Antioxidant and GC–MS analysis of *Annona reticulata* leaves extract against unsecure free radicals

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Abstract

Plants are excellent sources of secondary metabolites that has been used for preventing the human physiological system from unstable free radicals which leads to oxidation of macromolecules resulting in several degenerative diseases. The present study is aimed to investigate the antioxidant properties using various solvent extracts from *Annona reticulata* and total phenolic and flavonoid contents of chloroform, ethyl acetate, ethanol and aqueous extracts were quantified using standard methods. The results of the antioxidant properties of *A. reticulata* chloroform leaves extract revealed the potent free radical quenching properties were 99.2 ± 0.3 (DPPH); $71.0 \pm 2.1\%$ (ABTS) and effective reducing antioxidant power activity with concentration of the extract increased and the total phenolic and flavonoid contents obtained were 28.7 ± 0.31 $\mu\text{g}/\text{mg}$ and 135.28 ± 4.91 $\mu\text{g}/\text{mg}$ of dry weight was higher in ethanol extract compared to other solvent extracts. Finally, more than thirteen compounds were observed in gas chromatography mass spectrometry analysis. This study proved that the selected chloroform leaves extract from *A. reticulata* had the effectiveness for observed antioxidant properties against unsecure free radicals. It should be focused for the better development of integrative exogenous agonists for various reactive oxygen species and reactive nitrogen species under circumstances of oxidative stress.

Keywords *Annona reticulata* · Gas chromatography mass spectrometry (GC–MS) · Phenolic and flavonoid · Antioxidant

Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl
ABTS	2,2'-Azino-bis(3 ethylbenzothiazoline-6-sulfonic acid)
Fe(CN) ₆] ³⁻	Ferricyanide

enzymes called catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX) and superoxide dismutase (SOD) in physiological system [2]. The endogenous enzymes will fail to combat these radicals when the overproduction occurs under uncertain oxidative stress conditions, which can lead to high risk of inflammation, nephrotoxicity, neurological disorders, cardiovascular disorders, rheumatoid arthritis, liver damage, aging and cancer [3]. The exogenous antioxidant compounds rather than endogenous enzyme play a crucial role on free radical quenching effects. These antioxidants are not produced by human physiological system, but intake of fruits and vegetables in routine food habits, which serving as antioxidants properties will help to prevent these free radicals formation through the degradation or inactivation by donating the electron to the unpaired radicals [4, 5]. In this context, plant-derived antioxidant compounds are

1 Introduction

The human physiological system has naturally developed self defense mechanism against various free radicals, which encounters these free radicals without causing any oxidative stress mediated diseases [1]. The imbalance between oxidants and antioxidants in the circumstance of oxidative stress starts to accumulate large amount of major free radicals such as ROS and RNS. These important radicals are encountered by endogenous antioxidant

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getting as more importance for prevention of free radical formation, DNA mutation and lipid peroxidation. Moreover the plant-derived compounds such as phenolic acids, phenolic diterpenes, flavonoids and other important secondary metabolites are known to have strong antioxidant properties which can be used as alternative synthetic antioxidant products for these free radicals mediated pathogenesis [6, 7].

Annona reticulata is cosmopolitan in distribution, cultivated mainly in tropical and subtropical countries for used as traditional medicine to treat various diseases such as constipation, dysentery, bacterial infection, parasite and worm infestations, fever, dysuria, ulcer, haemorrhage, epilepsy, cardiac problem and also used as an insecticide [8–10]. Anti-proliferative activities of *A. reticulata* were documented in pharmacological studies [11]. Few studies were carried out on the antioxidant activity of *A. reticulata* using single organic solvent extract [12–15], but lacks of executing the complete antioxidative profile of *A. reticulata* leaves extract using various solvent system. Therefore, in the present study is aimed to investigate the complete screening and evaluation of free radical scavenging activity from the leaves extracts of *A. reticulata* using various solvent system, and quantifying antioxidant compounds of phenolic and flavanoid contents in each solvent extracts.

2 Materials and methods

2.1 Collection and preparation of plant materials

The *A. reticulata* leaves were collected and authenticated by Professor & Director. N. Mathivanan, Centre for advance studies in botany, University of Madras, India and voucher specimen No: CASBAH1018. The collected leaves were washed with distilled water and soaked in 0.9% saline solution to remove the air borne microbial contamination [16]. It was then kept for shade drying under laboratory condition ($28 \pm 2^\circ\text{C}$) for 1–2 weeks. The dried plant leaves were ground and powdered using mixer grinder. About 10 g of fine powder was subjected to sequential extraction using 100 mL of various solvents such as hexane, chloroform, ethyl acetate, ethanol and water by maceration with help of magnetic stirrer at room temperature (RT). The extraction time was allowed about 5 h for each solvent extraction. The extractions were allowed to dry at RT and the crude residues were obtained after complete evaporation of solvents. The samples were stored at 10°C until further use. The stock solutions were prepared by dissolving the obtained crude residues in dimethyl sulfoxide (5% DMSO) and it was further diluted in double distilled water with different concentrations i.e. 200–1000 $\mu\text{g/mL}$.

2.2 Determination of total phenolic contents

The total phenolic contents from solvent extracts viz. hexane, chloroform, ethyl acetate, ethanol and aqueous were determined using folin–ciocalteau reagent (FCR) method [17]. The reaction mixture was prepared by 1.0 mL (1 mg dry weight) of different solvent extracts with water after the complete dissolution in DMSO. Further these reaction mixtures 1 mL of folin–ciocalteau reagent (1:10 v/v) were added and incubated it at RT for 4 min. After the incubation period, the reaction mixtures were subjected to 800 μL of sodium carbonate solution (7.5% w/v) and agitated in vortex for 1 min. All the reaction mixtures were incubated in dark at RT for 2 h and OD values were taken at 765 nm. Three replicates for each solvent extract was performed to determine the total phenolic contents using standard tannic acid calibration curve and the concentration were indicated in terms of tannic acid equivalents (μg of TA/mg for extracts).

2.3 Determination of total flavonoid contents

The total flavonoid contents were determined using the protocol described by Chang et al. [18]. The reaction mixtures were prepared by 1.0 mL (1 mg dry weight) of different solvent extracts added with 1 mL of ethanol (95%), 100 μL of aluminium chloride (10%) followed by 100 μL of potassium acetate (1 M) were made up to 3 mL by the addition of double distilled water and incubate at RT for 30 min. After the incubation, the reaction mixtures were subjected to UV absorbance at 415 nm with suitable blank. Three replicates for each solvent extract was performed to determine the total flavonoid contents using standard quercetin calibration curve and the concentration were indicated in terms of quercetin equivalents (μg of QT/mg for extracts).

2.3.1 Antioxidant activity

2.3.1.1 DPPH free radical scavenging activity DPPH free radical scavenging activity was calculated by the method followed by Mensor et al. [19]. Briefly, 1 mL of different concentrations i.e. 200–1000 $\mu\text{g/mL}$ of various solvent extracts was prepared and mixed with 1 mL of DPPH solution (0.3 mM in ethanol). These mixtures were incubated in dark RT for 30 min after vigorous shaking and absorbance was taken at 516 nm with suitable negative control (NC) consist of 1 mL of DPPH with 1 mL of ethanol. Three replicates were performed for each solvent extract to determine the DPPH free radical scavenging activity. The

free radical scavenging activity (RSA) was calculated by the following formula.

$$\% \text{ RSA} = \frac{\text{Abs of DPPH} - \text{Abs of Sample}}{\text{Abs of DPPH (NC)}} \times 100$$

2.3.1.2 ABTS radical scavenging activity ABTS radical scavenging activity was followed by the method of Re et al. [20]. The ABTS solution was prepared by 1 mL of ABTS (7 mM) with 49 mL of potassium persulphate (2.45 mM) as an oxidative agent and kept it for overnight at dark RT. The working solution of ABTS was acquired by diluting the stock solution with 50% ethanol until the OD value reached 0.70 ± 0.02 at 734 nm. The 200 μL of different concentration of various solvent extracts (200–1000 $\mu\text{g/mL}$) were mixed with 1800 μL of ABTS solution, kept it incubation in dark RT and the absorbance were taken at 734 nm against suitable negative control. Three determinations for each solvent extract were performed. The inhibition of ABTS⁺ production was calculated by the following formula.

$$\% \text{ ABTS} = \frac{\text{Abs of ABTS} - \text{Abs of Sample}}{\text{Abs of ABTS (NC)}} \times 100$$

2.4 Reducing power assay

The reducing power was determined by the method proposed by Oyaizu [21]. 2.5 mL of different concentrations of various solvent extracts (200–1000 $\mu\text{g/mL}$) were mixed with 2.5 mL of 1% potassium ferricyanide followed by 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and incubated for 20 min at 50 °C. Later 2.5 mL of Trichloroacetic acid (10% w/v) was added to the reaction mixtures, which was then subjected to centrifugation at 3000 rpm for 10 min at RT. The collected supernatants were mixed with 2.5 mL of double distilled water and 0.5 mL of ferric chloride solution (0.1% w/v) in a test tube incubated for 10 min and the absorbance was taken at 700 nm against suitable blank. Three determinations for each solvent extract were performed. The high reducing power of various reaction mixtures was determined using the increasing absorbance values in spectrophotometer.

2.5 Gas chromatography: mass spectrometry (GC–MS)

The chloroform extract dried sample holding active ingredient was subjected to JEOL GC MATE II, which comprised of quadruple double focusing mass analyzer employed with photon multiplier tube detector and its capillary column about HP 5 ms, working in an electronic means at 70 eV with high purity of helium used as carrier gas at the constant flow rate of 1 mL/min. The injector temperature

was about 140 °C for 1 mL of injection and the ion source temperature was kept at 250 °C. The temperature of the oven was programmed from 50 to 250 at 10 °C/min and the mass spectra were taken at 70 eV.

2.6 Statistical analysis

All the data were carried out with three determinations and are exhibited in mean ($\bar{x}$) $\pm$ standard deviation (SD). IC_{50} concentration was calculated by probit analysis using software StatPlus Version 5.0.

3 Results

3.1 Total phenolic and flavonoid contents

The total phenolic and flavonoid contents of each solvent extracts were determined using standard calibration curve (Figs. 1, 2). The obtained results showed high variation in the distribution of phenolic and flavonoid contents among various solvent extracts (Table 1). The total phenolic contents (7.1 ± 0.64 , 8.42 ± 0.33 , 22.4 ± 1.46 , 25.6 ± 0.27 and 28.7 ± 0.31 $\mu\text{g/mg}$ of dry weight) and total flavonoid content (55.3 ± 4.72 , 145.2 ± 2.60 , 96.3 ± 3.53 , 106.7 ± 6.52 and 135.2 ± 4.91 $\mu\text{g/mg}$ of dry weight) were determined for aqueous, hexane, chloroform, ethyl acetate and ethanol solvent extracts respectively.

3.2 Antioxidant activity

Stable free radicals such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were used to determine the free radical scavenging activity. As shown in Tables 2 and 3, all solvent extracts have exhibited potent quenching effects towards the DPPH and ABTS free radicals. Among the various solvent extracts used, the chloroform extract has shown an effective free radical quenching properties with 99.2 ± 0.3 and $71.0 \pm 2.1\%$ at 1000 $\mu\text{g/mL}$ and its inhibitory concentration (IC_{50}) were 137.8 and 722.8 $\mu\text{g/mL}$ for DPPH and ABTS respectively.

On the other hand, *A. reticulata* leaves exhibited a high reducing power activity by increasing the optical density at 700 nm. Among the various solvent extracts used, the chloroform extract was shown to indicate high ferric-reducing activity in its final concentration (Fig. 3).

3.3 Characterization of antioxidant compounds

The GC–MS was performed to identify the potent antioxidant compounds from chloroform extract of *A. reticulata*. As shown in Table 4, thirteen bio-active compounds were

Fig. 1 Tannic acid standard calibration curve to find out quantity of phenolic content of various solvent extract *A. reticulata* leaves extract

Fig. 2 Quercetin standard calibration curve to find out quantity of flavanoid content from various solvent extract *A. reticulata* leaves extract

Table 1 Phenol and flavonoid content of *Annona reticulata* leaves extract

S. no	Extracts mg/dry weight	Phenol (µg)	Flavonoid (µg)
1	Aqueous	7.1 ± 0.64	55.3 ± 4.72
2	Hexane	8.42 ± 0.33	145.2 ± 2.60
3	Chloroform	22.4 ± 1.46	96.3 ± 3.53
4	Ethyl acetate	25.6 ± 0.27	106.7 ± 6.52
5	Ethanol	28.7 ± 0.31	135.2 ± 4.91

Values are mean ± SD of three biological replicates

identified from this extract. The major peak area percentage of the compounds such as 12.0 and 15.45 with the retention time 17.78 and 19.45 showed the presence of phytochemical constituents namely palmitic acid and oleic acid respectively in the GC–MS analysis (Fig. 4).

4 Discussions

Plant-derived antioxidant compounds aid in quiet unse- cure free radicals and prevent the oxidative stress-medi- ated pathogenesis by pairing the unpaired electron of free radical molecules [22]. The derivatives of both phenolic and flavonoid compounds served as potent antioxidants

Table 2 DPPH radical scavenging activity of *Annona reticulata* leaves extracts

Plant material (leaves)	Extracts	Concentration $\mu\text{g/mL}$	Percentage of radical scavenging $\pm$ SD	IC ₅₀ ($\mu\text{g/mL}$)
<i>A. reticulata</i>	Aqueous	200	4.3 $\pm$ 1.02	1642.93
		400	7.8 $\pm$ 2.0	
		600	13.2 $\pm$ 3.7	
		800	19.9 $\pm$ 1.6	
		1000	30.6 $\pm$ 2.1	
	Hexane	200	35.6 $\pm$ 1.2	549.20
		400	46.5 $\pm$ 2.3	
		600	51.7 $\pm$ 3.2	
		800	62.3 $\pm$ 5.4	
		1000	69.2 $\pm$ 2.3	
	Chloroform	200	80.0 $\pm$ 4.0	137.8
		400	92.9 $\pm$ 0.9	
		600	95.1 $\pm$ 1.0	
		800	96.8 $\pm$ 0.9	
		1000	99.2 $\pm$ 0.3	
	Ethyl acetate	200	40.9 $\pm$ 1.5	262.3
		400	53.0 $\pm$ 2.9	
		600	60.7 $\pm$ 2.9	
		800	70.2 $\pm$ 4.0	
		1000	83.2 $\pm$ 2.4	
	Ethanol	200	39.3 $\pm$ 8.7	210.4
		400	82.9 $\pm$ 2.5	
		600	85.1 $\pm$ 1.4	
		800	89.5 $\pm$ 1.1	
		1000	89.5 $\pm$ 1.1	

Values represent mean $\pm$ SE of 3 replicates and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey–Kramer HSD ($P \leq 0.05$)

activities involved in quenching of free radicals by blocking the chain reaction during various cellular free radicals formation. Quercetin, a flavonoid compound has the ability to chelate metal ions during DNA damage induced by cupric ion leading to inhibition of various oxidative radicals such as O_2^- , H_2O_2 and $\text{OH}^\cdot$ through a one-electron transfer mechanism [23]. Another class of flavonoid compound called anthocyanin which prevents the $\text{ROO}^\cdot$ generation during lipid peroxidation on lipoprotein membranes, etc. [24]. Phenolic derivatives such as tannic and gallic acids are rich in hydrogen-containing compounds which act against lipid oxidation and thus protecting the polyunsaturated fatty acids (PUFA), phospholipids (the major constituent of the cell membrane) and Low-density lipoprotein (LDL) by intercepting the chain-carrying $\text{ROO}^\cdot$ reaction [25]. To overcome these issues, many investigators have started to discover the unique structured plant-derived compounds are able to fight against number of reactive oxygen species (ROS) under various oxidative stress conditions [26]. These plant derived metabolites could be used for drug formulations in many pharmaceutical industries to enhance the

healthy lifestyle and detain the breakthrough of neuro-degenerative, cancer and other oxygen species related diseases [27]. Previous reports from this plant leaves extract have performed the antioxidant activities against a certain commercial available free radical molecules but inadequate details on plant screening using various solvent systems and other related characterization work is taken to further study in detailed. In connection, Baskar et al. [28]; Rout et al. [29] reported antioxidant properties using *A. reticulata* plant leaves extract against various free radicals with restricted number of solvent extracts screened, which was displayed the lacking of detailed investigation on these work. In another one study of Jamkhande et al. [30] also reported that, the antioxidant and antimicrobial activities against different uncharged molecule (Free radicals) as well as human pathogens but there was only a highly focused on antimicrobial activities and not a prerequisite investigation on antioxidant properties with help of *A. reticulata* leaves extract. Therefore to fulfill the detailed investigation of these highly medicinal plant properties against unsecure free radicals were studied

Table 3 ABTS activity of *Annona reticulata* leaves extracts

Plant material	Extracts	Concentration $\mu\text{g/mL}$	Percentage of radical scavenging $\pm$ SD	IC ₅₀ ($\mu\text{g/mL}$)
<i>A. reticulata</i>	Aqueous	200	13.2 $\pm$ 1.7	1078.16
		400	16.1 $\pm$ 1.3	
		600	29.4 $\pm$ 2.1	
		800	36.2 $\pm$ 0.9	
		1000	46.3 $\pm$ 0.3	
	Hexane	200	26.4 $\pm$ 0.9	1063.02
		400	33.8 $\pm$ 0.3	
		600	38.5 $\pm$ 1.5	
		800	41.6 $\pm$ 0.3	
		1000	45.7 $\pm$ 1.0	
	Chloroform	200	23.9 $\pm$ 2.3	722.8
		400	36.2 $\pm$ 1.1	
		600	48.84 $\pm$ 2.3	
		800	59.4 $\pm$ 3.3	
		1000	71.0 $\pm$ 2.1	
	Ethyl acetate	200	29.2 $\pm$ 0.7	1054.19
		400	32.5 $\pm$ 0.6	
		600	34.5 $\pm$ 0.5	
		800	40.7 $\pm$ 1.3	
		1000	48.5 $\pm$ 0.5	
	Ethanol	200	20.5 $\pm$ 1.6	780.5
		400	30.4 $\pm$ 3.3	
		600	44.9 $\pm$ 2.9	
		800	57.7 $\pm$ 2.8	
		1000	64.0 $\pm$ 3.0	

Values represent mean $\pm$ SE of 3 replicates and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey–Kramer HSD ($P \leq 0.05$)

Fig. 3 Ferricyanide reducing activity of various solvents extracts of *A. reticulata* leaves extract. Each value is the mean $\pm$ standard deviation

using screening of various solvents extracts and preliminary characterization of the bioactive compounds containing from *A. reticulata* chloroform extract.

Accordingly the present study, using *A. reticulata* leaves mediated obtained different solvent extracts were screened and produced a potent quenching effect against various free radicals for the chloroform extract compared to other solvent extracts were tested. The total phenolic and flavonoid contents from all the solvent extracts were documented in highly. The obtained high amount of phenolic and flavonoid contents from the solvent extracts such as hexane, ethyl acetate, ethanol and aqueous have failed to quench the DPPH, ABTS and Fe(III) free radicals predominantly, whereas the chloroform extract with low amount of phenolic and flavonoid contents were quenched the free radical formation effectively in an in vitro condition.

Similarly the previous documentation of Jaradat et al. [31] stated that the low level of flavonoid, phenol and tannin contents from aerial parts of *Trigonella berythea* plant species showed highest antioxidant activity rather

Table 4 Phytochemical constituents from chloroform extract of *A. reticulata* leaves

S. no	RT time	Compound name and formula	Molecular weight	Peak area %
1	16.7	Piperidine, 2-propyl-, (S)-(C ₆ H ₁₀ N ₂ O ₂)	127.2	4.294
2	17.2	Piracetam (C ₆ H ₁₀ N ₂ O ₂)	142.156	4.299
3	17.5	Benzene, (1-methyl butyl)-(C ₁₁ H ₁₆)	148.24	5.08
4	17.78	Palmitic acid (C ₁₆ H ₃₂ O ₂)	256.43	12.0
5	18.53	Mitoflaxone (C ₁₇ H ₁₂ O ₄)	280.27	6.31
6	19.45	Oleic acid (C ₁₈ H ₃₄ O ₂)	282.47	15.45
7	20.03	(1,1'bicyclopropyl)-2-octanoic acid, 2 hexyl-,methyl ester (C ₂₁ H ₃₈ O ₂)	322.525	9.04
8	23.78	3,4-dihydroxy-1,6-bis (3-methoxy-phenyl)-hexa-2,4-diene-1,6-dione ()	312.4	7.74
9	24.85	Cholesta-7,14-diene (C ₂₇ H ₄₄)	368.63	6.95
10	28.42	4H-1-Benzopyran-4-one, 2-(3,4 dimethoxyphenyl)-5-hydroxy-3,6,7-trimethoxy-(C ₂₀ H ₂₀ O ₈)	388.36	6.42
11	23.02	4-(4-nitrophenylazo)-benzoicacid, methoxycarbonylmethyl ester (C ₁₁ H ₁₂ O ₄)	208.2	6.78
12	21.38	2,6,10,14-Hexadecatrienoic acid (C ₁₆ H ₂₆ O ₂)	250.3	9.23
13	18.98	N-(4-Hydroxyphenyl)acetamide (C ₈ H ₉ NO ₂)	163.11	6.37

Fig. 4 GC–MS chromatogram for Chloroform extract of *A. reticulata* leaves extract

than the *Trigonella Arabica* plant species which contained high level of flavonoid, phenol and tannin contents and other secondary metabolites. This is could be a possible due to the presence of some other phytochemical constituent rather than phenolic and flavonoid contents from the extract, which reacts with folin-ciocalteu and other reagents which make interaction with free radicals to inactivate ROS and RNS, etc. [32]. We strongly implicit that the potent antioxidant properties due to

the palmitic and oleic acid presence as reported by many investigators previously [33, 34].

5 Conclusion

The present investigation from *A. reticulata* leaves extract documented potent antioxidant properties with phenolic and flavonoid contents from different solvent extracts. The chloroform extract of *A. reticulata* was characterized

by GC–MS analysis. Various compounds from the analysis were documented with assumption that the presence of one-compound protected all reactive species induced in unfavorable oxidative stress conditions. This extract from

Annona reticulata would be considered as potent anti-oxidant agents to enhance human healthy life from the oxidative stress induced diseases. In future, this work will be carried out, that which exact compound play as a potent antioxidant properties after purification from chloroform extract of *A. reticulata* and it would be analyzed further activity against any oxidative stress mediated disease like inflammation to reveal the effect of the purified bioactive compounds at the cellular level.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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