Microwave Assisted Extraction, Fractionation, Total Phenolic and Flavanoid Estimation and antioxidant potential of Annona reticulata Leaves

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Abstract:

The present study focuses on the microwave assisted extraction of Annona reticulata leaves in ethanol. The extract obtained was subjected to column chromatography and the fractionation was done to get different fractions. Preliminary phytochemical screening was done and the fractions were subjected to total phenolic and flavanoid content estimation. The crude extract contained 502 mg GAE/100g of phenolic content and among the fractions AM8 contained the maximum amount of phenolic content that is 360 mg GAE/100g. The flavanoid content present in the crude extract of the Annona reticulata was found to be 127mg QE/100g. Among all the tested fractions, the fraction AM8 showed more flavanoids i.e. 112 mg QE/100g and 118 AN/100g**.** The antioxidant activity has been studied in-vitro by using DPPH, hydrogen peroxide assay. DPPH, H_2O_2 and Nitric-oxide scavenging effect of Annona reticulata L. leaf extracts were found to be dose dependant with maximum inhibition at highest concentration. Methanolic extract of the leaves of Annona reticulata Linn. is found to have most potent anti-oxidant activity in DPPH, H_2O_2 Radical scavenging methods. IC50 value DPPH and H_2O_2 inhibition of methanolic extract at highest concentration (400µg/ml) are 62.58 ± 1.15, 68.27 ± 1.05 and 64.01 ± 1.02. The results suggest that all the tested extracts are having antioxidant property, but the methanol is having significantly higher flavonoid and phenol content. Due to presence of higher flavonoid and phenol content in methanol, it may be considered as the fraction with better pharmacological property in comparison to other tested extracts.

Keywords: Phenolics, flavanoids, quercetin, anonaine, Annona reticulate, anti-oxidant activity

Introduction

Flavanoids are the largest group of naturally occurring phenolic compounds. They occur both in free state and as glycosides. Flavones and flavonols are widely

distributed. They have a wide variety of biological activities. Flavanoids act as antioxidants acting against degeneration, cardiovascular disorders and also inhibit tumor development in animal models. Some are acting as heart stimulant [1]. They scavenge the free radicals such as superoxide and hydroxyl radicals.

Annona reticulata belongs to the family Annonaceae. It is also known as 'custard apple'. For the extraction of secondary metabolites from plants microwave assisted extraction is playing an important role. Microwave Assisted Extraction (MAE) can be used for the extraction of functional group. Here microwave energy is absorbed by the molecules. Strong penetrating force, high selectivity, high heating ability, less extraction time, reduced solvent consumption and energy requirements are the main advantages of this method [2,3].The application of microwave assisted extraction of secondary metabolites from plants also have been published [4,5].

The leaves of A. reticulata and Allium sativum (bulbs) were collected from regions of Karjat Dist-Raigad, Maharashtra, India in December-January, 2018-19. Plant materials were authenticated at "The Blatter Herbarium" - St. Xavier's College, Mumbai.

After identification and authentication of the plant, leaves of the plant were collected for the experimental process. The leaves were shade dried, made into coarse powder and the powdered material was initially defatted with petroleum ether and then subjected to cold maceration process for 72-h using 1:1 mixture of methanol and water as solvent to prepare hydro-alcoholic extract of Annona reticulata leave (percentage yield 20.5% w/w with respect to dried powder). The extract was filtered and concentrated by rotary evaporator. For the preparation of different fractions method was used [7-9]. About 30ml of ethanol was used as the solvent. The extract was made solvent free by distillation process and the resulting semisolid mass was vacuum dried to yield a solid residue. The experiment was repeated to get 20g of the extract. Isolation of the compounds through column chromatography was started by using 20g of the extract. Slurry of the extract was prepared in ethanol and the extract was uniformly packed over dry silica gel

(mesh size 230-400, 20g). Petroleum ether, Petroleum ether: ethyl acetate (4:3, 3:2, 2:3 and 1:4), ethyl acetate, ethyl acetate: methanol (4:3, 3:2, 2:3 and 1:4), acetone and methanol such different solvents in different ratios were used as the mobile phase. Similar small fractions were collected in small test tubes. TLC (silica gel F254) of all individual fractions were developed. It was then viewed under UV chamber. Based on the TLC results similar fractions were pooled. The fractions were dried in rotavapor under reduced pressure at a temperature of about 40±5º.Such 10 combined fractions were collected. Preliminary phytochemical screening was done by using the extract and fractions by standard methods [6].

MATERIALS AND METHODS

Plant material

The leaves of A. reticulata and Allium sativum (bulbs) were collected from regions of Karjat Dist-Raigad, Maharashtra, India in December-January, 2018-19. Plant materials were authenticated at “The Blatter Herbarium” - St. Xavier's College, Mumbai.

After identification and authentication of the plant, leaves of the plant were collected for the experimental process. The leaves were shade dried, made into coarse powder and the powdered material was initially defatted with petroleum ether and then subjected to cold maceration process for 60hours using same proportions of mixture of methanol and water as solvent to prepare hydro-alcoholic extract of Annona reticulata leaves (percentage yield 20.5% w/w with respect to dried powder). The extract was filtered and concentrated by rotary evaporator. For the preparation of different fractions method was used [7-9].

The sun dried and powdered leaves (76 g) of A. reticulata were successively extracted in a Soxhlet extractor at elevated temperature using 200 ml of distilled n-hexane (40-60)°C which was followed by petroleum ether, methanol, and chloroform. All extracts were filtered individually through filter paper and poured on petri dishes to evaporate the liquid solvents from the extract to get dry extracts. The dry crude extracts were weighed and stored in air-tight container with necessary markings for identification and kept in a refrigerator for future investigations.

Isolation of the compounds through column chromatography started by using 20g of the extract. A slurry of the extract was prepared in ethanol and the extract was uniformly packed over dry silica gel (mesh size 230-400, 20g). Petroleum ether, Petroleum ether: ethyl acetate (4:3, 3:2, 2:3 and 1:4), ethyl acetate, ethyl acetate: methanol (4:3, 3:2, 2:3 and 1:4), acetone and methanol such different solvents in different ratios were used as the mobile phase. Similar small fractions were collected in small test tubes. TLC (silica gel F254) of all individual fractions were developed. It was then viewed under UV chamber. Based on the TLC results similar fractions were pooled. The fractions were dried in rotavapor under reduced pressure at a temperature of about 40±5º.Such 10 combined fractions were collected. Preliminary phytochemical screening was done by using the extract and fractions by standard methods [6].

Determination of total phenolic content

Folin-ciocalteau reagent was used for the determination of total phenolic content and the standard used was gallic acid. The reagent (5ml) was mixed with 1ml of gallic acid at different concentrations and after 3 minutes 4ml of 2% sodium carbonate was added to each solution. After 30 minutes blue colour was developed. It was then read at 760nm. Different concentrations of the sample extract were treated in the similar manner. The test was repeated thrice. The concentration of the total phenol was expressed as mg/g of dry extract.

Determination of total flavanoids

Total flavanoid content was determined by aluminium chloride colorimetric assay [7]. Distilled water (4ml) was taken in 10ml of volumetric flask and an aliquot quantity of extract (1ml) or standard solution of quercetin (25-150µg/ml) was added to it. Sodium nitrite 5% (0.3ml) was added and after 5min,0.3ml of 10% aluminium chloride was added. After 5 min, 2ml of 1M sodium hydroxide was added and the volume was made up to 10ml with distilled water. The solution was mixed well and the absorbance was measured against the blank at 510nm. The total flavanoid content was expressed as mg quercetin equivalents (QE) and as mg quercetin equivalents (AN)).

Evaluation of Anti-oxidant Activity

DPPH Radical Scavenging Activity

The DPPH (1, 1-Diphenyl –2-picrylhydrazyl) assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple color). When Antioxidants react with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging antioxidant) and is reduced to the DPPH and as consequence the absorbance's decreased from the DPPH.

The stock solution of extracts and ascorbic acid (standard compound) were prepared in methanol to achieve the concentration of 1 mg/ml, then it was diluted to different concentration. 1 ml each of the diluted solutions were in a test tube and mixed with 1 ml of methanolic solution of DPPH in concentration of 1 mg/ml. After 30 min incubation in darkness at room temperature, the absorbance was recorded at 517 nm.

Hydrogen Peroxide Scavenging Activity

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al*., (1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (100µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40mM). Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide.

Results

In the present study the extraction of the plant Annona reticulata was carried out by microwave assisted method and the fractionation was done by column chromatography using petroleum ether, chloroform and ethanol at different ratios (Table 1). At different concentrations of gallic acid absorbance was calculated (Table: 2) and the standard graph was plotted (Fig.1).

Total phenolic content present in the extract and the fractions were calculated from the standard graph of the gallic acid. The results are shown in table (Table 3). It was found that the fraction AM 8 contained more amounts of the phenolic constituents. At different concentrations of quercetin and anonaine, absorbances were calculated (Table 4 and Table 5) and the calibration curve of quercetin and anonaine was plotted (Fig.2) and (Fig.3) respectively. The total flavanoid

content of the extract and the fractions were calculated from the standard graph of quercetin and was expressed as mg quercetin equivalents (QE). The results are shown in the table (Table 5).

Table 1: Preparation of different fraction with various fractions

Name of Extract fraction	Fractions	Colour and Consistency	Percentage yield (% W/W)
AR1	Petroleum ether	Orange dry mass	3.8
AR2	Petroleum ether:Ethyl acetate (4:1)	Light Green sticky mass	4.1
AR3	Petroleum ether:Ethyl acetate (3:2)	Greenish brown sticky mass	3.20
AR4	Petroleum ether:Ethyl acetate (2:3)	Greenish brown sticky mass	3.24
AR5	Petroleum ether:Ethyl acetate (1:4)	Greenish sticky mass	3.16
AR6	Ethyl acetate	Light Greenish dry mass	4.5
AR7	Ethyl acetate:Methanol (4:1)	Light Greenish dry mass	4.1
AR8	Ethyl acetate: Methanol (3:2)	Light Greenish sticky mass	5.2
AR9	Ethyl acetate: Methanol (2:3)	Greenish brown sticky mass	5.4
AR10	Ethyl acetate: Methanol (1:4)	Greenish brown dry mass	4.2
AR11	Acetone	Greenish dry mass	5.3
AR12	Methanol	Greenish dry mass	5.1

Table 2: Absorbance obtained at different concentrations of gallic acid

Sr. No.	Concentration of gallic acid (µg/ml)	Absorbance
1	100	0.786±0.0001
2	200	1.548±0.0002
3	300	2.099±0.0001
4	400	2.891±0.0002
5	500	3.594±0.0001

Table 3: Total phenolic content present in Annona reticulata methanol extract and fractions

Sample Extract	Absorbance	Total Phenolics mg GAE/100g
Extract	3.498	502
AR1	0.724	110
AR2	0.892	112
AR3	1.982	295
AR4	2.212	310
AR5	1.789	195
AR6	1.366	174
AR7	1.952	286
AR8	2.524	360
AR9	2.002	290
AR10	1.900	262
AR11	1.980	295
AR12	1.890	289

Table 4 : Absorbance obtained at different concentrations of quercetin

Sr. No.	Concentration of quercetin (µg/ml)	Mean Absorbance
1	25	0.242±0.0002
2	50	0.430±0.0002
3	75	0.612±0.0002
4	100	0.779±0.002
5	125	0.991±0.002
6	150	1.121±0.002

Table 5 : Total flavanoid content as quercetin present in Annona muricata ethanol extract and fractions

Sample	Absorbance	Total flavanoids (mg QE/100g)
Extract	1.020±0.0002	127

AR1	0.109±0.0002	2.4
AR2	0.198	13
AR3	0.598	62
AR4	0.664	80
AR5	0.412	45
AR6	0.314	30
AR7	0.775	97.5
AR8	0.889	112
AR9	0.332	34
AR10	0.682	84
AR11	0.702	88
AR12	0.692	86

Table 6 : Absorbance obtained at different concentrations of anonaine

Sr. No.	**Concentration of anonaine (µg/ml)**	**Mean Absorbance**
1	25	0.220±0.0002
2	50	0.401±0.0002
3	75	0.598±0.0002
4	100	0.802±0.002
5	125	921±0.002
6	150	1.141±0.002

Table 7: Total flavanoid content as anonaine present in Annona muricata ethanol extract and fractions

Sample	**Absorbance**	**Total flavanoids (mg AN/100g)**
Extract	1.005±0.0002	118
AR1	0.995±0.0002	2.2
AR2	0.189	12
AR3	0.572	57

AR4	0.654	71
AR5	0.410	41
AR6	0.301	28
AR7	0.781	98.1
AR8	0.881	110
AR9	0.321	40
AR10	0.675	84
AR11	0.695	86
AR12	0.682	84

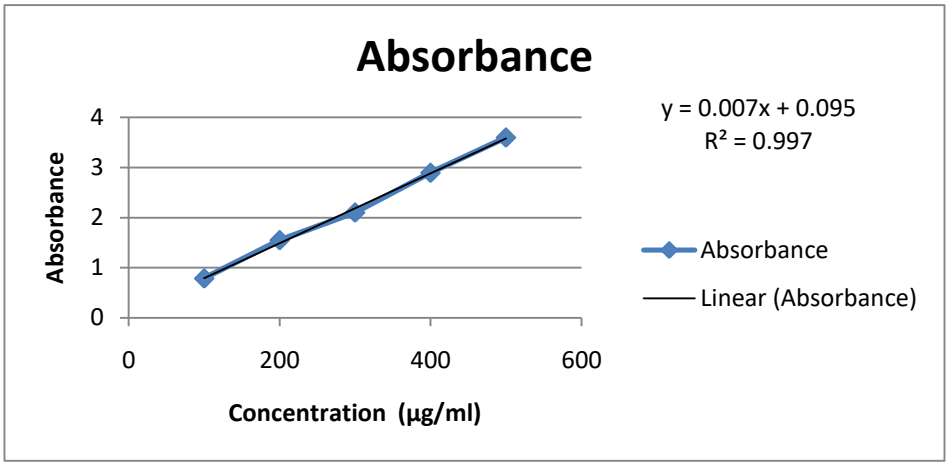

Figure 1: Standard plot for concentrations of Gallic acid absorbance

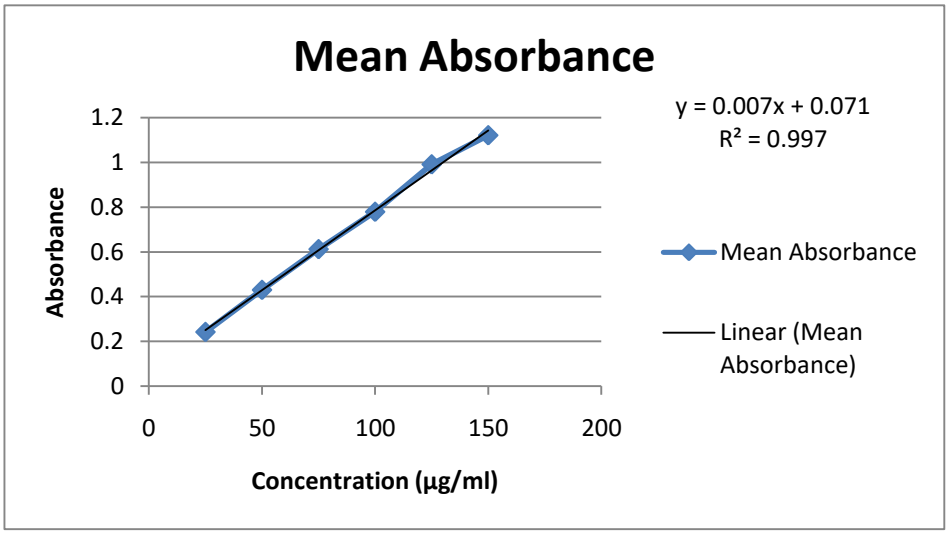

Figure 2: Standard plot for concentration of Quercetin

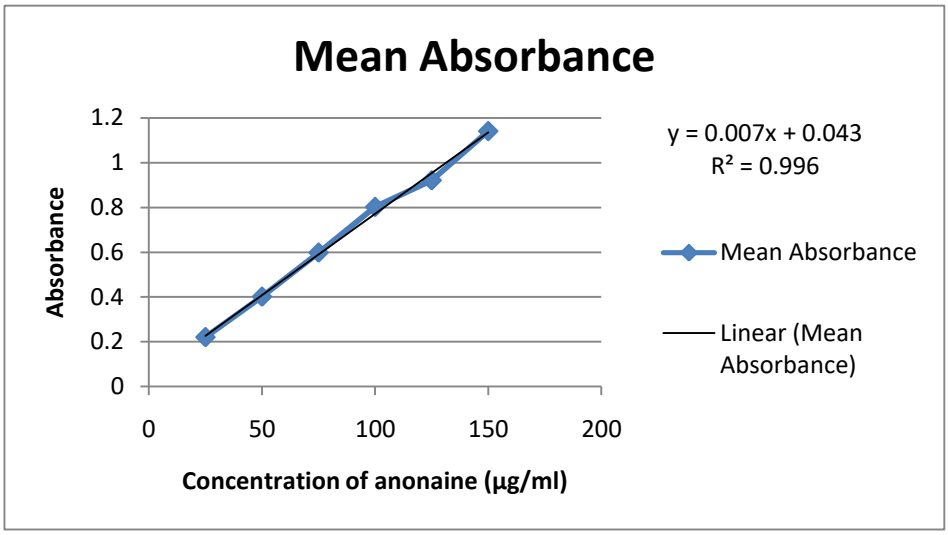

Figure 3: Standard plot for concentration of anonaine

TABLE 8: Effect of leaf extracts of annona reticulata linn. in DPPH antioxidant model

Sr. no.	Conc. (in µg/ml)	% Inhibition					
		Pet. ether extract	Ethyl acetate extract	Acetone extract	Methanol extract	Water extract	Standard (Ascorbic acid)
1	25	22.24±1.03**	30.35±1.11**	33.82±1.09**	51.57±1.02**	43.63±1.01**	65.19±1.01
2	50	30.54±1.12**	33.63±1.01**	45.08±0.97**	57.54±1.04**	53.10±1.04**	67.27±1.04
3	100	33.71±1.07**	46.76±1.01**	50.50±0.94**	64.97±1.05**	57.48±1.03**	75.68±1.05
4	200	40.05±1.18**	53.98±1.02**	58.62±0.98**	68.42±1.12**	62.84±1.02**	79.18±1.11
5	400	43.55±1.04**	60.98±1.01**	61.69±1.01**	72.58±1.15**	67.72±1.01**	82.46±1.02

Values are expressed as Mean ± SEM.; (n = 6); One Way ANOVA followed by Turkey – Kramer Multiple Comparison test; **p<0.01 *vs*. standard drug

TABLE 9: Effect of leaf extracts of annona reticulata linn. in H_2O_2 radical scavenging assay

Sr. no.	Conc. (in µg/ml)	% Inhibition					
		Pet. ether extract	Ethyl acetate extract	Acetone extract	Methanol extract	Water extract	Standard (Ascorbic acid)
1	25	26.21±1.01**	42.35±1.01**	49.19±1.03**	53.01±1.01**	49.30±0.95**	69.82±1.05
2	50	30.13±0.98**	45.72±1.05**	53.56±1.04**	56.92±1.05**	53.68±1.05**	73.74±1.01
3	100	34.51±0.94**	48.07±1.06**	57.04±1.03**	60.00±1.09	56.93±1.03**	79.33±1.01
4	200	38.57±1.02**	53.45±1.09	60.07±1.05**	64.89±1.07**	59.06±1.09**	81.82±1.04
5	400	45.16±1.06**	57.49±1.04**	62.14±1.01**	70.27±1.05**	62.65±1.04**	83.95±1.05

Values are expressed as Mean ± SEM.; (n = 6); One Way ANOVA followed by Turkey – Kramer Multiple Comparison test; **p<0.01*vs*. standard drug

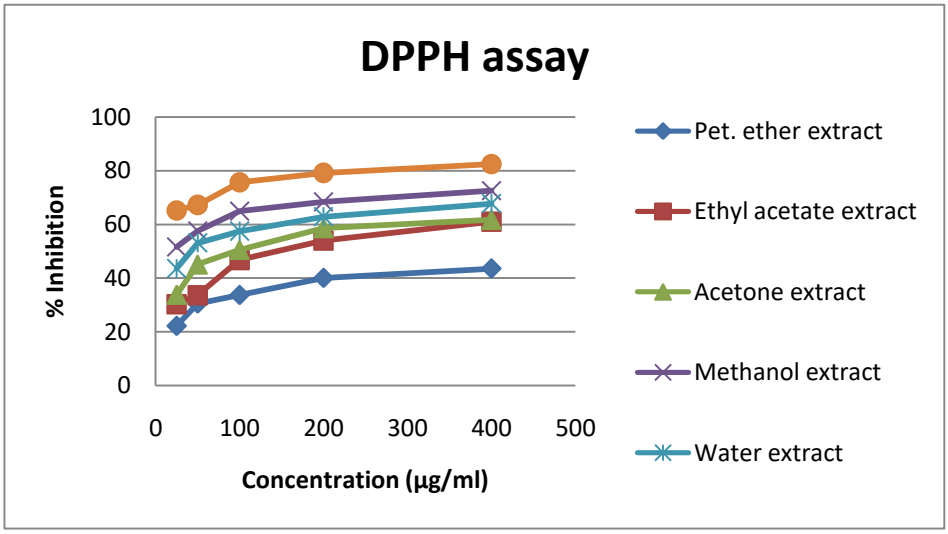

Figure: 4 comparative effect of leaf graph extracts of *a. Reticulata* linn. (sample) and ascorbic acid on DPPH assay

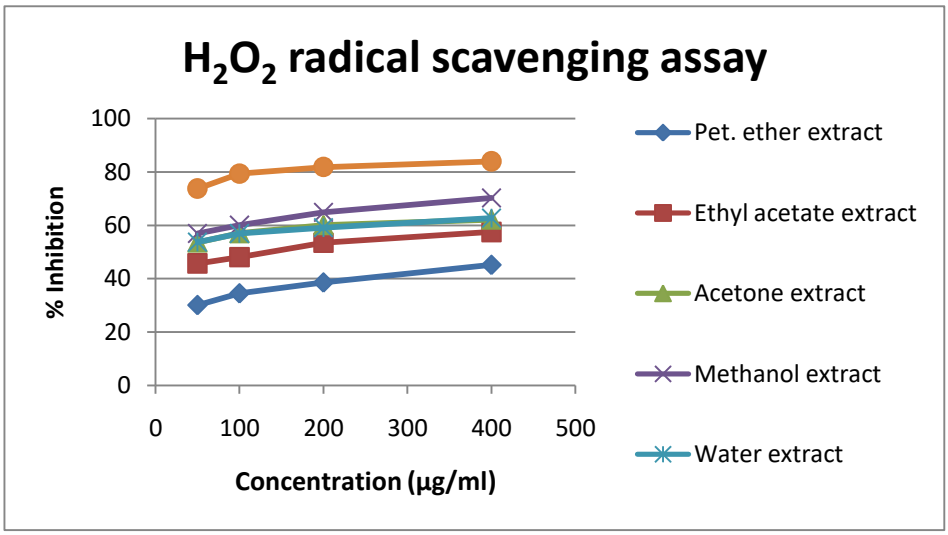

Figure: 5 comparative effect of leaf graph extracts of *a. Reticulata* linn. (sample) and ascorbic acid on H_2O_2 radical scavenging assay

Discussion

A wealth of studies had been conducted on Annona reticulata leaves due to various activities. But the promising activity is the anticancer effect. But most of the activities are concentrating on the extracts obtained from it. So the present study focused by using the fractions obtained from the extracts.

The microwave assisted method of extraction used in the present study is a widely accepted method for the removal of active components from the crude drug. The method helped to save time and the amount of solvents used. The literatures describe as the presence of phenolic content is the important criteria which attributes for many activities of the plant materials.

It is commonly known that the phenolic constituents and the flavanoids are related to various biological activities. They in turn affects the human health. So the present study was focused on the determination of total phenolic and flavanoid content. Since the ethanol extract and the fractions of Annona reticulata are showing more amount of phenolic and flavanoid, this may show various biological activities including the anticancer activity.

Conclusion

Many plants are showing various pharmacological activities due to the presence of phenolic and flavanoid contents. Annona reticulata is the plant coming under Annonaceae family having wide variety of biological activities. The microwave assisted ethanol extract of Annona reticulata leaves are showing the presence of phenolics and flavanoids. From this study it is clear that the fraction 8 of Annona reticulata ethanol extract is containing more amount of phenolics and flavanoids. So this fraction may show more antioxidant and anticancer activities. This fraction is to be taken for further study for the isolation of active constituents.

Conflicts of interest

Authors do not have any financial conflict of interests.

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