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Research Article

Evaluation of In-vitro Antioxidant and Antimutagenic Effect of *Annona squamosa* Leaves Extract

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Abstract

Green medicine, the drugs derived from plants attained a wide spread interest, as believed to be safe and dependable, compared with costly synthetic drugs that have adverse effects. In this study, *Annona Squamosa* (*A. squamosa*) have been investigated for their phytochemical, antioxidant, antimutagenic activities. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenol were determined by the well-known test protocol available in the literature. The extraction yield was found in the order: methanol>water>ethyl acetate>hexane. The extracts found to possess saponin glycosides, cardiac glycosides, phenolics, terpenoids, sterols, and flavonoids. The total phenolic content of hexane, ethyl acetate, methanolic and aqueous extract of *A. squamosa* were 3.04±0.37, 5.83±0.29, 31.42±2.35, and 27.21±1.06 GAE mg/g, respectively. The methanolic extracts exhibited highest amount of total polyphenol content compared to all other solvent extracts. The ethyl acetate, methanolic and aqueous extracts were evaluated for their antioxidant potential in terms of DPPH radical scavenging capabilities. The scavenging was found to dose dependent with IC_{50} value of 185.24, 103.09 and 160.17 µg/ml for the ethyl acetate, methanol and aqueous extracts respectively. The antimutagenic potential of the methanolic extract was studied using Ames test using T98 strain of *Salmonella typhimurium* applying NPD as the mutagen. The number of reverant colonies were counted using a digital colony counter and the reduction in colonies was calculated as the activity (%) of the extract at four doses. The reverent colonies were visible as lawn like structures on the surface of the plate and were significantly lower compared to positive control in the extract plate and the negative control. The extract was able to control the growth of reverant colonies upto 79.30% in comparison to the mutagenic control. The results show that the methanolic extract of *A. squamosa* leaf was able to exhibit a dose regulated antimutagenic activity by inhibiting the growth of His+ reverant colonies of *Salmonella typhimurium*.

Keywords: Green medicine, *Annona Squamosa,* Phytochemical, Antioxidant, Antimutagenic activities, *Salmonella typhimurium*

INTRODUCTION

Medicinal plants and plant derived products are known to play significant role in primary healthcare and in the management of various ailments since long times. Bioactive plant extracts are the integral part of traditional system of medicine and often exhibit multiple biological activities including antioxidant and antimutagenic activities. This has led the researchers for the systemic evaluation of various biological properties of medicinal plants. Reactive oxygen species (ROS) are known to be involved in the onset of cancer and other pathophysiological conditions. The most susceptible target of free radicals are the lipids, proteins and DNA that causes the formation of malondialdehyde, 4-hydroxynonemal, carbonyl moieties, and DNA damage; leading to disturbance in genetic stability. They may cause DNA-protein crosslinking, alterations in nitrogenous bases and strand breaking leading to DNA mutations. The DNA damage caused by the different types of ROS is implicated in the complex process of mutagenesis and considered as primary step involved in the onset of different type of cancers. This has led to the discovery and characterisation of various antioxidant and antimutagenic agents. Several studies have demonstrated the role of medicinal plants in chemoprevention of ROS associated problems. Many plant-derived phenolic compounds such as flavonoids, anthocyanins, catechins, etc., among other secondary metabolites, have shown strong antioxidant and antimutagenic activities. A direct correlation between phenolics and antioxidant activity of the plant has been reported earlier. Various mechanisms proposed for these phenolics against ROS action includes the reduction reaction, proton donation, metal chelation and acting as singlet oxygen and nitrogen quencher. The precise mechanism of bioactive plant extracts and phytocompounds exhibiting antimutagenic activity is still unclear. It has been proposed that antimutagenic activity is due to the ability of phytocompounds to form a stable complex with mutagens and neutralizing them, secondly it can activate the cellular detoxification system and, thirdly by neutralizing ROS directly (acting as an antioxidant). Recent studies have also highlighted the role of plant extracts/phytocompounds as protective barrier between DNA and an attacking mutagen or carcinogen. Considering the damaging health effects of ROS and free radicals, there is need to systematically evaluate the rich diversity of Indian medicinal plants for potential broad-spectrum antimutagenic and antioxidant properties. Previously, we have reported the antioxidant and antimutagenics activities of several medicinal plant such as *Carum copticum, Murraya koenigii, Punica granatum, Psidium guajava, Piper cubeba, Syzygium cumini*[1]. The *A. squamosa* are family Annonaceae that was reported with 130 genus and almost 2500 species [2], where it can be found in

tropical climate of Africa, Australia, South America and Indonesia for food consumption and traditional herbs. *A. squamosa* has been used to treat diarrhea, influenza, cough, colon infection and the leaves was used as wound treatment [3,4]. Besides, *A. squamosa* can be used for other food processing [5], juice, candy and sweets[6]. Research on *A. squamosa* has been carried out based on its leaf, bark, roots, fruits and seeds, where *A. squamosa* reported to demonstrate an antioxidant, anticancer, antidiabetic, antihypertension, hepatoprotective, antiparasitic, antimalaria, insecticide and antimicrobes [7]. The secondary metabolite of *A. squamosa* extracts contain several components such as saponin, alkaloid, coumarin, terpenoid, tannin, phenol and flavonoid. The secondary metabolite is potential as antioxidant, which containing phenolic groups. In addition, Al-Nemari et al. (2020) reported that methanolic extract of *A. squamosa* leaves contain sesquiterpenes, diterpene alcohol, triterpene and ketone. However, most of the component are sesquiterpenes hydrocarbon. Coupled with our continuous interest of pharmacological screening of Indian medicinal plants, in this study we aimed to investigate the antioxidant and antimutagenic activities of the methanolic extracts of leaves of *A. squamosa.*

MATERIALS AND METHODS

Plant materials

Collection and identification of the plant material

The presence of phenolics and flavonoids in *A. squamosa* has been reported in literature and the plant parts have been widely investigated for various pharmacological actions. Only a single report of antimutagenic action of the plant was found. This was the main reason for the selection of the plant for the present investigation. The leaves of *A. squamosa* were collected from the local places of Bhopal, Madhya Pradesh in the month of November and authenticated.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals and solvent used in this study were of analytical grade. The pathogenic microbes used in the current study were obtained from Microbial Culture collection, National Centre Forcell Science, Pune, Maharashtra, India.

Preparation of the plant material

The authenticated plant leaves were washed with distilled water, dried under shade and powdered using a blender at low speed. The powdered leaves were stored in air tight container until taken for use.

Extraction of leaves

The powdered leaves were used for the extraction process. 114 gm of powder was evenly packed in the extractor of the Soxhlet apparatus and extracted successively with various solvents of increasing polarity including hexane, ethyl acetate, methanol and water by hot continuous extraction process for about 7-12 h. The extracts were filtered while hot through to remove any impurity. The extracts were concentrated by rotary vacuum evaporation to reduce the volume to 1/10. The concentrated extracts were transferred to 100 ml beaker and the remaining solvents were evaporated on water bath. The oleo-resinous extracts were collected and placed in desiccators to remove the excessive moisture**.** The dried extracts were stored in desiccators for further processing [8, 9].

Phytochemical screening

Phytochemical screening to detect the presence of bioactive agents was performed by standard procedures[10, 11]. After the addition of specific reagents to the solution, the tests were detected by visual observation of colour change or by precipitate formation.

Total phenolic content

The extraction of phenolic compounds was based on a modified method by *Olufunmiso et al* [12]. Briefly 100 mg dried extract was macerated overnight with 10 ml of methanol and filtered. The solution was stored at 4°C in amber bottles and served as the stock solution (10 mg/ml) for subsequent analyses. For total phenolic content determination, 200 µl of sample was mixed with 1.4 ml purified water and 100 µl of Folin-Ciocalteu reagent. After 2 min of incubation at room temperature 300 µl of 20% Na_2CO_3 aqueous solution was added and the mixture allowed to incubate in dark for 2 h. The absorbance of the colored solution was measured at 765 nm with a UV-Vis spectrophotometer. Standard solutions of gallic acid (10-100ppm) were similarly treated to plot the analytical curve. The control solution contained 200 µl of methanol and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of the dry sample.

DPPH scavenging assay

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH. Briefly, 1mM solution of DPPH and extract solution (50-250µg/ml) were prepared in ethanol separately. 1.5ml of the extract solution was added to 1.5 ml of DPPH solution. The absorbance of the purple color of solution was measured at 517 nm against the corresponding blank solution which was prepared using 3 ml ethanol. The control sample used was 3 mL of DPPH. The assay was performed in triplicates. Percentage inhibition of free radical DPPH was calculated based on control reading by following equation[13].

$$\text{DPPH scavenged (\%)} = \frac{(A_{con} - A_{test})}{A_{con}} \times 100$$

A_{con} - is the absorbance of the control reaction

A_{test} - is the absorbance in the presence of the sample of the extracts.

Antimutagenic assay

Ames test

The antimutagenic activity of the extract was determined by using Salmonella histidine point mutation assay proposed by Maron and Ames (2015)[14]. *Salmonella typhimurium* strains (MTCC-98) were purchased from Institute of Microbial technology, Chandigarh and cultured using nutrient broth. Two modes of experimentation were followed for testing antimutagenic action *viz.*, co-incubation and pre-incubation. In co-incubation, 0.1 ml of bacterial culture, 0.1 ml of 4-nitro-o-phenylenediamine (NPD, 200µg/ml in DMSO) and 0.1 ml of extracts (100, 500, 1000 and 2500 µg/ml) were added to 2 ml of top agar containing 0.5 mM histidine/biotin. In the pre-incubation mode of experimentation, equal volumes of the mutagen and the extracts were mixed in sterile capped tubes and allowed to stand for 30 min at 37°C under continuous shaking and 0.2 ml of this was added to 2ml of soft agar with 0.1 ml of fresh *Salmonella* culture. The plates were tilted and rotated quickly to ensure speedy and uniform spreading of top

agar and incubated at 37°C for 30 min. The soft agar was poured on a minimal glucose agar plate. The plates were tilted and rotated quickly to ensure speedy and uniform spreading of top agar and incubated at 37°C for 48 h. Concurrently, a positive control (where mutagen but no extract was added) and a negative control (where no mutagen was added) were also set[15]. Revertant colonies in each case were counted after 48 h. The activity of each extract was expressed as the percentage decrease of reverse mutation.

$$Activity\ (\%) = {a - b\ \times 100}/{a - c}$$

Where a=No. of histidine revertants induced by mutagen (NPD); b = No. of histidine revertants induced by mutagen in the presence of extract; c = No. of revertants in the negative control

RESULTS AND DISCUSSIONS

The crude extracts so obtained after each of the successive soxhlation extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The extraction yield of the leaf using different solvents is presented in Figure 1. The extraction ability of different solvents was found in the order: methanol>water>ethyl acetate>hexane. The extracts found to possess saponin glycosides, cardiac glycosides, phenolics, terpenoids, sterols, and flavonoids Table 1. The total phenolic content of the hexane, ethyl acetate, methanolic and aqueous extract of *A. squamosa* were evaluated by Folin-Ciocalteau method. The total phenolic content of hexane, ethyl acetate, methanolic and aqueous extract of *A. squamosa* were 3.04±0.37, 5.83±0.29, 31.42±2.35, and 27.21±1.06 GAE mg/g, respectively. The methanolic extracts exhibited highest amount of total polyphenol content compared to all other solvent extracts Table 2 & Figure 2. The ethyl acetate, methanolic and aqueous extracts were evaluated for their antioxidant potential in terms of DPPH radical scavenging capabilities. The scavenging was found to dose dependent with IC_{50} value of 185.24, 103.09 and 160.17µg/ml for the ethyl acetate, methanol and aqueous extracts respectively Table 3 & Figure 3. The antimutagenic potential of the methanolic extract was studied using Ames test using T98 strain of *Salmonella typhimurium* applying NPD as the mutagen. The number of reverant colonies were counted using a digital colony counter and the reduction in colonies was calculated as the activity (%) of the extract at four doses. The reverant colonies were visible as lawn like structures on the surface of the plate and were significantly lower compared to positive control in the extract plate and the negative control. The extract was able to control the growth of reverant colonies up to 79.30% in comparison to the mutagenic control Table 4, 5 & Figure 4, 5.

Table1: Phytochemical screening of *Annona squamosa* leaf extracts

Chemical Tests	Observation	Hexane extract	Ethyl acetate extract	Methanolic extract	Aqueous extract
Alkaloids					
Mayer's reagent	cream colour precipitate	-	-	+	++
Hager's reagent	yellow colour precipitate	-	-	+	++
Wagner's reagent	reddish brown precipitate	-	-	+	++
Dragendorff's reagent	reddish brown precipitate	-	-	+	++
Glycosides					
Froth test	Frothing is seen	**-**	**-**	**++**	**++**
Kedde's Test	No color	-	-	-	-
Bontrager's Test	Rose pink or red color in the ammonical layer not found	-	-	+	+
Keller-Kiliani	No color in acetic acid layer	-	-	-	-
Phenols/Tannins					
Ferric chloride	Blue green color	-	-	+	++
Gelatin Solution	White precipitate	-	-	++	++
Alkaline reagent test	Yellow to red precipitate	-	-	+	++
Vanillin HCl test	Purplish red color	-	-	+	++
Flavonoids					
Shinoda test	red color	+	+	+	++
Alkaline reagent test	Yellow color that turns red on acidification	+	+	+	++
Zinc HCl reductino test	red color	+	+	++	++
Proteins					
Millon's Test	white precipitate, turns red on heating	-	-	+	+
Ninhydrin Test	Violet color	-	-	+	+
Sterols/triterpenoids					
Liberman-Burchard Test	Brown ring at junction Upper layer turns green	-	-	+	+
Salkowski Test	Yellow color in lower layer	-	-	+	+

Table 2: Total phenolic content

Extract	Total phenolic content (GAE mg/g)
Hexane	3.04±0.37
Ethyl acetate	5.83±0.29
Methanol	31.42±2.35
Aqueous	27.21±1.06

Data expressed as gallic acid equivalent (GAE) mg per gm of the extract, Values are mean ± SEM of triplicate determinations

Table 3: DPPH Scavenging potential of ethyl acetate, methanol, aqueous extract

Conc (µg/ml)	DPPH Scavenging %			
	Ascorbic acid	Ethyl acetate Extract	Methanol Extract	Aqueous Extract
50	91.5±0.18	9.2±0.25	24.1±0.61	16.7±0.35
100	-	25.1±0.91	47.7±0.85	25.4±0.61
150	-	33.6±0.68	84.3±0.75	46.8±0.87
200	-	58.5±0.29	102.5±0.37	61.1±0.54
250	-	69.4±0.37	121.1±0.36	82.9±0.62

Values are mean ± SEM of six determinations

Table 4: No of reverant colonies of T98

Plate observed	Dose (µg/ml)	No. of reverant colonies
Positive control	-	1013 ± 23.007
Negative control	-	69.66 ± 6.658
Co-incubation with extract	100	836 ± 9.848
	500	753.33 ± 12.013
	1000	612.33 ± 11.060
	2500	265 ± 9.165
Pre-incubation with extract	100	931 ± 91.65
	500	774 ± 9.643
	1000	648 ± 7.000
	2500	283 ± 9.165

Table 5: The percent reduction in the reverant colony formation was calculated as the activity percent of the extract at various doses

Observed Plate	Dose (µg/ml)	Activity (%)
Positive control	-	
Negative control	-	
Co-incubation with extract	100	18.82
	500	27.57
	1000	42.51
	2500	79.30
Pre-incubation with extract	100	8.75
	500	25.38
	1000	38.73
	2500	77.40

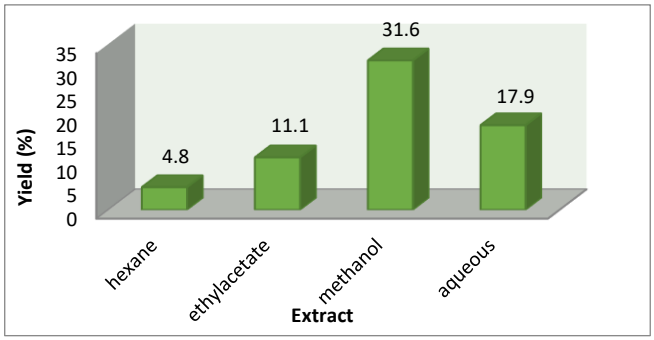

Figure 1: Extraction yields of various solvents

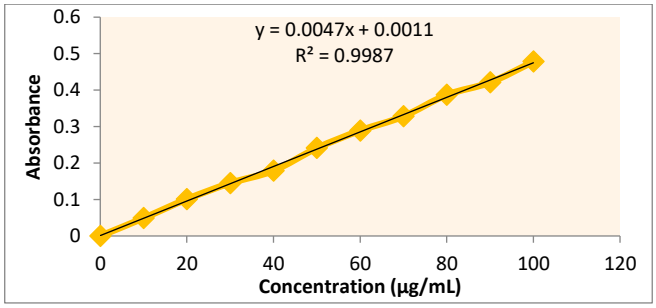

Figure 2: Calibration curve of gallic acid

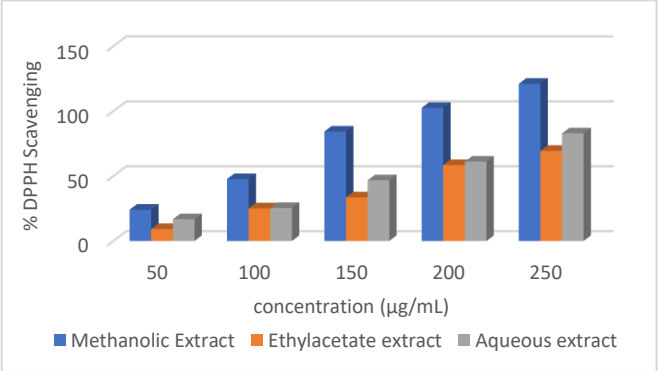

Figure 3: % DPPH scavenging potential of various extracts of *Annona squamosa*

Figure 4: Reverant colonies in positive control

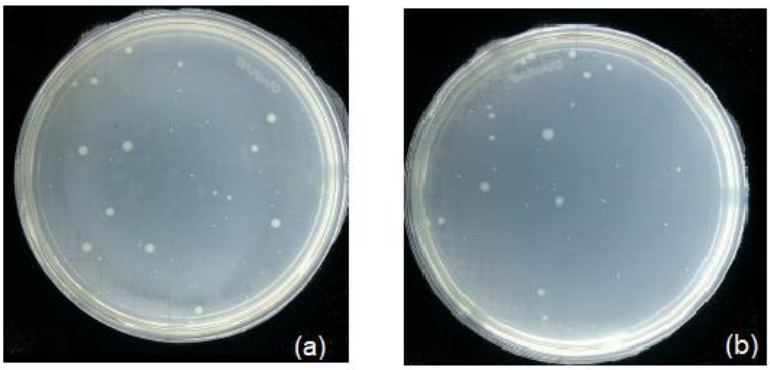

Figure 5: Reverant colonies in (a) negative control (b) extract treated

CONCLUSION

In the present work Ames test was utilized to study the antimutagenic potential in the leaf extract of *A. squamosa*. The antioxidant activity revealed high DPPH scavenging potential in the methanolic extract and hence the methanolic extract was used for antimutagenic study. The results show that the methanolic extract of *A. squamosa* leaf was able to exhibit a dose regulated antimutagenic activity by inhibiting the growth of His+ reverant colonies of *Salmonella typhimurium*.

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