition in mean ulcer index dose dependently. Probably these effects may be due to presence of bioflavonoid and antioxidant property further study requiresrevealing the exact mechanism of action of Annona squamosa.

CONCLUSION

As aqueous extract of Annona squamosaleaves scavenged free radicals In-vitro in different models and it has decreased gastric secretion, gastric acidity and mean ulcer index significantly and dose dependently in Indomethacin induced ulcer model in rat. Thus from the observations and results obtained it can conclude that the aqueous extract of Annona squamosa leaves is having antioxidant property and good antisecretory antiulcer activity.

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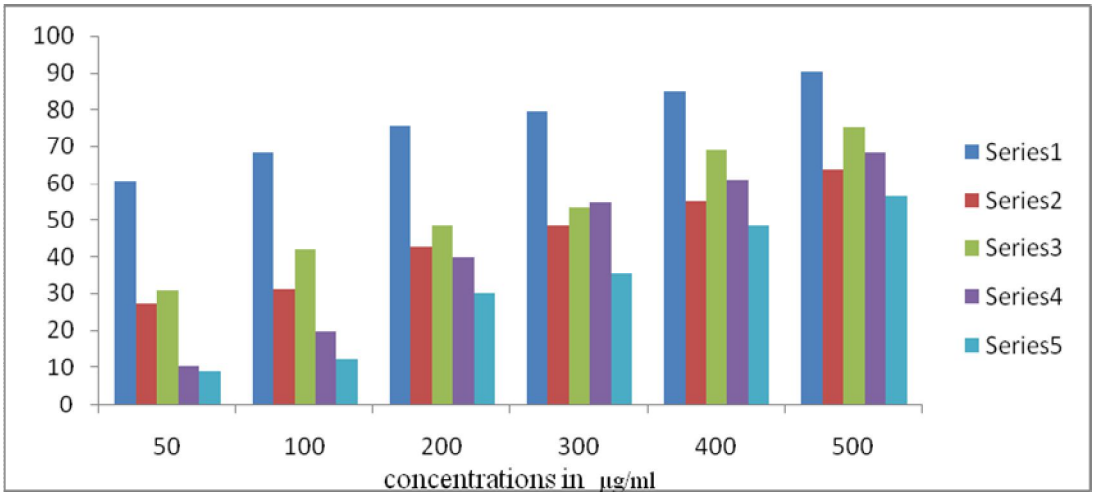
Table I: Invitro antioxidant activity of aqueous extract of Annona squamosa leaves, by various models,(values are given in mean ±SE)

Concentration in µg/ml	DPPH	Nitric Oxide	Hydroxyl Radical	Superoxide Radical	Lipidperoxidation inhibition
50	60.54±2.51	27.07±1.28	30.58±1.17	10.26±0.44	8.63±0.34
100	68.13±1.28	31.15±0.90	41.95±0.25	19.6±0.26	12.08±0.46
200	75.59±1.07	42.61±1.07	48.29±0.52	39.57±0.59	29.88±0.50
300	79.58±0.69	48.48±1.20	53.31±0.88	54.73±0.43	35.14±0.26
400	85.14±1.51	54.99±1.05	68.94±0.59	60.94±0.35	48.42±0.28
500	90.16±1.70	63.92±0.72	75.20±0.55	68.19±0.25	56.33±0.50

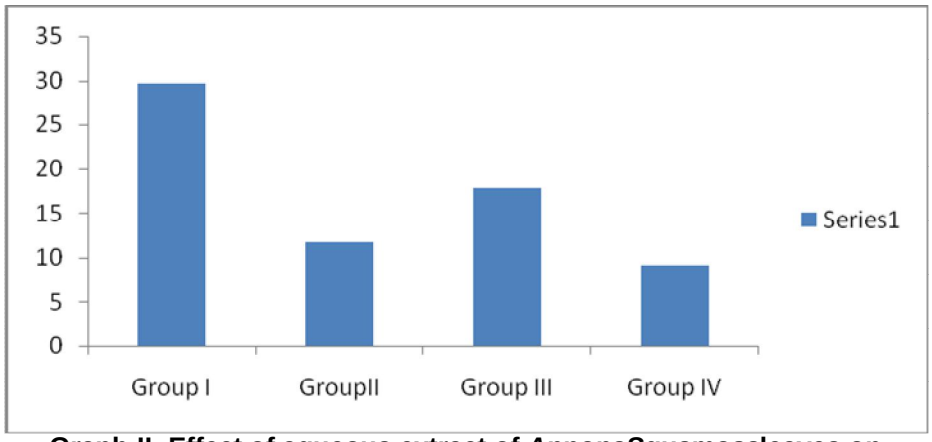
Table II: Effect of aqueous extract of *Annona Squamosa* leaves on indomethacin induced gastric mucosa damage

Groups	Gastric Content (ml)	pH (gastric juice)	Total Acidity (meq/L)	Mean Ulcer Index	% Inhibition of Ulcer
Group I (treated with 1% sodium CMC orally)	5.48±0.52	1.80±0.08	98.66±24.54	29.63±1.54	-
Group II (treated with omeprazole 10mg/kg orally)	2.68±1.08	3.03±0.15	35.83±15.30	11.66±0.87	60.64%
Group III (treated with aqueous extract of *Annona Squamosa* leaves 150 mg/kg orally)	4.26±0.76	2.05±0.18	77.5±7.14	17.83±1.53	39.82%
Group IV (treated with aqueous extract of *Annona Squamosa* leaves 300 mg/kg orally)	3.05±0.44	2.63±0.30	48.66±14.78	9.16±3.10	69.08%



Graph- I: Invitro antioxidant activity of aqueous extract of Annonasquamosa leaves, by various models (% inhibition given in the graph)

Series 1: DPPH
Series 2: Nitric Oxide
Series 3: Hydroxyl Radical
Series 4: Superoxide Radical
Series 5: Lipidperoxidation



Graph-II: Effect of aqueous extract of *AnnonaSquamosa*leaves on indomethacin induced gastric mucosa damage. (Ulcer index)

Series 1: Mean ulcer index

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