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Phytochemical differences of *Annona squamosa* and *Annona reticulata* spp. and evaluation of their anti-cancer properties in colon cancer: a comparative study

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Abstract- Phytochemicals are plant products produced as a result of primary or secondary metabolism which is used by plants for various purposes such as pigment or as a defence against insects and predators. Phytochemicals are being increasingly utilized for medicinal purposes due to the presence of bioactive compounds in them. The phytochemical analysis of the leaf extracts was performed both qualitatively and quantitatively with the respective methods and the differences in the phytochemical status of both the plant extracts were determined. The anti-oxidative capacity of the leaf extracts was measured by DPPH radical scavenging activity. The presence and characterization of bioactive active compounds were assessed by Thin Layer Chromatography. The anti-tumorigenic potential of the leaf extracts was assessed by the tetrazolium dye method viz. MTT assay. The anti-tumorigenic potential of the leaf extracts was analyzed in three colon cancer cell lines viz., HCT-15, COLO-205, and HT-29 and in normal human keratinocyte (HaCaT) cell line.

Key words: Phytochemicals, 2,2, Diphenyl picryl hydrazine, MTT, inhibitory concentration, TLC

INTRODUCTION

Colon cancer is the second most frequently observed cancer in women and the third most commonly observed cancer in men worldwide.[1-4] Colon cancer usually originates from the inner lining of the large intestine or colon and can traverse up to the rectum. The tumour can spread via lymph nodes and blood vessels to the adjoining tissues and organs such as the lung, abdominal cavity or pelvis wall. The risk factors associated with colon carcinoma primarily include obesity, consumption of foods that are low in fruits and vegetables and behavioural disorders such as physical inactivity and smoking.[5] The treatment modality depends on the type and resectablilty of the tumor. The choice of treatment begins after the preliminary examination via radiological imaging techniques such as CT scan and MRI to assess the location and to determine tumor grading along with a series of pathological tests.[2] The course of treatment also takes into consideration the status of cancer as to whether it is a primary non-metastatic tumor or a metastatic tumor. The primary non-resectable tumors are generally treated by radiotherapy or chemotherapy but the non-resectable metastatic tumors are treated with a combination of chemotherapy as well as adjuvant therapy.

A relentless quest to discover a suitable approach to combat this dreaded disease is unending and hence, newer methods are being constantly sought. Bioactive compounds obtained from the phytochemicals are proving to be a good

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alternative to the chemical compounds used in the therapy as these chemical compounds have a long history of side effects. The phytochemicals can prove beneficial in terms of affordability and minimizing the side effects as these are naturally occurring compounds.

Phytochemicals are naturally occurring biologically active compounds produced by the plants and are used mainly for their defence against biotic and abiotic stresses and are also responsible for the presence of color, fragrance and taste of the plants. The phytochemicals are produced by various plant parts such as root, stem, leaves, fruits or seeds. The phytochemicals present in the leaves of the plant are responsible for imparting pigment to the leaves such as anthocyanin, similarly, the phytochemical produced by the fruits such as lycopene impart pigment. The phytochemicals when present on the outer layer of the plant mainly impart pigment to the plant parts.[6] The phytochemicals are classified mostly as phenolics, alkaloids, saponins, terpenoids, flavonoids, glycosides and tannins.

Annona squamosa and *Annona reticulata* are plants of the Annonaceae family and are native to West Indies but cultivated throughout India.[7] Both species tend to grow well in the tropical and sub-tropical climates with the former more adaptable to tropical climates. *Annona squamosa* mainly grows as shrubs with lateral branching and grows up to the height of about 3 meters while *Annona reticulata* grows like a tree with a height of about 6.0-7.5 meters. [8,9] The leaves of both *Annona squamosa* and *Annona reticulata* are simple, alternate with acute tips with the latter having slightly larger and glabrous leaves. The fruits of *A. squamosa* are green in color and range from round to heart-shaped structures. The fruit has a sweet and edible pulp with a high glycemic index. The fruits of *A. reticulata* appear yellow and are nearly heart-shaped and turn yellowish-red on ripening and generally have a somewhat repulsive taste and are most popular in the preparation of desserts.

The *Annona* spp. has been studied widely across the globe and they have been reported to have various medicinal properties and have been used in traditional medicine as well. The *Annona* spp. has a long history of ethnobotanical uses and have been shown to possess insecticidal, anti-microbial, anti-diabetic, antioxidant, anti-helminthic, anti-ulcerative, cardioprotective as well as anti-carcinogenic properties among various other medicinal properties studied so far.[8,10] The medicinal properties of *Annona squamosa* have been spread across the whole plant such as root, stem, leaves and seeds as well. The crushed leaves of the plant are sniffed to beat hysteria and fainting spells, also the leaf decoction is used to treat dysentery and the leaves are also used as a paste to treat wounds and ulcers as known from the traditional practices.[11] *Annona reticulata* also possess many medicinal properties such as the dried or unripe fruits are used to prepare decoctions which are beneficial in the treatment of dysentery and diarrhoea also; the leaves are implied in the treatment of ulcers. The decoction of leaves is also useful in the treatment of malaria and syphilis. The root bark is implied in toothache and epilepsy as known from the traditional knowledge and practices.[12]

The secondary metabolites are mostly synthesized as byproducts of primary metabolism and constitute less than 1% of the dry weight of the plant.[13] The secondary metabolites produced by the plants generally belong to three categories; namely alkaloids, terpenoids and flavonoids. The production of secondary metabolites by plants was first recognized as non-essential but they have served a greater purpose to the plant as the production of volatile floral scents and pigments have evolved to attract insect pollinators hence aiding in fertilization. Also, the synthesis of toxic chemicals and pigments has helped the plants to overcome pathogens and herbivores.[13,14] Secondary metabolites also help the plants to adapt to adverse environmental conditions and overcome abiotic stresses.[13] The secondary metabolites obtained from plants are beneficial to the plants and are also of importance commercially. Secondary metabolites have been widely exploited commercially as food flavours, food additives as well as pharmaceuticals. Many of the secondary metabolites have been used as drugs such as atropine from *Atropa belladonna*, vinblastine and vincristine from *Pappeaveur somniferum* and various taxol drugs are used by the pharmaceutical industry in the production of cardiac steroids such as diosgenin from *Digitalis lanata* for ages. So, the present study aims to discover the phytochemical constituents including secondary metabolites present in the *Annona* species which could be further implied in the production of anti-cancer compounds in the most natural and affordable ways.

An understanding of the pigments present in the plants can be valuable for predicting their possible role in the plant system and knowing their mechanism of action. Thin-layer

chromatography (TLC) is one of the inexpensive and valuable tools to separate the pigments in plants.[15] It also has other wider applications in the separation of amino acids[16] and bacterial metabolites[17]. TLC is performed in glass plates or alumina sheets coated with silica gel. TLC plate is dipped in a solvent system which depends on the study of interest. Usually, a mixture of organic solvents is in use for the separation of a wide number of metabolites. The most commonly used mixtures comprise butanol, acetone, petroleum ether, chloroform and methanol in different proportions according to the study.

Both the plants produce various secondary metabolites in addition to being a popular sweet edible tropical fruit. The medicinal properties of the plant are because of the presence of these secondary metabolites hence a comprehensive exploration of these is beneficial to assess the status and amount of these metabolites in the plant to be used in various applications. In addition to various medicinal properties possessed by these plants, they are also known to possess anti-tumorigenicity in some plant parts. Thus, the study of these properties will open new avenues for diagnostics in the field of medicine apart from their use in traditional medicine.

The present study focuses on the differences in the status of phytochemical constituents of leaves of A.squamosa and A. reticulata and their capacities in demonstrating anti-tumorigenesis.

MATERIALS & METHODS

Materials

The solvents for extraction viz, methanol and ethanol were purchased from Merck (USA). 2, 2, Diphenyl picryl hydrazine (DPPH) was purchased from Sigma Aldrich (USA). Rutin trihydrate was purchased from Hellwa Analytica, Gallic acid was purchased from Fluka, Ascorbic acid and Tannic acid was purchased from Himedia(Mumbai, India) and all other chemical and reagents were of analytical grade. The cell culture media RPMI 1640, DMEM was purchased from Himedia Laboratories. The cell lines were obtained from NCCS, Pune.HCT 15 and COLO205 cell lines were grown in RPMI 1640 and HT-29 and HaCaT cell lines were maintained in Dulbecco's Modified Eagle Media(DMEM). The media was supplemented with 100U/l of penicillin-streptomycin solution (Thermo Fischer).

Preparation of extracts

The fully matured leaves of *Annona squamosa* and *Annona reticulata* were collected from the Ranchi University campus. The leaves were washed thoroughly with tap water followed by distilled water and dried in shade for few days. The dried leaves were then ground in a blender and aqueous, methanolic and ethanolic extracts were prepared. For methanolic and ethanolic extracts, 10gm of powdered leaves were dissolved in 100ml of methanol and ethanol respectively and kept in a shaker for 48hours. The extracts were then filtered using Whatman filter paper No.1 and further evaporated using a rotary vacuum evaporator and stored in screw-capped bottles in the refrigerator. The aqueous extracts were prepared using 10gm of dried leaf powder dissolved in 100ml distilled water and kept in the water bath at 70-80°C for 2 hours and filtered using a muslin cloth. This process was repeated at an interval of 2 hours and the extract finally obtained was autoclaved and kept in the refrigerator for further use.[18]

Phytochemical evaluation of *Annona squamosa* and *Annona reticulata* leaf extracts

The aqueous, methanolic and ethanolic extracts were tested for the presence of polyphenols, tannins, flavonoids, saponins, phytosterols and fixed oils and fats following the standard methods.[19-21]

Test for saponins

The aqueous extract was used to test for the presence of saponins. To perform this test, the aqueous extract was dissolved with distilled water and shaken in a graduated tube for 15 minutes. The formation of a 1cm layer of foam indicated the presence of saponins.[21] Both of the leaf extracts confirmed the presence of saponins.

Test for phytosterols

The test for the presence of phytosterols was performed using the method described by Salkowski's test.[22,23] To perform this test, a small quantity of ethanolic extract was dissolved in 5ml chloroform and to it, a few drops of concentrated sulphuric acid was added. The solution was then allowed to stand for some time. The formation of a brown ring indicated the presence of phytosterols. Both methanolic and ethanolic extracts of *A.squamosa* and *A.reticulata* confirmed their presence.

Test for tannins

Dried powdered leaves were taken for this study. About 0.5gm of dried powdered leaves were dissolved in 20ml of distilled water and kept in a water bath for few minutes. The above mixture was then filtered using filter paper and to this few drops of 0.1% of ferric chloride was added.[24] The development of brownish-green or a blue-

black colouration indicated the presence of tannins. Leaf powders from both plants confirmed this test.

Test for Flavonoids

The presence of flavonoids was detected by the alkaline reagent test as well as the lead acetate test and Ferric chloride test.[25] The methanolic and ethanolic extract was treated with few drops of 1N sodium hydroxide solution. The formation of intense yellow color which becomes colorless on the addition of 1N hydrochloric acid indicated the presence of flavonoids. Both methanolic and ethanolic extracts confirmed this test. The presence of flavonoids was also tested with a ferric chloride test. For this, few drops of ferric chloride were added to the extracts and the development of intense green color indicated the presence of flavonoids. Both the extracts of *A. squamosa* confirmed this test. The extracts of *A. reticulata* did not show the presence of flavonoids with ferric chloride test but confirmed the presence of flavonoids with alkaline reagent test and lead acetate test.

Test for terpenoids

The presence of terpenoids was performed with the Salkowski's test.[21] For this test, 2ml of chloroform was added to the aqueous and methanolic extract. To the extracts, 3ml of concentrated sulphuric acid was carefully added. A reddish-brown coloration of the interface indicated the presence of terpenoids. The aqueous extract and methanolic extract were used for this test and while the aqueous extract gave a positive result to this result, the methanolic extract did not conform to this result.

Test for cardiac glycosides

The presence of cardiac glycoside was performed with the Keller-Killani test.[26] To perform this test, the aqueous extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. To this solution, 1ml of concentrated sulphuric acid was added. The appearance of a brown ring at the interface indicates the deoxy sugar characteristic of cardenolides. The appearance of a violet ring below the brown ring and a greenish ring in the acetic acid layer confirms the presence of cardiac glycosides. Aqueous extracts of both plants confirmed this test.

Test for alkaloids

To test the presence of alkaloids in the extracts, the individual extracts were dissolved in dilute HCl and was filtered. The filtrate was then subjected to different reagents to ascertain the presence of alkaloids.[27] The Hager's test was performed for this test. The filtrate was treated with a saturated solution of picric acid (Hager's reagent) and a yellow colored precipitate confirmed the presence of alkaloids.

Test for carbohydrates

The presence of carbohydrates was detected by the Molisch's test and to perform this, 2 gm of the extract was dissolved in 5ml of distilled water and was then filtered.[23] The filtrate was then treated with 2 drops of alcoholic α-naphthol solution in a test tube and to it conc. H_2SO_4 was added from the side of the test tube. The development of a violet ring at the junction of the two liquids confirmed the presence of carbohydrates.

Determination of total phenolic content

The determination of total phenolic content in the extracts was performed by the methods described by Nazish Siddique *et al.* (2017) with slight modifications.[28-30] A standard solution of gallic acid was prepared in methanol and a series of dilutions in the range of 50-450µg/ml was taken and a standard calibration curve was prepared. 0.5 ml of different extracts were taken and dissolved in methanol for this study. After incubation of 45 minutes, the absorbance was recorded at 765nm and the concentrations of extracts were obtained from the calibration graph and the results were expressed as mg/gm GAE.

Determination of total flavonoid content

The determination of total flavonoids was performed by the aluminium- trichloride method with slight modifications.[29,31,32] A standard solution of rutin was prepared in methanol for this study. Serial dilutions ranging from 50-400µg/ml was prepared and a calibration curve was prepared. After incubation for 30 minutes, the absorbance was recorded at 415nm. The concentrations of extracts were obtained from the graph and the results were expressed as mg/gm RE.

Determination of total tannin content

The determination of total tannins was estimated by the method described by Tambe *et al.* with slight modifications.

Briefly, 2.5 grams of dried powder was taken and dissolved in 25 ml of distilled water and boiled in a water bath for 2 hours. The contents were filtered and the process was repeated in 2 hours. The extract was then centrifuged and the supernatant was taken for further analysis. 0.1 ml of the supernatant was taken and to it, 7.5ml of distilled

water was added and then to it, 2.5ml of Folin-Ciolcateau reagent was added. The solution was then mixed properly and to it, 1ml of 35% sodium carbonate was added. Again, 1ml of distilled water was added to the mixture and vortexed. The solution was incubated at room temperature for 30 mins. The absorbance of the solution was read at 725nm.[33] A calibration curve of tannic acid was prepared (0-100µg/ml).

Determination of antioxidant activity

The antioxidant capacity of the extracts was measured spectrophotometrically by the DPPH method. 100µM DPPH was prepared in methanol and ascorbic acid was used as a standard for this method. The methanolic and ethanolic extracts were *A. squamosa* and *A. reticulata* were prepared in methanol and the readings were taken at 517nm at t=0 and after incubation at room temperature for 30 minutes in dark.[34] The radical scavenging activity was assessed by the percentage inhibition of extracts at the time intervals and expressed by the following formula.

% inhibition = A0-A1/A0*100

Thin Layer Chromatography

The presence of bioactive compounds in the extracts was studied by thin-layer chromatography. The free radical scavenging activities of the crude extracts were also determined by the thin layer chromatographic method.[35] The solvent system used for the characterization of radical scavenging activities in the crude extract was a mixture of petroleum ether: chloroform: methanol (49:50:1) which was further sprayed with 0.2% DPPH solution in methanol and was incubated in dark for 30 mins.[35] The presence of a yellow or colorless spot against a purple background confirmed the radical scavenging activity of the crude extracts. The solvent system used for flavonoids was chloroform: methanol (19:1) and was detected in UV rays at 365nm. The spots were detected in the UV light as orange bands. The presence of phenolic compounds was detected using solvent system of chloroform: methanol (9:1) ratio and which was further sprayed with Folin Ciocalteu reagent. The terpenoids was detected using a solvent mixture of butanol: acetic acid: water (4:1:5) and the plates were dried and sprayed with vanillin-sulfuric acid spray followed by heating for 10 minutes at 110°C. Presence of pink or violet spots confirmed the presence of terpenoids.

RESULTS & DISCUSSION

Qualitative Phytochemical test

Table1- Phytochemical analysis of leaf extracts of *A.squamosa* and *A. reticulata*. The presence of phytochemicals is presented with ++ in both plant extracts.

Sl. no.	Phytochemical	Methanolic extract	Ethanolic extract	Aqueous extract
1.	Alkaloids	--	++	--
2.	Terpenoids	--	--	++
3.	Flavonoids	++	++	--
4.	Tannins	--	--	++
5.	Cardiac Glycosides	--	--	--
6.	Phytosterols	++	++	--
7.	Saponins	--	--	++
8.	Carbohydrates	++	--	--
9.	Phenolic compounds	++	--	--
10.	Proteins & amino acids	--	--	--

Quantitative estimation of Phytochemicals

Determination of total phenolic content

Table 2- Phenolic content of extracts expressed as milligram equivalent of Gallic acid (mg/GAE). Results are expressed as mg/GAE ± S.D. n=3. p > 0.05.

Sl.no.	Extract	Concentration (mg/GAE)
1.	Methanolic (*A.squamosa*)	88±0.06
2.	Ethanolic (*A.squamosa*)	65.5±0.11
3.	Methanolic (*A.reticulata*)	35±0.02
4.	Ethanolic (*A.reticulata*)	15±0.05

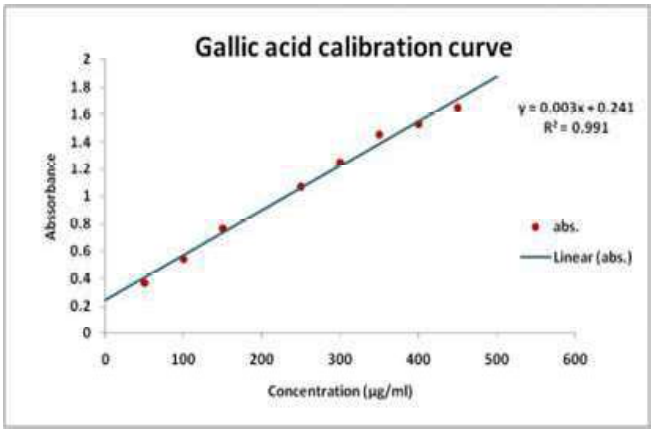


Fig.1- Calibration curve for Gallic acid used as a standard for total phenolic content in extracts.

Determination of total Flavonoids content

Table 3- Flavonoids content of extracts expressed as milligram equivalent of Rutin (mg/Rut). Results are expressed as mg/RTE ± S.D. n=3. P > 0.05.

Sl.no.	Extract	Concentration (mg/Rut)
1.	Methanolic (*A.squamosa*)	34.5±0.02
2.	Ethanolic (*A.squamosa*)	30.5±0.02
3.	Methanolic (*A.reticulata*)	0.46±0.006
4.	Ethanolic (*A.reticulata*)	0.42±0.03

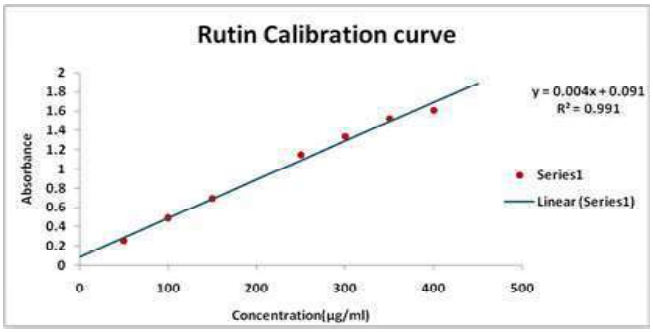


Fig. 2- Calibration curve of Rutin used as a standard for determination of total flavonoids content in extracts

Determination of total tannin content

The total tannin content in *A. reticulata* was found to be 0.011mg/TAE±0.004 and for *A. squamosa* it was found out to be 0.001mg/TAE ±0.007.

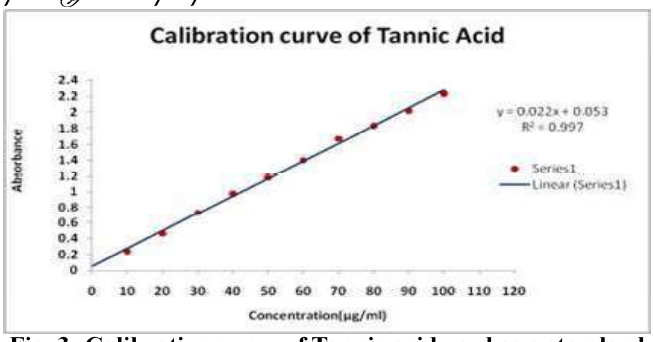


Fig. 3- Calibration curve of Tannic acid used as a standard for determination of total tannin content in extracts.

Determination of antioxidant activity

Table 4- Determination of antioxidant activity expressed as a percentage of inhibition of DPPH radical

Sl.no.	Extract	% Inhibition of DPPH radical (t=30 mins)
1.	Ascorbic acid (Standard)	96.91%
2.	Methanolic (*A.squamosa*)	38.51%
3.	Ethanolic (*A.squamosa*)	70.0%
4.	Methanolic (*A.reticulata*)	37.71%
5.	Ethanolic (*A.reticulata*)	89.5%

Assessment of anti-tumorigenesis potential of extracts

The anti-tumorigenic potential of the extracts is described by the half-maximal inhibitory concentration (IC50) values.

Table 5- Determination of half-maximal inhibitory concentrations of extracts/drugs in different cell lines

Sl.no.	Extracts/ Drugs	HT29	COLO205	HCT15	HaCaT
1.	Doxorubicin	19.51±4.38µM	13.1±1.78 µM	27.87±2.79µM	20.74±1.94 µM
2.	Aqueous (*A.squamosa*)	299.93±2.03µg/ml	207.09±6.64µg/ml	173.09±0.92µg/ml	124.74±0.66µg/ml
3.	Methanolic (*A.squamosa*)	67.34±3.25 µg/ml	59.85±2.91µg/ml	76.08±1.60µg/ml	54.21±2.41 µg/ml
4.	Ethanolic (*A.squamosa*)	63.75±1.71 µg/ml	52.43±2.17µg/ml	37.29±1.22µg/ml	58.18±2.91 µg/ml
5.	Aqueous (*A.reticulata*)	140.20±3.95µg/ml	102.85±6.64µg/ml	250.3±4.35µg/ml	285.41±5.90µg/ml
6.	Methanolic (*A.reticulata*)	47.15±1.41 µg/ml	67.39±10.50µg/ml	59.57±3.90µg/ml	54.92±0.86 µg/ml
7.	Ethanolic (*A.reticulata*)	54.30±2.15 µg/ml	53.74 ±0.46µg/ml	56.72±1.61µg/ml	51.69±2.35 µg/ml

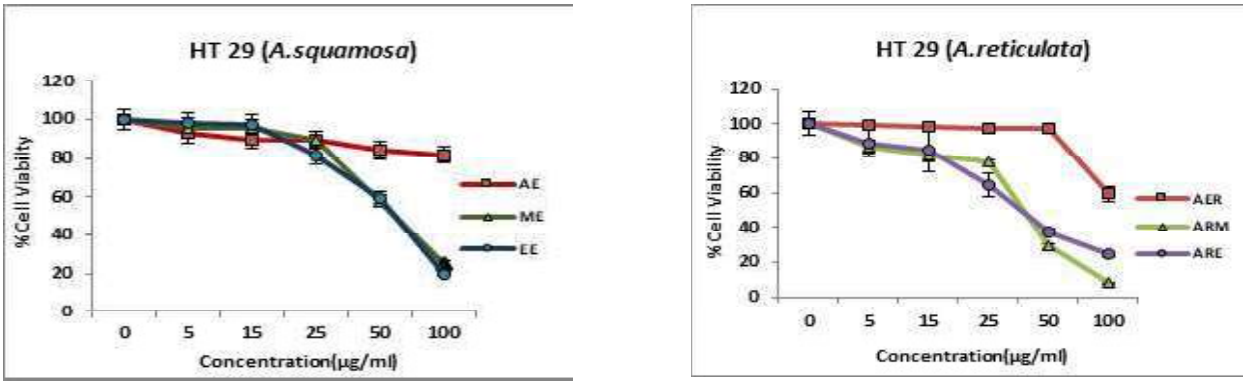
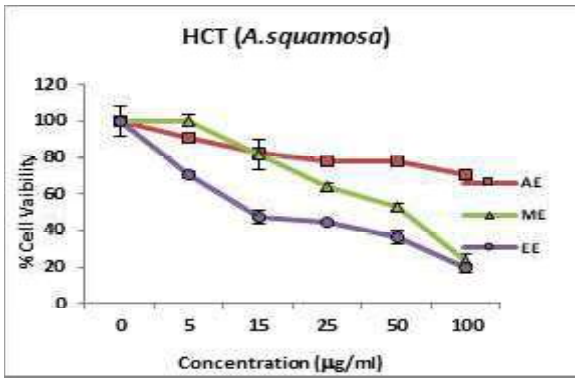


Fig. 4- MTT assay of extracts of *A.squamosa* (a) and *A. reticulata* (b) in HT-29 cell line.



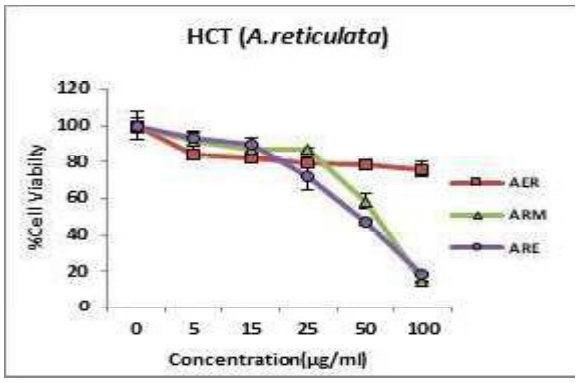
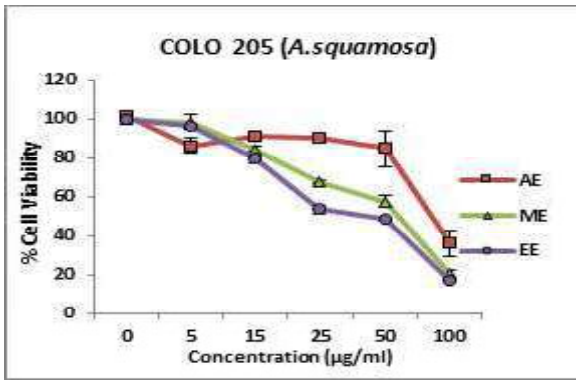


Fig. 5- MTT assay of extracts of *A.squamosa* (a) and *A. reticulata* (b) in HCT-15 cell line.



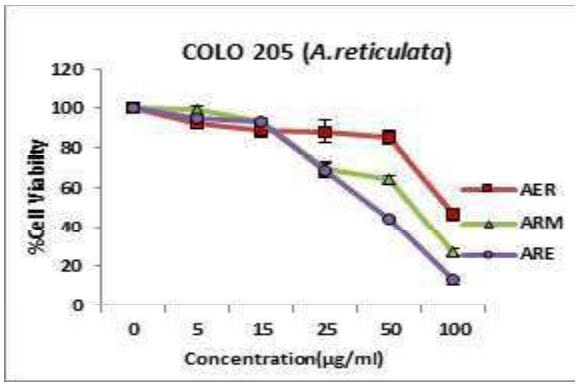
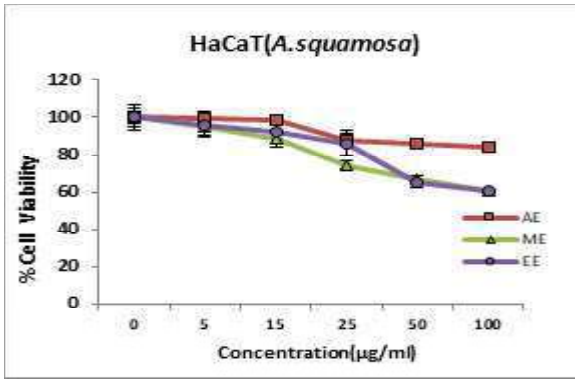


Fig. 6- MTT assay of extracts of *A.squamosa* (a) and *A. reticulata* (b) in COLO-205 cell line.



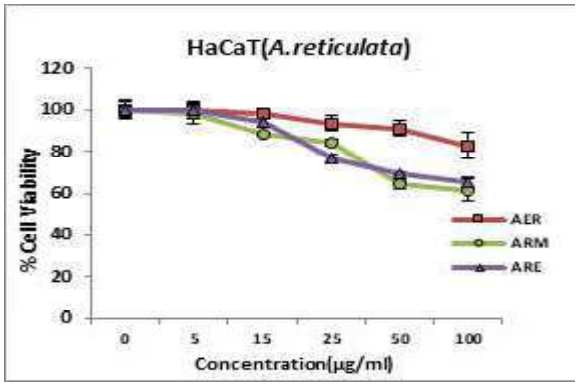


Fig. 7- MTT assay of extracts of *A.squamosa* (a) and *A. reticulata* (b) in HaCaT cell line.

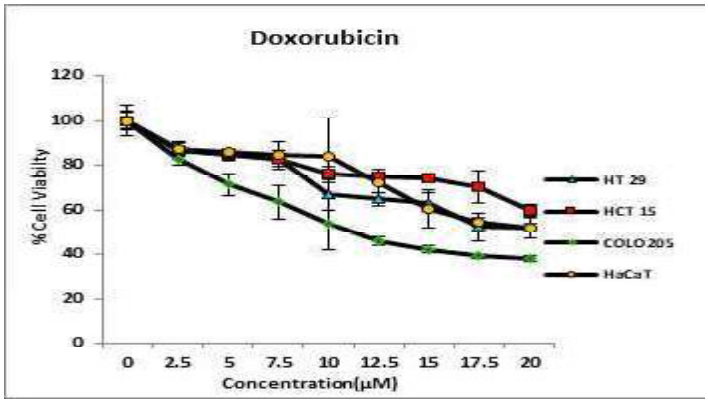
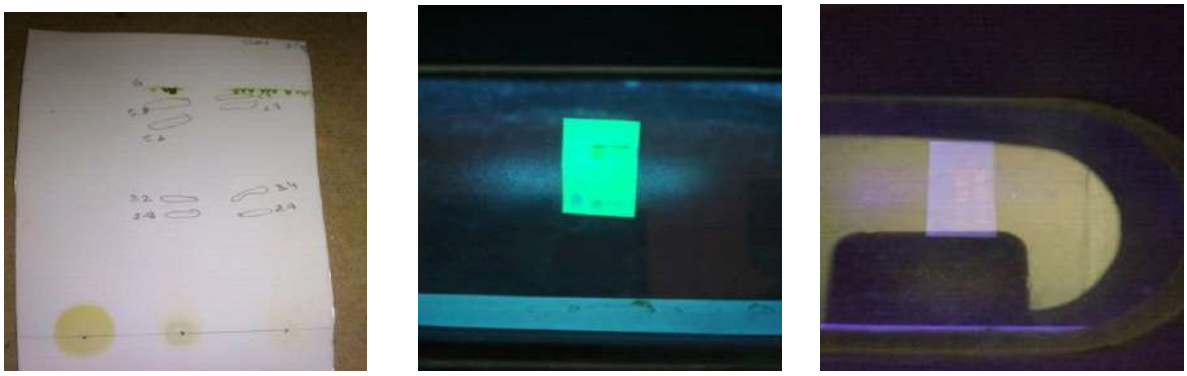


Fig. 8- MTT assay of extracts of Doxorubicin in different colon cancer cell lines.

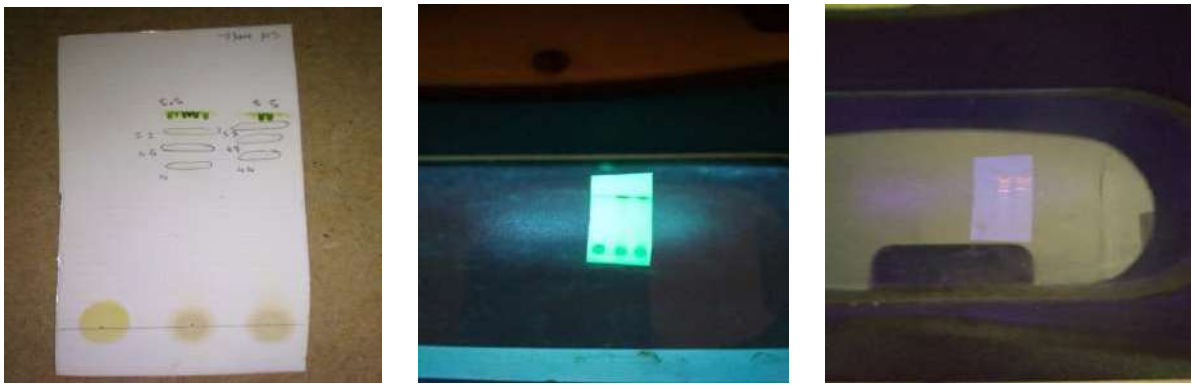
Thin Layer Chromatography

TLC was performed for the detection of the presence of phenolics, terpenoids, flavonoids. Flavonoids were detected as orange bands in near UV range but terpenoids and phenolics were not detected. Also, radical scavenging properties of the extracts were confirmed as white or yellow spots after spraying with DPPH solution which corresponded to its bleaching effect as a mark of the scavenging activity of the extracts



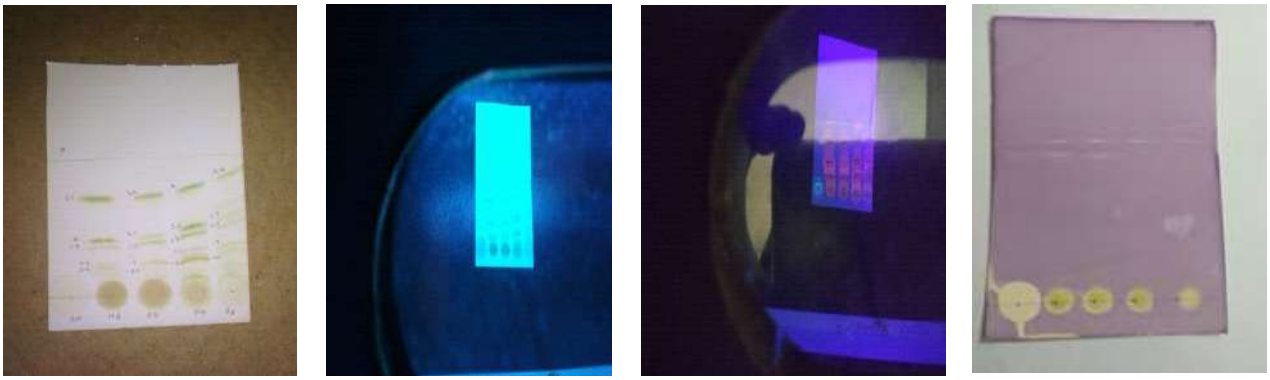
(a) (b) (c)

Fig .9- TLC for detection of flavanoids in organic extracts of *A. reticulata* at (a) 400 nm, (b) 365nm and (c) 254nm. Rutin was taken as a standard for flavonoids.



(a) (b) (c)

Fig .10- TLC for detection of flavanoids in organic extracts of *A. squamosa* at (a) 400 nm, (b) 365nm and (c) 254nm. Rutin was taken as a standard for flavonoids.



(a) (b) (c) (d)

Fig. 11- TLC for radical scavenging activity in organic extracts of *A. squamosa* and *A. reticulata* at (a) 400 nm, (b) 365nm and (c) 254nm. Ascorbic acid was used as a standard antioxidant. (d) shows the radical scavenging properties of the extracts after spraying with 0.2% DPPH solution.

Thin Layer Chromatography

Table 6: The retention factor (Rf) values of plant pigments

(i) TLC for flavonoids

Sl. no.	Extracts	Pigments (no.)	Color	Rf value
1.	Methanolic extract (*A.squamosa*)	1	Grey-greenish	0.40
		2	Yellow	0.72
		3	Yellow	0.83
		4	Green	0.94
2.	Ethanolic extract (*A. squamosa*)	1	Grey – greenish	0.43
		2	Yellow	0.82
		3	Yellow	0.89
		4	Green	0.96
3.	Methanolic extract (*A.reticulata*)	1	Grey-greenish	0.46
		2	Yellow	0.53
		3	Yellow	0.86
		4	Green	0.96
4.	Ethanolic extract (*A.reticulata*)	1	Grey – greenish	0.48
		2	Yellow	0.56
		3	Yellow	0.96
		4	Green	0.98

(ii) TLC for radical scavenging activity

Sl. no.	Extracts	Pigments (no.)	Color	Rf value
1.	Methanolic extract (*A.squamosa*)	1	Yellow	0.16
		2	Yellow-green	0.18
		3	Grey	0.24
		4	Green	0.40
		5	Dark green	0.70
2.	Ethanolic extract (*A. squamosa*)	1	Yellow	0.18
		2	Yellow-green	0.20
		3	Grey	0.24
		4	Green	0.38
		5	Dark green	0.74
3.	Methanolic extract (*A.reticulata*)	1	Yellow	0.24
		2	Yellow green	0.34
		3	Grey	0.44
		4	Green	0.52
		5	Dark green	0.80
4.	Ethanolic extract (*A.reticulata*)	1	Yellow	0.28
		2	Yellow-green	0.38
		3	Grey	0.50
		4	Green	0.58
		5	Dark green	0.88

(iii) TLC for radical scavenging activity after spraying with DPPH

Sl.no.	Pigments	Rf value
1.	Ascorbic acid	0.96
2.	Methanolic extract (*A.squamosa*)	0.85
3.	Ethanolic extract (*A. squamosa*)	0.71
4.	Methanolic extract (*A.reticulata*)	0.62
5.	Ethanolic extract (*A. reticulata*)	0.71

DISCUSSION

This study is aimed to identify the phytochemicals present in the leaves extracts of two *Annona* spp. viz., *Annona squamosa* and *Annona reticulata*. Both of these plants being tropical possess several phytochemicals which have been used in ethnobotany in varied applications as cited by literature. The phytochemicals are identified qualitatively and its subsequent quantitative estimation using standards has been done. The aqueous and organic extracts; namely methanolic and ethanolic extracts were prepared as per standard protocols and all these extracts were subjected to qualitative phytochemical detection. The phytochemicals identified in both ethanolic and methanolic extracts include saponins, phytosterols, phenolics and flavonoids. The presence of alkaloids was detected in methanolic extracts of both plants but ethanolic extracts did not give positive results for this test. The aqueous extracts tested positive for tannins and terpenoids. The presence of cardiac glycosides and fixed oils and fats were not detected in any of the organic or aqueous extracts. The presence of proteins and amino acids and carbohydrates was also not observed.

The quantitative estimation of phenolic compounds was done by taking gallic acid as a standard phenol and the methanolic and ethanolic extracts of both plants was determined. The phenolic content of methanolic extract of *A. squamosa* was found out to be 88±0.06mg/ GAE (Gallic acid equivalent) and for *A. reticulata* it was observed to be 35± 0.02mg/GAE. The total phenolic content of ethanolic extract of *A. squamosa* was 65.5±0.11mg/GAE and for *A. reticulata* it was 15±0.05mg/ GAE. The total flavonoid content of the extracts was also determined by taking rutin as a reference standard. The flavonoid content of methanolic extract of *A. squamosa* was found out to be 34.5± 0.02mg/RUT(Rutin equivalent) and for *A. reticulata* it was 0.46±0.006mg/RUT. The flavonoid content of the ethanolic extract was 30.5±0.02mg/RUT and 0.42± 0.03mg/RUT for *A. squamosa* and *A. reticulata* respectively. The total tannin

content was estimated with dry powdered leaves of *A. squamosa* and *A. reticulata* with tannic acid as a reference standard and it was found out to be 0.001±0.007mg/TAE (Tannic acid equivalent) and 0.011±0.004mg/TAE in *A. squamosa* and *A.reticulata* respectively. The radical scavenging activity of the organic extracts was assessed by the DPPH method taking ascorbic acid as a standard radical scavenger. The percentage of radical activity was calculated and it was found out to be 96.91% for ascorbic acid. The methanolic and ethanolic extracts of both plant leaf extracts also scored well. The total radical scavenging activity after 30 mins incubation in dark was 38.51% and 37.71% for the methanolic extract of *A. squamosa* and *A. reticulata* respectively. The ethanolic extracts of both plants showed relatively higher radical scavenging activity after incubation in dark for 30 mins and it was 70.0% and 89.5% was *A. squamosa* and *A. reticulata* respectively.

After evaluation of phytochemical status and radical scavenging activity, the extracts were assessed for their potential in minimising tumorigenicity in cell lines. For this study, four human colon cancer cell lines and one normal human keratinocyte cell line were subjected to MTT assay and a standard FDA approved drug was employed as a reference compound. The organic and aqueous extracts potential for tumorigenicity was determined and their corresponding half-maximal inhibitory concentration (IC50) values were calculated. The IC50 values correspond to the concentration of drugs that can cause 50% inhibition of tumorigenicity in cell lines. According to this study, the aqueous leaf extracts of both plants showed somewhat higher IC50 values in all the cell lines. The IC 50 value of aqueous extracts of *A. squamosa* was reported to be 299.93±2.03µg/ml, 207.09±6.64µg/ml, 173.09±0.92µg/ml in HT29, COLO205 and HCT15 cell line respectively and 124.74±0.66µg/ml in HaCaT cell line. The IC 50 values for methanolic extract in *A. squamosa* was observed to be 67.34±3.25 µg/ml in HT29, 59.85±2.91µg/ml in COLO205, 76.08±1.60µg/ml HCT15 and 54.21±2.41 µg/ml in HaCaT cell lines. Similarly, the IC50 values for ethanolic extract were calculated to be 63.75±1.71 µg/ml, 52.43±2.17µg/ml, 37.29±1.22µg/ml and 58.18±2.91 µg/ml in HT29, COLO205, HCT15 and HaCaT cell line respectively. The leaf extracts of *A. reticulata* were also prepared and was tested for their tumorigenic potential in colon cancer lines and normal human keratinocyte cell lines. The aqueous extracts had IC50 values as 140.20±3.95µg/ml, 102.85±6.64µg/ml, 250.3±4.35µg/ml and 285.41±5.90µg/ml in HT29, COLO205, HCT15 and HaCaT cell lines respectively. The methanolic extracts of leaf extracts of *A.reticulata* showed IC 50 values as 47.15±1.41 µg/ml, 67.39±10.50µg/ml,59.57±3.90µg/ml and 54.92±0.86 µg/ml in HT29, COLO205, HCT15 and HaCaT cell lines respectively. Similarly, IC50 values for leaf ethanolic extracts of A.reticulata *was calculated as 54.30±2.15 µg/ml, 53.74* ±0.46µg/ml, 56.72±1.61µg/ml and 51.69±2.35 µg/ml in HT29, COLO205, HCT15 and HaCaT cell lines respectively. The half-maximal inhibitory concentration was also calculated in Doxorubicin which was taken as a reference drug during this study and the IC50 values were calculated to be 19.51±4.38µM, 13.1±1.78 µM, 27.87±2.79µM and 20.74±1.94 µM in HT29, COLO205, HCT15 and HaCaT cell lines respectively.

Thin-layer chromatography was performed for organic leaf extracts of *A. squamosa* and *A. reticulata* for the presence of flavonoids, terpenoids, phenolic compounds and also for radical scavenging activity of the extracts. The radical scavenging activity of the extracts gave positive results on staining with DPPH as yellow or white spots and their corresponding Rf values were recorded. The chromatogram for Flavonoids also gave positive results when they were exposed to UV light at 254nm. The spots were also observed in visible light and short-range UV light at 365nm. A total of four numbers of spots were observed at 365nm and four orange bands were observed at 254nm which confirmed the presence of Flavonoids in the extracts and their corresponding Rf value were noted. Ascorbic acid was taken as standard for radical scavenging activity of the extracts and it was run parallel to the organic extracts and their relative Rf values were calculated and presented in the table. A total of five bands of different colors were observed in this study which was found to be white or yellow color bands against a purple background which confirmed the radical scavenging activity of the extracts along with ascorbic acid which showed only one band or spot in the chromatogram. The presence of terpenoids and phenolics were also checked but they did not show any spot in the chromatogram after spraying with Vanillin- sulphuric acid and Folin-Ciocalteu reagents respectively.

CONCLUSION

Plants are known to possess various phytochemicals and this study aims to point out the differences in the

content of phytochemicals between two *Annona* species; viz; *A. squamosa* and *A. reticulata*. Also, the type and quantity of phytochemicals differ in both aqueous and organic extracts and thus an attempt was made to uncover the phytochemicals present in the leaf extracts of both plant species. As per our findings, we found out that the ethanolic extracts possess higher amounts of some phytochemicals such as flavonoids than the methanolic extracts but the phenolic content was higher in the methanolic extracts of both extracts. Also, the leaf extracts of *A. reticulata* showed comparatively lower amounts of phenolics as compared to the leaf extracts of *A. squamosa*. The tannin content was checked for the aqueous extracts as the organic extracts did not show their presence which was relatively quite low for both plants. One interesting finding was in the case of radical scavenging activity where the ethanolic extracts of both plants showed higher radical scavenging activity as compared to the methanolic extracts and were quite similar to the standard ascorbic acid used. The TLC was also somewhat similar for both the plant extracts for flavonoids and radical scavenging activity. The anti-tumorigenic potential of the extracts also showed some interesting values and they were comparatively lower for ethanolic extracts as compared to the methanolic extracts with minor exceptions and thus can be used as effective drugs for further characterization of the anti-cancerous activity of the extracts. Thus, we conclude that several phytochemicals are present in *A. squamosa* and *A.reticulata* leaf extracts which can be exploited for use in therapeutics and also in the field of cancer biology although this needs further research.

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