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Screening Of Total Phenolics, Antihemolytic, Antioxidant Properties, And Gc-Ms Profiling Of Extracts Of *Annona Squamosa* L. Leaves.

Sonawane S.R.[1*]

[1*]Research Scholar, Department of Zoology, D.B.F Dayanand College of Arts and Science, Solapur (rutugandh.25@gmail.com)

***Corresponding Author:- Sonawane S.R.**

[*]Research Scholar, Department of Zoology, D.B.F Dayanand College of Arts and Science, Solapur (rutugandh.25@gmail.com)

ABSTRACT

The present study was carried out for the identification of phytoconstituents present in *Annona squamosa* L. leaves and also to determine the total phenolic content, and antioxidant and antihemolytic properties of leaf extract. The total phenolic content of the methanolic extract was carried out by using Folin Ciocalteu method which was 256.99±8.78 (mg/g GAE). The antihemolytic activity carried out on rat erythrocytes revealed 87.76% percentage inhibition activity (IC_{50} 163.40µg/mL). The extract of *Annona squamosa* L. was found to have the most potent anti-oxidant properties in DPPH and H_2O_2 radical scavenging methods. IC_{50} value DPPH and H_2O_2 inhibition of extract were 189.39 µg/mL and 219.01 µg/mL respectively. The GC-MS study was also carried out and which showed the presence of phytochemicals like Neophytadine (RT: 24.39), 2-Pentadecanone, 6,10,14-trimethyl-(RT:21.74).

INTRODUCTION

Annona squamosa L. belongs to the family Annonaceae and is commonly known as custard apple. It is an important tropical fruit cultivated in South and Central America, West Indies, Peru, Brazil, India, Mexico, Bahamas, Egypt, and Bermuda. Leaves of *Annona squamosa* L. plants have been studied for their health benefits, which are attributed due to the presence of several phytochemical compounds such as alkaloids, steroids, saponins, terpenoids, and phenolics,. *Annona squamosa* L. extract has been studied for several biological activities, including antimicrobial, anticancer, antioxidant, antidiabetic, antiobesity, and hepatoprotective functions (Ma *et al*.,2017; Nugraha *et al*., 2019).

Reactive oxygen species (ROS) are oxygen-free radicals and play a dual role of being both toxic and beneficial to biological systems (Islam *et al.,*2012). ROS can destroy cellular macromolecules like proteins, membrane lipids, and DNA causing hazardous effects such as reduced enzyme activity, dysfunction of structural proteins, lipid peroxidation, and oxidative DNA damage. Oxidative stress is a phenomenon describing a disturbance in the equilibrium between reactive oxygen species

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(ROS) production and the antioxidant defense mechanisms (McCord, 2000). This oxidative stress may lead to various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia, aging, and regulation of intracellular signal transduction. Erythrocytes are tremendously susceptible to oxidative damage as they are continuously exposed to high concentrations of oxygen (Halliwell and Gutteridge 19840. ROS generation in cellular metabolism leads to the peroxidation of cell membrane lipids causing membrane damage and the consequent hemoglobin leakage. Peroxidation of cellular membranes induced by ROS is a major event in oxidative damage in living systems(Yunfeng *et al.,* 2006). It is believed that due to the presence of high content of polyunsaturated fatty acids in the erythrocyte cell membrane, these serve as an acceptable model to study lipid peroxidation. Many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective antioxidants having the potential to neutralize free radicals but they may possess some side effects and toxic properties to human health (Mengi and Deshpande 1999). Therefore, many researchers are interested in medicinal plants for the evaluation of antioxidant phytochemicals compounds such as phenols, alkaloids, flavonoids, and tannins as their potential role in the prevention of numerous human diseases. The present experiments are carried out to evaluate the antihaemolytic and antioxidant potential of extracts obtained. *Annona squamosa* L. leaves in-vitro. Efforts are also made to estimate the phenolic content of the extracts.

MATERIAL AND METHODS

Collection and identification of medicinal plants

Annona squamosa L. plant fresh leaves were collected from Solapur region. Identification was by a botanist at D.B.F. Dayanand College of arts and science, Solapur. Washed thoroughly with tap and distilled water and shaded and dried for 4-5days.

Extraction of medicinal plant parts

Shade-dried leaves were powdered using an electric grinder and powdered plant material was extracted using pure cold methanol for successive 7 days (1 kg/lit), as per the methodology described by Dama *et al.*1999. The contented extracts were cleaned through Whatmann's filter paper. Extraction was done using the methodology agreed upon by Dieu *et al.,* 2019.

Total phenolic content:

The total phenolics content of crude extracts of selected plant was determined using the Folin-Ciocalteu reagent and expressed in terms of Gallic acid equivalent (mg/g dry wt) (Ebrahimzadeh *et al.* 2010).

Partial purification of crude medicinal extracts using Column chromatography

All extracted and screened medicinal plant extracts were partially purified by column chromatography using Hexane - Acetone eluents, a methodology described by Dama *et al.* 1999.

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Antihaemolytic activity:

For the present research work, male wistar rats were obtained from 'Crystal biological solutions, Pune, and housed as per CPCSEA guidelines laid down by (CPCSEA, 2003). All animals were fed with adequate pelleted food supplied and fresh water.

Preparation of rat erythrocytes: Rats in the body weight range of 200-250g. The animals were sacrificed under anesthesia and blood is collected by heart puncture in heparinized tubes. Erythrocytes were isolated, stored, and centrifuged (1500×g, 10 min) at 4ºC, erythrocytes were separated from the plasma and buffy coat and are washed three times by centrifugation in 10 volumes of 10 mM phosphate buffered saline (pH 7.4; PBS). Washed erythrocytes stored at 4ºC and used within 6 h for further studies.

The antihaemolytic activity of extract against H_2O_2-induced hemolysis: The rat erythrocyte hemolysis is performed with H_2O_2 as a free radical initiator. The inhibition of rat erythrocyte hemolysis by the extract was evaluated according to the procedure described by Ebrahimzadeh *et al* 2010. The absorbance of the resulting supernatants was measured at 540 nm by spectrophotometer to determine the hemolysis. The inhibitory effect of the extract was compared with standard antioxidant Vitamin C. Percentage of hemolysis is calculated by taking hemolysis caused by 100 µM H_2O_2 as 100%. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

Antioxidant Activity:

DPPH radical-scavenging activity:

The antioxidant capacity of active fractions of plant extract was confirmed by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay according to a standard method by Dehpour *et al.,* 2009. The absorbance is recorded at 517 nm. Vitamin C was used as standard control. IC_{50} values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radicals.

Inhibition (%) = [(Ac − As)/Ac] × 100,

where, As is the absorbance of methanolic extract at different concentrations. Ac is the absorbance of control. The experiment is repeated for three times.

Hydrogen peroxide scavenging activity

The scavenging activity of active fractions extracts towards hydrogen peroxide radicals was determined by the modified method of Eslami *et al,* 2009. The absorbance of H_2O_2 at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H_2O_2.

H_2O_2 Scavenged (%)= [(Ao −A1)/Ao] × 100,

where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard. The experiment was repeated in triplicate.

Statistical analysis: Experimental results were expressed as means ± SD. All measurements were replicated three times.

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GC-MS Analysis of Plant Extracts

The mass spectrum of unknown components was compared with the spectrum of the known components stored in the NIST and Wiley library. Interpretation of the mass spectrum of GC-MS was done using a database of NIST library having more than 75,000 compounds (software-Turbomas 5.2). The name, molecular weight, and structure of components were then ascertained. The relative percentage was calculated by comparing its average peak area to the total area.

RESULTS

Collection and identification of medicinal plants

For the present research work, medicinal plants were collected from western parts of Maharashtra, India. The collected plant materials were identified by a botanist from the Department of Botany, and the Herbarium sheaths were deposited in the Department of Zoology, D.B.F Dayanand College of Arts and Science Solapur. The shade-dried plant material was then ground in fine powder for extraction.

Extraction of medicinal plant parts

Extraction of plant materials was done as per the methodology discussed by Dama *et al.* 1999.

Partial purification of crude extracts using Colum chromatography

The extracted plant extract was partially purified by using column chromatography, the methodology described by Dama *et al*. 1999. Using Hexane - Acetone eluents, five fractions were obtained. From these, fractions C, D, and E show high bio-active metabolites in it hence for the present work these fractions were chosen for further animal studies.

Animal collection and maintenance of albino mice

For the present research work 33 male wistar rats. The animal experiments conducted during the present study were approved by the Institutional Animal Ethical Committee (IAEC). The animal study was carried out at Crystal Biological Solutions, Pune (MS) report no: CRY/2021/025; CPCSEA Registration No. 2030/PO/RcBiBt/S/18/CPCSEA.

Total phenolic content:

Total phenolic compounds of crude extract of Lam. plant, determined by Folin Ciocalteu method, were reported as gallic acid equivalent.

Plant extract	Total phenolic content (mg/GAE/gm)
Annona squamosa L.	256.99±8.78

Partial purification of crude medicinal extracts:

All extracted medicinal plant extracts were partially purified by using column chromatography using hexane acetone fractions. From these, fractions with high bioactive metabolites were chosen for further animal studies (Valasange, 2019).

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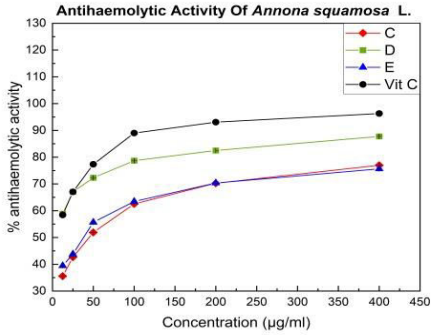
Antihaemolytic activity: The active fractions of selected medicinal plants were screened for antihaemolytic activity at a concentration range from 12.5ug/mL to 400ug/mL. The result indicates that *Annona squamosa* L. exhibited a good antioxidant potential of 87.76 ±0.15% at a concentration of 400 µg/mL as compared to extracts compared with Vitamin C as a standard.



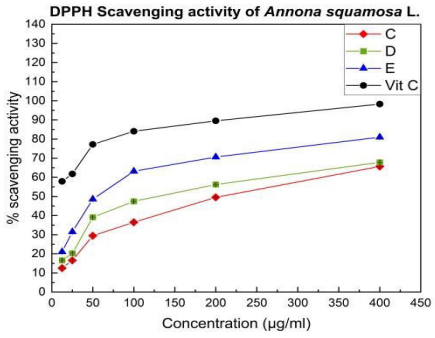
The *Annona squamosa* L plant extracts possess higher antihaemolytic activity in fraction D least fraction C against H_2O_2-induced hemolysis on rat erythrocytes as compared with standard Vitamin C.

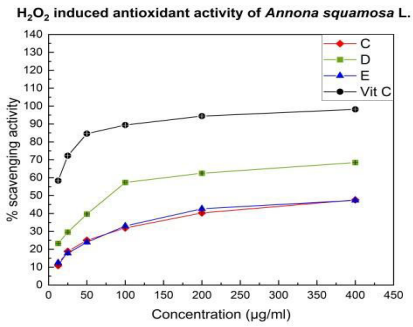
Antioxidant Activity: The active fractions of selected medicinal plants were screened for antioxidant activity at a concentration range from 12.5ug/mL to 400ug/mL.

DPPH radical-scavenging activity:-

When DPPH, a stable free radical becomes paired off in the presence of a hydrogen donor and is reduced to the DPPHH and as a consequence decrease in absorbance from the DPPH to DPPHH (Sharma and Garg 2009).

Hydrogen peroxide scavenging activity:





Antioxidant activity: *Annona squamosa* L. plant extracts possess higher DPPH Scavenging activity in fraction E and least in fraction C as compared with standard Vitamin C, whereas fraction D possesses higher H_2O_2 scavenging activity and least activity was recoded in fraction E and C as compared with standard Vitamin C.

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Determination of IC_{50} values

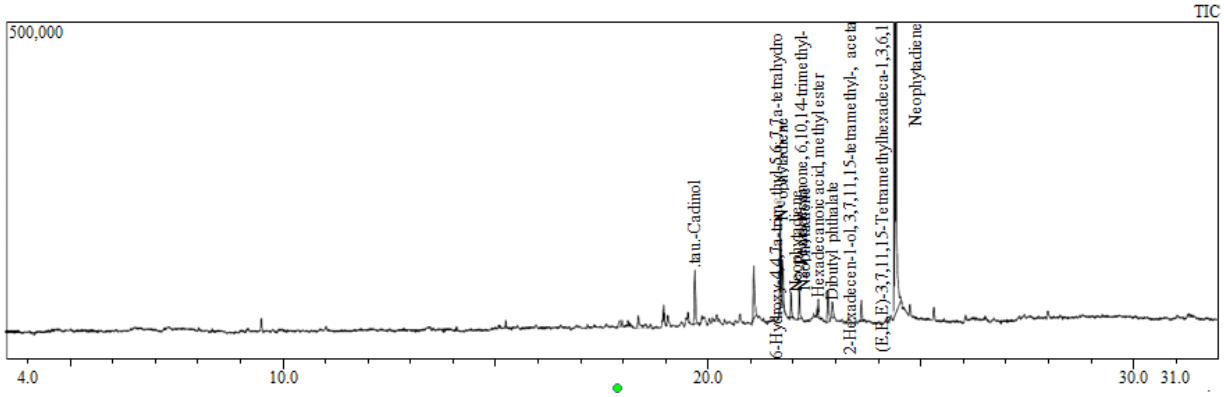
Table 4: IC_{50} values of antihemolytic and antioxidant activity of *Annona squamosa* L. plant extracts

Plant	IC_{50} (µg/ml)		
	DPPH	H_2O_2 scavenging activity	Antihemolytic activity
Annona squamosa L.	163.40	219.01	189.39
Vit. C	145.01	143.68	144.93

GC-MS Analysis of Plant Extracts

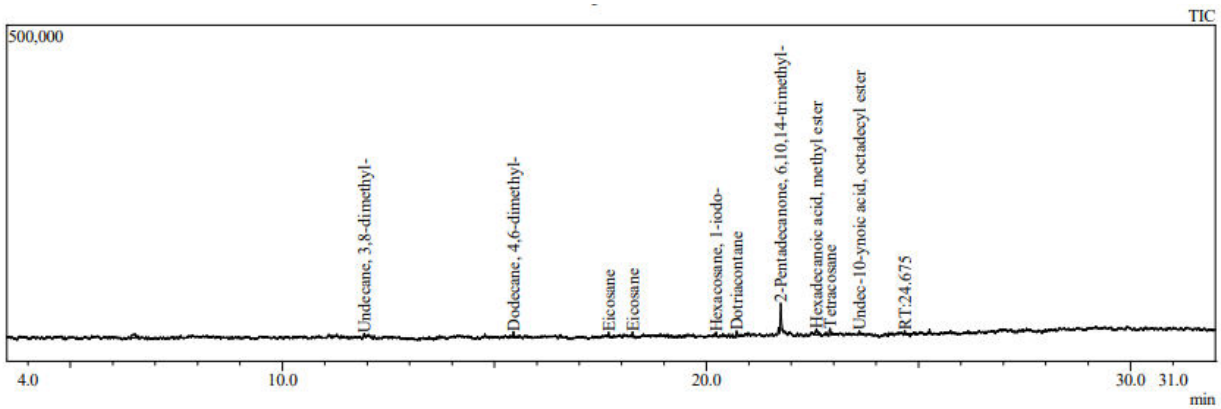
Annona squamosa L. plant extract fractions with the highest antihaemolytic and antioxidant activity were subjected to GC-MS analysis.

The GC-MS Chromatogram of *Annona squamosa* L. Fraction D.



The GC-MS chromatogram of *Annona squamosa* L. shows compounds present in plant extract as follows-. Cadinol (RT: 19.688), 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydro benzofuran-2 (4H)-one (RT: 21.075), 2-Pentadecanone, 6,10,14-trimethyl- (RT: 21.74), Hexadecanoic acid, methyl ester (RT: 22.59), 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, acetate, [R-[R*,R*-(E)]]- (RT: 22.81), (E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene (RT:23.60), Neophytadiene (RT:24.39) with percentage area 69.33%.

The GC-MS Chromatogram of *Annona squamosa* L. Fraction E



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the GC-MS chromatogram of *Annona squamosal* L. shows compounds present in plant extract as follows-Undecane, 3,8-dimethyl- (RT:11.93), Dodecane, 4,6-dimethyl-(RT:15.44), Eicosane (RT: 17.69), Eicosane (RT: 18.26), Hexacosane, 1-iodo-, Dotriacontane (RT:20.22), 2-Pentadecanone, 6,10,14-trimethyl-(RT: 21.75), Hexadecanoic acid, methyl ester (RT: 22.59), Tetracosane (RT: 22.91), Undec-10-ynoic acid, octadecyl ester (RT: 23.60).

DISCUSSION

The toxicity of substances in the body depends on the nature of the substances, dose, and time of exposure. The direct hemolysis of these toxic substances may be due to several mechanisms, causing hemolysis through the dissolution of the erythrocyte plasma membrane which ruptures due to increased fragility or due to osmotic lysis caused by increased permeability of the plasma membrane and ultimately cell damage (Shobana and Vidhya, 2016). The medicinal plant *Annona squamosa* L. extract reported antihaemolytic activity in different concentrations in different fractions manner. Among all grades, the maximum inhibition of rat erythrocyte hemolysis was reported in Fraction D at a concentration of 400 µg/mL as 87.76 ±0.15% and for Vitamin C it was 96.30 ±0.93% with an IC_{50} value of 189.39 µg/mL. Findings from our study agree with the findings of Rengasamy *et al.,* 2013 where the extracts of various plants tested showed low hemolytic effects against human erythrocytes. Some reports improve that high total phenol and tannin contents in the extract led to its potent antihemolytic activity (Robertis and Robertis, 1995).

Cellular damage in the human body may induce the generation of increased levels of free radicals. Various disorders including cancer, myocardial infarction, atherosclerosis, and neurogenerative disorders are mainly correlated to these free radicals (Swamy *et al.,* 2015). Antioxidants are chemical compounds having the ability to prevent various oxidative stress-related cell damages mediated by ROS. Many antioxidant molecules from several medicinal plants are identified and will be beneficial in the treatment of several human diseases (Mohanty *et al.,* 2014). In this study, DPPH and Hydrogen peroxide radical scavenging activity was observed to be concentration dependent and corroborates with the previous reports on several other plant species (Swamy *et al.,* 2015; Gonbad *et al.,* 2015 and Naz *et al.,* 2013). The reduction of DPPH radical by *Annona squamosa* L. plant extract indicates that, among all fractions, Fraction E exhibited good antioxidant potential of 80.99 ±0.16% at a concentration of 400 µg/mL and Vitamin C showed 98.29 ±0.39% with IC_{50} value 163.40 µg/mL. The H_2O_2-scavenging activity of *Annona squamosa* L. medicinal plant indicates that, among all fractions, Fraction D exhibited the moderate antioxidant potential of 68.47 ±0.32% at a concentration of 400 µg/mL and Vitamin C showed 98.16 ±0.06% with 219.01 µg/mL IC_{50}.

The superior radical scavenging potential of plant extracts may be interrelated to the presence of various antioxidants such as polyphenolic compounds. The GC-MS of active fraction revealed the presence of phytochemicals like Neophytadine (RT: 24.39), 2-Pentadecanone, 6,10,14-trimethyl-(RT:21.74). Neophytadiene is considered to be a good analgesic, cardioprotective properties, antipyretic, anti-inflammatory, antimicrobial, and antioxidant compound (Bhardwaj, 2020).

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CONCLUSION

The observations and findings from the above study depict a significant linear correlation between the concentration of phenolic compounds and performed phytochemical activities. *Annona squamosa* L. extract showed the potential ability to scavenge free radicals and also effective against oxidative stress-induced hemolysis as it showed an elevated level of phenolic compounds contributing such properties. GC-MS study also contributes to the identification of potent phytochemicals responsible for bioactivities.

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