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ORIGINAL ARTICLE

Secondary Metabolite Profiling of Soursop Fruit and Leaves in Water, Ethanol and Methanol Extracts

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ABSTRACT
The leaves and fruits of Annona muricata (L) have been studied for pharmaceutical purposes with little a attention paid to the study of secondary metabolites present in its fruits and leaves. The present experiment was therefore conducted to evaluate the level of secondary metabolite present in different solvent extraction. Most of the phenolic acids and flavonoids were higher in ethanol extract when compared with other solvent extraction. A. muricata leaves contain more of secondary metabolites than its fruits. Vanillic, syringic, ferulic and salicylic acids were the major phenolic acids present in leaves. Whereas ferulic acid was the major phenolics present in fruits. The leaves also showed significantly higher concentration of flavonoids in the ethanol extract of leaves when compared to the fruits. Terpenoids and anthocyanins were higher in the leaves while organic acid and fatty acids were significantly higher in the fruits. The results of our present study indicate that soursop leaves exhibited significantly higher secondary metabolites when extracted with ethanol compared to the fruits. These findings suggest that the ethanol extracts of leaves could be used as a antioxidant source against inflammatory diseases.
Keywords: *Secondary Metabolite, Phenolics, Organic acids, Antioxidant.*

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INTRODUCTION

An antioxidant is any substance which is capable of delaying, preventing the oxidative damage of lipids, protein and nucleic acids by reactive oxygen species, which include reactive free radicals such as superoxide, hydroxyl, peroxyl, alkoxyl and non- radicals such as hydrogen peroxide and hypochlorous [1]. Thus, antioxidants have gained numerous attentions in the past few years, especially within the food, biological and agrochemical fields. There is increasing evidence that the consumption of vegetables and fruits is associated with a reduced risk of degenerative disease such as cancer, cardiovascular disease and cataracts [2]. This association is often attributed to the natural antioxidant present in fruits and vegetables, such as vitamin C and E, carotenoids, phenolic acids and flavonoids, which prevent free radical damage [3]. Epidemiological studies have established a positive correlation between the intake of fruits and vegetables and prevention of diseases like atherosclerosis, cancer, diabetes, arthritis and also ageing [4]. In Annona muricata L. (A. muricata) is one of the tropical fruits that demonstrate antioxidant properties. This plant contains annonaceous acetogenins in the twigs, unripe fruit, seeds, roots, and bark tissues, which display antitumor, pesticidal, antimalarial, anthelmintic, piscicidal, antiviral, and antimicrobial effects, thus suggesting many potentially useful applications. Ripe A. muricata pulp extract contains three prominent acetogenins: asimicin, bullatacin, and bullatalicin. Previous research on A. muricata was focused on the leaves, seeds and roots for pharmaceutical purposes [5],[6]. Little attention has been paid to the study of the pulp and peel of A. muricata fruit. This study was therefore conducted to

compare the antioxidant properties, phytocontituents, proximate and mineral compositions of the peel and pulp of *A. muricata*.

MATERIAL AND METHODS

Chemicals

The following chemicals were used in this study 2, 2-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide [K3 Fe (CN)], gallic acid (GA), ascorbic acid (AS), and $FeCl_3$, were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA), and ammonium molybdate and sodium carbonate were from Merck Chemical Supplies (Darmstadt, Germany). All the other chemicals used, including the solvents, were of analytical grade.

Preparation of plant extracts

The soursop (*Annona Muricata* L.) leaves and ripe fruits were harvested and washed thoroughly under running tap water then oven dried for one week at 40-60^{0}C. The dried leaves and fruit pulp were uniformly ground using an electric grinder. The powdered material (100g) was extracted for 3 days in 500 mL of distilled water, ethanol and methanol. The separated extracts were then filtered through Whatman No.1 filter paper and the filtrates were evaporated to dryness using a rotary evaporator at 40^{0}C. The semisolid extract was then dried at room temperature and stored at -20^{0}C until further use.

Extraction or phenolic acids and flavonoids: The individual phenolic acids and flavonoids for LC-MS analysis was isolated from 80% methanol as previously described by [7] and [8] with slight modification. 10g of sample was homogenized in methanol (80%), centrifuged and made up to 50 mL. Evaporated 20 mL of extract near to dryness under vacuum at 45°C and then diluted to 5 mL with water later extracted thrice with petroleum ether then in 40 mL of ethyl acetate using separating funnel. The aqueous layer was discarded and the ethyl acetate extract was evaporated to dryness under vacuum at room temperature. To the dried residue, 4 mL of 2N NaOH was added and allowed to hydrolyzing for overnight. Once acidifying to pH 2 using 5 mL 2N HCl, again re-extracted with 50 mL ethyl acetate. Ethyl acetate layer again re-extracted twice with 25 mL of 0.1N NaHCO3. The ethyl acetate layer which carried the flavonoids evaporated to complete dryness under vacuum, the residue was dissolved in 2 mL MS grade methanol filtered through 0.2µm nylon filter prior to inject in LCMSMS for flavonoids estimation. The aqueous layer further acidified to pH 2 with 5 mL 2N HCl and extracted thrice with 25 mL ethyl acetate, the ethyl acetate layer was dried completely in rotary evaporator and the residue was dissolved in 2 mL MS grade methanol filtered through 0.2µm nylon filter prior to inject in LCMSMS for phenolic acid estimation.

LC and MS-MS conditions: The phenolic acids and flavonoids were resolved on the analytical column BEH-C18 (2.1 x50 mm, 1.7 µm) from waters India ltd., protected by a Vanguard BEH C-18 (Waters, USA) with the gradient flow of organic and aqueous phase with the flow rate of 0.3mL/min. The column temperature was maintained at 25°C during analysis and the sample injection volume was 2µL. The eluted phenolic acids and flavonoids through monitored by a PDA detector and the UPLC column effluent pumped directly without any split into the TQD-MS/MS (Waters, USA) system optimize for the phenolic acids and flavonoids analysis.

Anthocyanin profiling by LCMS: Extraction of anthocyanins by following the method described by [9] with a slight modification. 5g of sample was homogenized in a pestle and mortar using 1% acidified methanol under dark condition. Made up the volume to 50 mL using acidic methanol, 5 mL of the extract was taken and dried under vacuum evaporator and dissolved with mobile phase. Filtered through 0.2 µm nylon filter prior to injection in LCMS.

LC and MS-MS conditions: Anthocyanins were resolved on the analytical column BEH-C18 (2.1 x 50 mm, 1.7 µm) from waters India ltd., protected by a Vanguard BEH C-18 (Waters, USA) with the gradient flow of organic and aqueous phase with the flow rate of 0.25mL/min. The column temperature was maintained at 25°C during analysis and the sample injection volume was 2µL. The eluted anthocyanins through monitored by a PDA detector and the UPLC column effluent pumped directly without any split into the TQD-MS/MS (Waters, USA) system optimize for the anthocyanins analysis.

Organic acids profiling by LCMS: The extraction procedure of organic acids follows as described earlier by [10], [11] with slight modification. Homogenized the 5g of sample using 10 mL of 80% methanol and sonicated for 30 min. The extract was then allowed to cool at room temperature and centrifuged at 10000 rpm for 15 min, collected the supernatant and the traces of methanol was completely removed by using vacuum evaporator. The extract was then transferred to separating funnel and extracted with 3-4 times 25 mL ethyl acetate. The lower aqueous phase was collected and a trace of organic solvent was completely removed using vacuum evaporator. The eluted solution obtain from the cartridge was dried completely under the flow of nitrogen. Reconstituted the same in the mobile phase (Solvent A and

Solvent B; 50:50) and filter through a nylon membrane filter with a pore size of 0.45µm follow by 0.2µm, 4 µL inject into the LC-MS/MS for analysis.

LC and MS-MS conditions: The initial gradient composes of 100% aqueous phase (A) and 0% organic phase (B), holed for 0.5 min. At 5.0 min, the gradient was changed to 95% aqueous phase and 5% organic phase, holed for 0.5 min then system was returned to the initial conditions at 6 min and this condition was holed for 1 min to equilibrate before the next injection. The flow rate was 0.1 mL/min. The analytical column was used 2.1 X 50mm UPLC BEH- Amide column (Waters) with 1.7µm particles, protected by a Vanguard 2.1 X 5mm BEH-Amide with 1.7µm particle size guard column (Waters) and the column temperature was maintained at 25°C. The sample injection volume was 4 µL. The elute organic acids monitor using a PDA detector and the UPLC column effluent was pumped directly without any split into the TQD-MS/MS (Waters, USA) system, which was optimize for the identification and quantification of organic acids analysis.

GC-FID analysis of fatty acids: GC-FID analysis of fatty acid methyl esters was carried out using a Varian-3800 Gas chromatograph system equip with flame ionization detector (FID) on a fused silica capillary column (VF-5 Factor Four, Lake Forest, CA, USA), 30 m × 0.25 mm i.d and 0.25µm film thickness. The temperature program for the column is as follows: Initial oven temperature is set to 100°C for 4 min, increase by 3°C per min up to 220°C and hold for 4 min. The temperature is further increase to 260°C at the rate of 5°C per min and hold for 10 min. Injector and detector temperatures are maintain at 250°C and 260°C respectively. Helium at a flow rate of 1 mL/min is used as the carrier gas. Flow rates of H2 and air maintain at 20 mL/min and 250 mL/min respectively. Injection is initially complete in split-less mode follows by split mode (1:30) after1.5 minutes.

Gas chromatography-mass spectrometry (GC-MS): GC-MS analysis performs on Varian-3800 gas chromatograph couple with Varian 4000 GC-MS-MS ion trap mass selective detector. Fatty acids are separate on VF-5MS fused silica capillary column (Varian, USA) (30 m × 0.25 mm id with 0.25 µm film thickness) by applying the same temperature program as described above for GC-FID analysis. The carrier gas is helium at a flow rate of 1mL/min; injector temperature, 260°C; ion source-temperature, 220°C; trap temperature, 200°C and transfer line temperature, 260°C. Mass detector conditions are: EI-mode at 70 eV with full scan range, 50–450 amu. Fatty acids are identified by comparing the relative retention times of FAME peaks with those of reference standards (Sigma-Aldrich, USA) and also by comparing the spectra with those available in Wiley and NIST-(2007) spectral libraries [12]. The total quantity of FAME is estimated as the sum of all GC-FID peak areas in the chromatogram and individual compounds are quantify by comparing the known individual FAME procured as standard.

Statistical analysis

The experimental results were expressed as mean ± SEM of three replicates. The data were subjected to one-way analysis of variance (ANOVA) followed by student's *t*-test has been applied to calculate the statistically significant differences between the control groups and the test samples. The IC_{50} value was calculated by interpolation from linear regression analysis using XLSTAT software. A value of $p \leq 0.05$ was termed to be significant.

RESULTS

Table 1 shows the results of phenolic acid profiling present in the soursop leaves and fruit in different solvent extracts which was done by LCMS analysis. The highest concentration of phenolic acid was observed in leaves sample compared to fruit. Ethanol extract of leaves samples recorded higher (3410.5µg/100g FW) the concentration compared to methanol (2947.89 µg/100g FW) and aqueous (723.37µg/100g FW) extracts (Table 1). Aqueous extract of fruit pulp showed lower (41.46µg/100g FW) level of phenolic acids while ethanol extract had higher the concentration (105.26µg/100g FW). Protocatechuic acid was found as major phenolic acid present in soursop leaves sample at the concentration of 679.25µg/100g FW, while the Gentisic acid was observed lower the level (0.211µg/100g FW). Ferulic acid was a major phenolic acid in fruit pulp sample at the concentration of 33.36µg/100g FW in ethanol extracts, while gallic acid was present in least concentration. The result presents in Table 2 the MRM details of phenolic acid analysis. Figure 1 shows the chromatogram details of the sample extracts. Table 3 indicates the results of flavonoids present in leaves and fruit pulp samples which was done by LCMS method. The highest flavonoid content was observed in leaves compared to fruit samples. Ethanol extracts of leaves and fruit showed higher concentrations of flavonoids as compared to methanol and aqueous extracts. Luteoline (301.49µg/100g FW) was the major flavonoid present in leaves and more apigenin (62.93µg/100g FW) was observed in fruit samples (Table 3). Fruit aqueous extract had 80% less flavonoids as compared to aqueous leaves extract. The ethanol extract of leaves had 88% higher flavonoids content. Catechin and Myrcetin content were least in leaves and fruit (7.46µg/100g FW,

1.72µg/100g FW) respectively. The result presents in Table 4 the MRM details of flavonoids analysis. Figure 2 shows the chromatogram details of the sample extracts. The results of terpenoids profiling of soursop leaves and fruit samples in different solvent extracts is represented in Table 5. Maximum concentration of total terpenoids was observed in the ethanol extract of leaves (46.66 ng/g FW) and fruit (13.87 ng/g FW). Aqueous extract of the test samples showed very lower level of terpenoids as compared to ethanol and methanol extracts. δ-cadinene was the predominant terpenoid in both leaves and fruit pulp sample at 27.37 ng/g FW, 9.87 ng/g FW respectively (Table 5). β-Guaiene and β-elemene were observed very lower the concentration in both leaves and fruit pulp sample 0.265 ng/g FW, 0.019 ng/g FW respectively. Soursop leaves methanol extract recorded 18% lower terpenoid content as compared to ethanol extract. Terpenoids could positively influence its antioxidant activity. Table 6 depicts anthocyanin presents in soursop leaves and fruit samples which were done by LCMS analysis. Total three (cyanidin-3-glucoside, Isopeonidin 3-O-arabinoside and Petunidin) anthocyanin were observed in the test samples in different solvent extracts. Aqueous extracts both leaves and fruit pulp samples showed no anthocyanin peaks (Table 6). Ethanol extract of soursop leaves recorded maximum (0.808 mg/g FW) anthocyanin content as compared to methanol extract (0.601 mg/g FW). Lower the concentration was observed in soursop fruit sample as compared to leaves. Methanol extract of fruit sample showed lower (0.0199 mg/g FW) the concentration than ethanol extract (0.0414 mg/g FW). Anthocyanin plays an important role in the antioxidant activity, and exists lower pH range and better electron delocalization contributes the maximum in the antioxidant activity. The result of the level of organic acids in soursop leaves and fruit samples is represented in Table 7. The maximum concentration of organic acids was observed in different extract of fruit pulp sample as compared to leaves. Both leaves and fruit pulp ethanol extract contained relatively high concentration of organic acids 1.64 mg/ g FW, 5.01 mg/ g FW respectively. Malic acid was found as major organic acid in soursop fruit with the concentration of 2.03 mg/ g FW while Shikimic acid recorded lower (0.035 mg/ g FW) the level. In leaves, citric acid was observed maximum concentration (0.589 mg/ g FW) while oxalic acid content was lower the level (0.0088 mg/ g FW). Fruit organic acids such as malic and citric acids are the most abundant organic acids may have the positive health benefits as antioxidants nature and have the ability to chelate harmful metals. Table 8 shows the result of fatty acid profile of soursop leaves and fruit pulp samples, which was done by GCMS analysis. Mostly, saturated, monounsaturated and polyunsaturated fatty acids were reported. Leaves sample had 62.7% lower level of fatty acids as compared to fruit pulp sample. The saturated palmitic (C16:0) acid (145.8 mg/100g FW) was found in higher quantities in leaves and fruit samples as compared to the polyunsaturated and monounsaturated fatty acids. Palmitic (Hexadecanoic, C16:0) and stearic (octadecanoic, C18:0) acids were the major saturated fatty acids. Other saturated fatty acids found were caprylic (C8:0), dodecanoic (C12:0), tridecanoic (C13:0) and hexacosanoic (C26:0) acids. The polyunsaturated fatty acids such as linoleic (C18:2) and linolenic (C18:3) were found highest concentration 62.28 mg/100g FW, 2.33 mg/100g FW respectively. Oleic acid is a monounsaturated fatty acid and was observed at maximum concentration in fruit pulp (32.35 mg/100g FW) and leaves (8.03 mg/100g FW) samples.

Table 1. Profiling of phenolic acids in different extract of soursop leaves and fruits

Phenolic acids (mg/g FW)	Leaves			Fruit		
	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol
Chlorogenic acid	0.005±0.0002	3.01±0.24	4.74±0.35	0.001±0.0009	0.004±0.0002	0.91±0.08
Vanillic acid	92.48±1.65	452.9±6.89	456.7±7.32	0.46±0.028	1.78±0.13	2.31±0.35
Syringic acid	80.70±2.01	468.84±5.89	575.55±7.28	1.87±0.24	3.61±0.44	3.57±0.39
Ferulic acid	60.07±1.22	508.42±6.32	547.36±4.97	19.50±1.34	26.12±1.86	33.36±2.98
Caffeic acid	56.76±1.79	186.66±2.86	198.13±3.02	0.06±0.004	1.05±0.16	2.65±0.34
Gallic acid	2.55±0.11	8.65±0.79	11.00±1.03	0.006±0.0003	0.02±0.006	0.003±0.0001
p-coumaric acid	39.34±1.55	104.5±2.78	191.46±3.75	4.74±0.56	15.39±1.06	17.65±1.66
o-coumaric acid	69.57±2.14	156.99±3.76	168.73±5.24	5.21±0.63	14.00±1.22	17.49±1.88
2,4-dihydroxy benzoic acid	0.62±0.05	2.51±0.31	3.37±0.24	0.008±0.0006	0.05±0.003	0.06±0.003
Gentisic acid	0.15±0.02	0.18±0.025	0.21±0.018	0.003±0.0001	0.002±0.0001	0.010±0.002
Protocatechuic acid	146.35±2.86	536.24±6.47	679.25±8.55	2.53±0.35	17.71±1.23	20.60±2.47
t-cinnamic acid	19.79±1.39	67.14±1.89	87.51±2.26	0.54±0.04	1.34±0.24	2.03±0.34
p-OH benzoic acid	24.12±1.66	121.56±2.79	145.57±3.04	1.07±0.21	1.56±0.30	2.83±0.28
Salycylic acid	130.83±2.85	330.14±3.57	340.87±4.05	0.73±0.05	1.37±0.21	1.75±0.35

Table 2. MRM details for phenolic acids

Compound	Formula/ Mass	Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode
Caffeic acid	180	178.90	30	135.05	16	ES-
2,4-Dihydroxybenzoic acid	154	152.90	28	65.02	18	ES-
Chlorogenic acid	354	352.97	22	191.10	18	ES-
Ferulic acid	194	192.90	26	134.02	14	ES-
Gallic acid	170	168.90	28	125.03	12	ES-
Gentisic acid	154	152.90	24	108.98	12	ES-
o-Coumaric acid	164	162.90	22	119.06	12	ES-
p-Coumaric acid	164	162.90	24	119.05	14	ES-
p-Hydroxybenzoic acid	138	136.90	26	93.01	12	ES-
Protocatechuic acid	154	152.90	26	109.05	16	ES-
Salicylic acid	138	136.90	28	93.10	14	ES-
Syringic acid	198	196.97	26	182.07	10	ES-
t-Cinnamic acid	148	146.90	26	103.05	10	ES-
Vanillic acid	168	166.97	26	108.01	20	ES-

Table 3. Profiling of flavonoids in different extract of soursop leaves and fruits

Flavonoids (µg/g FW)	Leaves			Fruit		
	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol
Umbelliferone	23.04±1.02	116.51±2.65	153.42±1.88	2.85±0.25	17.65±1.02	25.72±1.45
Apigenin	14.10±0.58	217.73±3.24	245.59±2.49	11.65±0.88	52.37±1.67	62.932.07
Neringenin	15.99±0.87	139.17±2.34	187.78±3.59	17.92±1.05	37.06±1.67	48.51±2.13
Luteoline	71.14±1.47	256.93±3.65	301.49±5.34	1.04±0.08	2.60±0.31	3.01±0.54
Catechin	2.41±0.27	4.10±0.56	7.46±0.87	0.73±0.09	1.10±0.12	1.85±0.23
Hesperitin	30.48±1.26	101.74±3.04	123.18±2.67	2.09±0.32	6.43±0.88	8.50±1.17
Quercetin	19.07±0.79	51.94±1.47	89.50±1.95	1.27±0.16	1.75±0.26	3.12±0.43
Myrcetin	4.93±0.51	35.54±1.57	40.37±1.85	0.55±0.04	1.01±0.12	1.72±0.31
Rutin	2.10±0.18	8.42±0.74	14.57±1.21	0.70±0.06	1.12±0.14	2.06±0.41
Kaempferol	50.03±1.64	207.14±4.81	253.27±6.12	0.96±0.10	7.15±0.86	8.88±1.01
Epicatechin	18.61±0.98	39.24±1.87	47.80±1.31	2.30±0.31	7.08±0.98	10.16±0.66
Epigallo catechin	7.20±0.82	40.52±1.37	44.11±1.86	10.52±0.75	14.74±1.09	19.59±1.59

Table 4. MRM details for flavonoids

Compound	Formula/ Mass	Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode
Apigenin	270	268.97	46	107.04	30	ES-
Catechin	290	289.03	38	245.05	12	ES-
Hesperetin	302	300.97	42	286.15	16	ES-
Kampherol	286	284.97	54	145.5	36	ES-
Leutoline	286	284.97	54	150.99	26	ES-
Myrcetin	318	317.03	42	151.06	28	ES-
Neringenin	272	271.03	34	151	16	ES-
Quercetin	302	301.03	36	151.12	20	ES-
Rutin	610	609.1	60	300.2	42	ES-
Umbelliferone	162.14	161.04	42	133.07	18	ES-
Epicatechin	290.27	289.05	20	245.15	15	ES-
Epigallo catechin	306.27	305.05	20	219.05	15	ES-

Table 5. Profiling of terpenoids in different extract of soursop leaves and fruits

Terpenoids (ng/g FW)	Leaves			Fruit		
	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol
α-Pinene	0.104±0.020	0.282±0.03	0.341±0.04	0.083±0.007	0.121±0.009	0.127±0.007
β-Guaiene	0.102±0.011	0.224±0.025	0.265±0.013	0.061±0.004	0.189±0.02	0.238±0.031
δ-cadinene	6.871±1.02	23.49±1.85	26.37±1.97	2.741±0.34	8.023±0.76	9.874±0.89
α-muurolene	0.074±0.005	0.113±0.012	0.342±0.041	0. 042±0.003	0.197±0.024	0.246±0.034
β-Caryophyllene	1.472±0.12	4.879±0.62	6.355±0.77	0.756±0.064	1.689±0.18	1.874±0.27
τ-cadinol	1.004±0.09	4.672±0.48	6.485±0.87	0.214±0.018	0.675±0.057	0.768±0.064
α-cadinol	0.723±0.08	4.793±0.61	5.003±0.62	0.087±0.006	0.452±0.037	0.555±0.049
α-humulene	0.104±0.009	0.223±0.031	0.476±0.057	0.078±0.005	0.110±0.008	0.168±0.024
β-Elemene	0.087±0.006	0.425±0.028	1.023±0.11	0.006±0.0004	0.014±0.002	0.019±0.003

Table 6. Profiling of anthocyanins in different extract of soursop leaves and fruits

Anthocyanins (mg/g FW)	**Leaves**			**fruit**		
	Aqueous	**Methanol**	**Ethanol**	**Aqueous**	**Methanol**	**Ethanol**
cyanidin-3-glucoside	0.003±	0.042±	0.068±	0.001±	0.0028±	0.012±
Isopeonidin 3-O-arabinoside	0.001±	0.138±	0.187±	0.003±	0.0021±	0.0034±
Petunidin	0.003±	0.421±	0.553±	0.002±	0.015±	0.026±

Table 7. Profiling of organic acids in different extract of soursop leaves and fruits

Organic acids (mg/ g FW)	**Leaves**			**Fruit**		
	Aqueous	**Methanol**	**Ethanol**	**Aqueous**	**Methanol**	**Ethanol**
Oxalic acid	0.0031±	0.0074±	0.0088±	0.072	0.147±	0.201±
Maleic acid	0.023±	0.057±	0.066±	0.468	0.792±	0.917±
Citric acid	0.283±	0.441±	0.589±	0.234	0.558±	0.764±
Tartaric acid	0.172±	0.374±	0.401±	0.253	0.708±	0.861±
Malic acid	0.305±	0.462±	0.535±	0.897	1.770±	2.03±
Ascorbic acid	0.004±	0.008±	0.010±	0.240	0.561±	0.623±
Shikimic acid	0.003±	0.011±	0.013±	0.018	0.030±	0.035±
Fumeric acid	0.002±	0.008±	0.013±	0.091	0.106±	0.143±

Table 8. Profiling of fatty acids of soursop leaves and fruits

	Fatty acids (mg/100g FW)	**Fruit**	**Leaves**
C8:0	CAPRYLIC	0.43±	0.18±
C10:0	CAPRIC	0.02±	0.01±
C12:0	LAURIC	0.03±	0.008±
C13:0	TRIDECANOIC	1.34±	0.70±
C14:0	MYRISTIC	3.72±	1.99±
C15:0	PENTADECANOIC	0.86±	0.28±
C16:0	PALMITIC	145.8±	72.8±
C16:1	PALMITOLEIC	0.64±	0.12±
C17:0	HEPTADECANOIC	5.83±	1.58±
C18:0	STEARIC	49.12±	11.93±
C18:1	OLEIC	32.35±	8.03±
C18:2	LINOLEIC	62.28±	17.06±
C18:3	LINOLENIC	2.33±	0.35±
C19:0	NONADECANOIC	0.07±	0.004±
C20:0	ARACHIDIC	4.82±	0.86±
C20:1	EICOSENOIC	2.47±	0.35±
C21:0	HENEICOSENOIC	0.34±	0.09±
C22:0	BEHENIC	2.47±	0.79±
C22:1	ERUCIC	0.57±	0.13±
C23:0	TRIEICOSENOIC	0.08±	0.02±
C24:0	TETRAEICOSENOIC	0.005±	0.001±
C26:0	HEXAEICOSENOIC	1.29±	0.73±

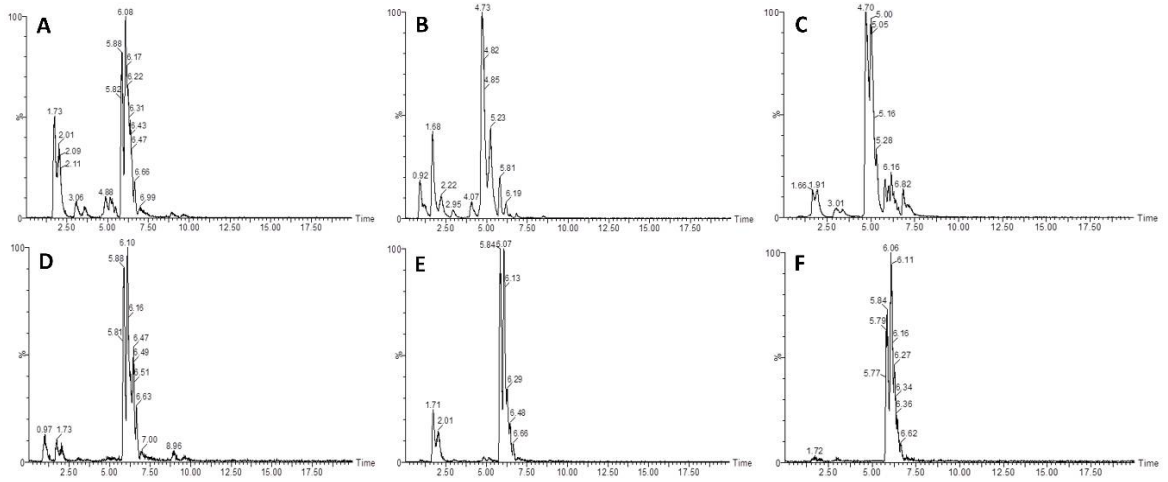

Figure 1. Chromatogram details of phenolic acids present in soursop leaves and fruit extract analyzed by LCMS. A,B and C indicates samples of leaves ethanol, methanol and aqueous extracts. D,E and F showed the results of fruit pulp ethanol, methanol and aqueous extracts

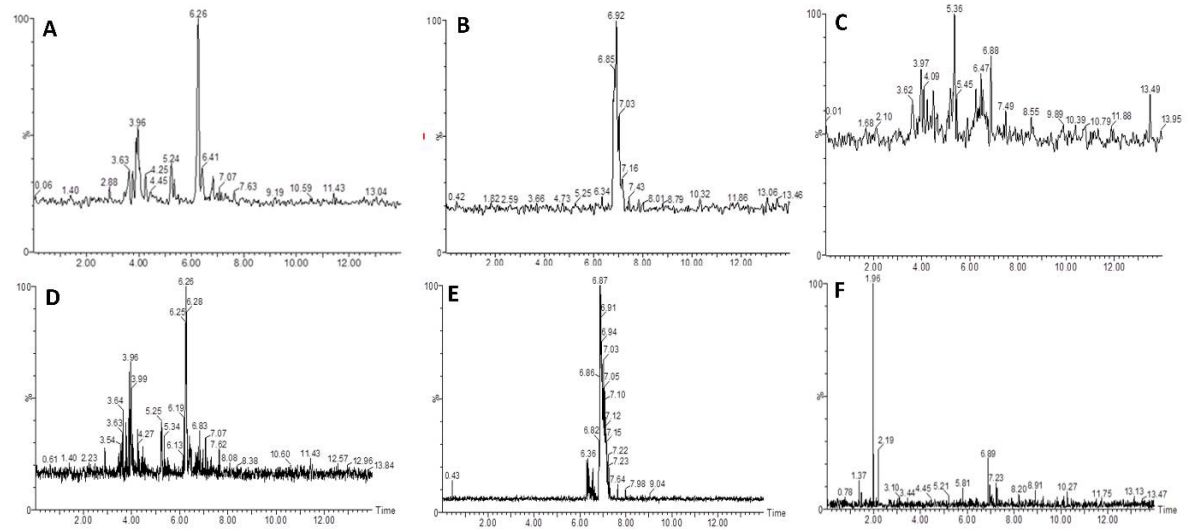

Figure 2. Chromatogram details of flavonoids present in soursop leaves and fruit extract analyzed by LCMS. A, B and C indicates samples of leaves ethanol, methanol and aqueous extracts. D, E and F showed the results of fruit pulp ethanol, methanol and aqueous extracts

DISCUSSION

Most of the phenolic compounds of the soursop leaf extracts evaluated in the present study corresponded vanillin, gallic acid and eugenol were minor fractions (Table 1). Rutin (the rhamnoglucoside of the flavonoid quercetin) was the principal compound in A. muricata leaves. This result has been determined in previous studies, such as that of [13] who reported high concentrations of rutin in A. muricata leaves, even higher than other sources. In an investigation purified rutin and other metabolites from A. muricata leaf fractions, rutin showed significant antihyperglycemic activity. The other compounds found in the present study have been extracted from the stems, leaves, roots and fruits of other Annonaceae, including vanillin, gallic acid, eugenol and naringenin. The antioxidant capacity of the extracts is influenced by the pH and chemical nature of the solvents used in the extraction, as less polar solvents show a greater capacity to dissolve bioactive antioxidant substances. The antioxidant activity of AE by DPPH test was the lowest among all of the samples included in this study. This low antioxidant capacity could be related to the low contents of phytochemicals such as phenolics and flavonoids in these extracts (Table 1). In previous research by [14] that evaluated A. muricata pulp using the DPPH radical assay, the values of antioxidant activity were different than those found in the present study. This is mainly because the

leaves are more metabolically active than the fruit, and their secondary metabolism produces antioxidant compounds as a response to environmental factors. On the other hand, it is observed that in the antioxidant capacity for ethanol extract measured by DPPH and ABTS, it was higher than other solvent extracts. These results indicated that the phenolic compounds in the Table 1 contributed positively to their antioxidant capacity by reducing the levels of free radicals. The TPC varied in the range of 10.92–244.61 mg CE/g in leaf extracts; 20.75–187.48 mg CE/g in peel extracts; 19.84–50.15 mg CE/g in pulp extracts; and 5.06–202.17 mg CE/g in seed extracts (Table 1). MeOH extracts had a much Extract TPC (mg CE/g) TFC (mg CE/g) higher TPC than the extracts obtained using other solvents (except fruit pulp extracts). In the case of fruit pulp, EtOAc was a more effective ($p<0.05$) phenolic compound extract- ant. Hxn extracts had the lowest TPC ($p<0.05$). Converting TPC of extracts by extraction yields, it can be noted that peels and leaves were the richest sources of phenolic compounds, followed by pulp and seeds. Higher TPC of soursop fruit pulp compared to that of seeds was in line with literature data. Moreover, higher TPC in the peels than in the pulp of fruits of different Annona species (*A. cherimola* L. and *A. squamosal* L.) was previously reported [15]. The TFC of extracts is shown in Table 1. When the results were compared based on the extraction solvent used, MeOH and EtOAc extracts had the highest TFC. On the other hand, as could be expected, Hxn was the least effective solvent for flavonoid extraction. [15] reported that TFC/ TPC ratios of Annona fruit peel and pulp ranged from 0.3 to 0.6. In our study, similar values were obtained for MeOH extracts, but TFC/TPC ratios of EtOAc extracts were significantly higher, i.e. at about 0.9. This indicates good selectivity of EtOAc for flavonoid extraction from soursop fruits and leaves. In previous studies, the presence of flavonoids belonging to subclasses of flavan-3-ols and flavonols was determined in soursop leaves, fruit pulp, and peels [16]. Besides flavonoids, hydroxycinnamic acid derivatives were identified in leaves and pulp [17]. In turn, phenolic terpenoids were found in soursop seeds.

CONCLUSION

Phytochemical screening of leaves ethanol extracts revealed it to be rich in secondary metabolic compounds. The use of Annona muricata in traditional medicine is validated by presence of these phytochemicals of known health benefits. LC-MS Analysis revealed that, more phytochemicals such as phenolic acids, flavonoids, terpenoids and anthocyanins were present in leaves ethanol extract. The *in vitro* antioxidant activity of leaves and fruit extracts of Annona muricata revealed a significant antioxidant activity in ethanolic leaves extract. These results indicated that soursop leaves can inhibit hepatic cancer cell proliferation and activate apoptosis pathway. Hence, it is anticipated that *Annona muricata* would be a potentially useful pharmaceutical material to manage liver cancer.

COMPETING INTERESTS

The authors have declared that no competing interest exists

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