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Research Article

Antioxidant potential of fruit peel waste of two species of *Annonaceae* and detection of spathulenol and β-Pimaric acid as major bioactive compounds by GC-MS.

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ABSTRACT
Methanolic (M), hydroxymethanolic (AQ: M) and aqueous (AQ) fruit peel extract of *Annona squamosa* and *Annona reticulata* were investigated for their therapeutic potential. For this purpose, the total phenolic content, total flavonoid content and free radical scavenging activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH), Ferric ion reducing antioxidant power (FRAP) and 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays were studied. Methanolic fruit peel extract of *A. squamosa* and *A. reticulata* showed highest total phenolic content (27.99±0.33 mgGAE/g dry material) and total flavonoid content (9.00±0.20 mgQE/g dry material), respectively. DPPH, ABTS and FRAP result indicated high antioxidant potential of fruit peel waste. This is the first report on GC-MS analysis of methanolic fruit peel extract of *Annona* and the results revealed the presence of three main therapeutically important bioactive compounds viz β-Pimaric acid, Spathulenol and 4H-Pyran-4-one in both the species with concentration of 24.32, 14.65 and 7.92%, respectively in *A. squamosa* and 18.58, 9.53 and 12.13%, respectively in *A. reticulata*. These compounds are reported to show antioxidant, antibacterial, antiproliferative, anticancerous, anti-inflammatory and immunomodulatory activities. Other compounds detected in these extract were n-Hexadecanoic acid (6.05%), n-Heptacosane (6.0%), 1H- Napthol[2,1-b]pyran-7-carboxylic acid, 3-ethenyldodecahydro-3,4a,7, 10a- tetramethyl -methyl ester (14.17%), 1,2-Benzenediol (8.33%) and 9,12-Octadecadienoic acid (ZZ)- (7.83%). The present report indicates a strong possibility of effective utilization of fruit peel waste for extraction of active principles.

KEYWORDS
Annona reticulata, *Annona squamosa*, antioxidant activity, GC-MS analysis, fruit peel waste, secondary metabolite.

1. INTRODUCTION

Annona squamosa is an evergreen, small, well-branched shrub. It is commonly known as "sitaphal" in Hindi and "custard apple" in English. *Annona reticulata* is a low, erect deciduous tree with rounded crown. It is commonly known as "Ramphal" in Hindi and Bullock's heart" in English. The Sitaphal and Ramphal are ancient, mystical, exotic and highly distinctive fruits of *Annonaceae* family. These fruits are in great demand and are highly consumed by a large number of population for their unique delicious taste and nutritional value. Apart from being rich in carbohydrates (23.5%), proteins (1.6%), vitamins (A, B_6 and C), minerals (Iron, calcium, magnesium, potassium, phosphorus and copper) and amino acids like arginine, glutamine, serine, leucine, tyrosine and tryptophan[1]. These fruits also possess great therapeutic and medicinal potential. Presence of large number of various secondary metabolites like phenols, steroids, tannins, glycosides, terpenes, flavonoids and polyphenolic compounds in these fruits has been reported[2, 3]. The fruit contains special class of compounds called acetogenins that possesses the anticancer properties[4]. The cytotoxic property is due to potent inhibition of NADH: ubiquinone oxidoreductase (complex1) of the mitochondrial electron transport system which results in inhibition of oxidative phosphorylation and lowering of ATP levels such that cell growth is inhibited[5]. In addition, the fruits have also been reported to show antidysentric, vermifuge, antihelmintic, styptic, insecticide and immunosuppressive properties[6].

According to the market research report, global market competition of custard apple industry has increased significantly in the international market around the world including USA, EU, Japan, China and India[7]. The fruits are in great demand in food, agro and dairy product industries, where pulp is utilized to make juice, ice cream and rabadi. In view of the importance of these fruits in food industry its exploitation for medicinal purpose is limited. Fruit peel however is considered as inedible and is therefore discarded which adds to the agricultural waste. Agro waste disposal is a serious environmental problem. Peel makes around 30-50% of the total fruit weight in many fruits. Since the fruit peels are also known to be rich in bioactive compounds like fibres, oil, pectin, phenols, flavonoids, enzymes, tannins, terpenoids and antioxidants[8, 9, 10], their utilization in herbal formulations could be an innovative technique for peel waste management. Previous study has reported high antioxidant activity in fruit peel of *A. crassiflora*[11]. Very little information is available on phytochemical characterization of fruit peel of *A. squamosa* and *A. reticulata* except for the presence of phenols and flavonoids[12, 13]. The present study was aimed at exploring the therapeutic potential of peels of *A. squamosa* and *A. reticuala* by evaluating its antioxidant activity, total phenols and flavonoid content and GC-MS based metabolite profile.

2. MATERIALS AND METHODS

2.1. Plant Material

The fruits of *A. squamosa* and *A. reticulata* were collected from the APMC local market, Navi Mumbai, Maharashtra, India. The fruits were taxonomically identified and authenticated by Dr. Jossy Varghese, Dept. of Botany, B.K. Birla College, Kalyan. The peels were separated from fruits and air dried in shade separately.

2.2. Fruit Peel Samples and Extraction Procedure

The dried fruit peels were powdered in mixer and grinder. 5 g of sample were extracted using 50 ml solvents; methanol, aqueous: methanol (1:1) and aqueous two times for 48 hours by the plant tissue maceration method[14]. The extract will be decantated, filtered using whatman no. 1 paper and concentrated under pressure in a rotary evaporator at 40°C and redissolved in the solvent to determine the concentration. The concentrated extracts were stored in refrigerator at -20°C till further used. Percentage extraction yield *A. squamosa* and
A. reticulata in different solvent was calculated.

2.3. Chemicals and Reagents

All the reagents used in the study were of analytical grade. Folin-Ciocalteu reagent sodium carbonate, gallic acid, ascorbic acid, quercetin, sodium nitrite, aluminium chloride, ferric chloride, sodium acetate, ammonium persulphate, potassium persulphate, glacial acetic acid and butylated hydroxy toluene (BHT) were purchased from Sigma Chemicals Co., (St. Louis, MO, USA); 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ) and 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Himedia, Mumbai, India.

2.4. Determination of Total Phenolic Content (TPC)

The total phenolic content was determined in peel extract of both species using the Folin-Ciocalteu reagent as per the standard protocol[15]. 1 ml of various concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0) of the extract or standard (Gallic acid) was added to a 25 ml volumetric flask containing 9 ml of distilled water. The total phenolic content was determined as mg GAE/g extract on comparison with a standard gallic acid graph. A reagent blank consisting of distilled water was prepared. 1 ml of Folin-Ciocalteu reagent (1N) was added to the mixture and shaken. After 5 minutes, 10 ml of 7% sodium carbonate was added to the mixture. The volume was then made up to the mark. After incubation for 90 minutes at room temperature in dark, the absorbance against the reagent blank was determined at 765 nm using a UV visible biochrom spectrophotometer. This was compared to the standards curve of Gallic acid concentration and expressed as mg of Gallic acid equivalents per g (mg GAE/g) of dry powder.

2.5. Determination of Total Flavonoid Content (TFC)

The aluminium chloride colorimetric assay was used to measure the total flavonoid content of the plant extracts[15]. 1 ml of various concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0) of the extract or standard (quercetin) was added to a 10 ml volumetric flask containing 4 ml of distilled water. 0.3 ml of 5% sodium nitrite ($NaNO_2$) was added to the flask and after five minutes, 0.3 ml of 10% aluminium chloride (Al_2Cl_3) was added. The volume was made up to the mark with distilled water. The solution was mixed and allowed to stand for 30 minutes at room temperature. The absorbance of reaction mixture was measured against the blank at 510 nm using a UV visible spectrophotometer. The total flavonoid content was quantified according to the standard curve prepared for quercetin and concentration of flavonoid was expressed as mg of quercetin equivalents per g (mg QE/g) of dry powder.

2.6. Antioxidant Assays

2.6.1. DPPH Radical Scavenging Activity

Antioxidant activity of the extract was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [16]. 0.1 ml of various concentrations (0, 20, 40, 60, 80 and 100 µl) of the extract was added to a

2.7 ml of methanol and then 0.2 ml of 0.1% freshly prepare methanolic DPPH was added. The mixture was shaken and left to stand at room temperature in the dark. After 30 minutes the decrease in the absorbance was measured at 517 nm against a methanol blank. The antioxidant activity of the sample was compared with known synthetic standard of (0.16%) of butylated hydroxy toluene (BHT) as a positive control. The concentrations of the samples for 50% inhibition of DPPH (IC_{50}) were obtained from the graph of DPPH RSA% (radical scavenging activity percentage) versus a concentration of the sample in milligram per milliliter (mg/ml). The ability to scavenge DPPH radical was calculated using the formula:

DPPH RSA (%) = Abs of control – Abs of sample/ Abs of control *100

Where, Abs control is the absorbance of the control solution (sample without extract) and Abs sample is the absorbance of the test sample (sample with extract).

2.6.2. Ferric ion reducing antioxidant power assay (FRAP)

The FRAP method measures the change in absorbance that occurs when the (2,4,6-tripyridyl-s-triazine) TPTZ-Fe^{3+} complex is reduced to the TPTZ- Fe^{2+} form in the presence of antioxidant compounds[17]. The FRAP reagent was prepared by mixing 10 mM TPTZ solution in 40 mM HCL, 20 mM $FeCl_3$ and 0.3 M acetate buffer (pH 3.6) in ratio 1:1:10. The above reagents were mixed together and incubated at 37°C for 30 minutes. 1 ml of various concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0) of extract were allowed to react with 0.2 ml of the FRAP reagent. The final volume of reaction mixture was made up to 3 ml with distilled water. The reaction mixture was kept in the dark for 30 minutes at 37°C. The absorbance was measured at 593 nm using spectrophotometer. The antioxidant content based on ferric ion reducing ability was calculated using a standard curve of ascorbic acid at 593 nm. The FRAP result was expressed as mg of ascorbic acid equivalent antioxidant capacity per g of dry powder (mg AEAC/g).

2.6.3. ABTS Radical Scavenging assay (ABTS)

ABTS assay is one of the most used assays for the determination of the concentration of free radicals[18]. It is based on the neutralization of a radical-cation arising from one electron oxidation of the synthetic chromophore 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). The stock solution of 7 mM ABTS and 2.45 mM ammonium persulphate solution was made separately. The working solution was made by mixing the two stock solutions in equal quantity and allowing them to react for 12-16 h at room temperature in dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an OD of 0.707±0.001 at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Fruit peel extracts (1ml) were allowed to react with 1 ml of ABTS solution and the OD was taken at 734 nm after 6-7 min using the spectrophotometer. All the readings were taken in triplicate. The ABTS scavenging capacity of the extract was calculated as:

% Inhibition = Abs of control-Abs of sample/ Abs of control*100

Where, Abs control is the absorbance of the control solution (sample without extract) and Abs sample is the absorbance of the test sample (sample with extract).

2.7. Detection of bioactive compounds by GC-MS

The methanolic peel extract of both species of *Annona* were subjected to gas chromatography-mass spectral analysis on an Agilent Technologies with model 7890A GC System, equipped with a model JEOL The AccuTOF GCv JMS-T100GCV mass selective detector (EIMS, electron

energy, 70 eV) and an Agilent chem. station data system. The GC column was an HP-5ms fused silica capillary with a EB-5 (5% phenyl methyl polysiloxane), film thickness of 0.25 µm, a length of 30 m and an internal diameter of 0.25 mm. The carrier gas was helium with a flow rate of 1.0 ml/min. Inlet temperature was 250°C and MSD detector temperature was 270°C. The mass spectrometer was operated in electron impact ionization (EI) mode with 70eV energy. The mass range was 50-700 a.m.u. and the ion source temperature was 200°C. The GC oven temperature program was used as follows. 80°C initial temperature for 2 min then programmed at 10°C/min to 270°C and held for 10 min. Concentrated methanolic fruit peel sample of both species were used. 1 µl injection using a splitless injection technique was used. Identification of components was achieved based on their retention indices (RI) and by comparison of their mass spectral fragmentation patterns with those reported in the literature and stored on the MS libraries [National Institute of Standard and Technology (NIST) 05; Mass Finder database (G1036A, revision D.01.00 and Wiley 7 Mass Finder] [19]. The bioactive components detected from fruit peel extract of both the species are compiled (Table- 5, 6).

2.8. Statistical analysis

The Statistical analysis was performed using SPSS 14.0 software. All data were expressed as the mean ± S.D. (Standard Deviation) and all the experiments were performed in triplicate. An analysis of variance (ANOVA) was performed to assess the statistical significance of the differences between the samples. Duncan's multiple range test (DMRT) was performed for comparison of means, considering a confidence level of 95% ($p < 0.05$). Linear regression analysis R^2 was performed on the standard and test extracts. The IC_{50} values were calculated by linear regression analysis using Microsoft Excel. Linear correlation was performed to assess the relationship between the total phenols, flavonoids and antioxidant assays following Pearson's method.

3. RESULTS AND DISCUSSION

3.1. Percentage of extraction yield

The percentage yield was highest in methanolic extract in *A. squamosa* (28.8%) and *A. reticulata* (18.4 %) as compared to hydroxymethanol (1:1) and aqueous extracts (Table-1). Thus methanol served as the best solvent for extracting principle components in the present study. Our results are in agreement with studies that also reported methanol as a best solvent for exhibiting the highest extraction yield in citrus fruit peel and pineapple[9, 10].

3.2. Total Phenol and Flavonoid content (TPC and TFC)

The plants belonging to genus *Annona* are rich in phenolics and flavonoids compounds and are therefore known for their medicinal value[20]. The present investigation indicated the good amount of phenol and flavonoids present in fruit peel extract. Values of TPC and TFC were significantly different ($p<0.05$) across the two species and the solvent system used. As compared to other solvents methanolic extracts showed maximum values of TPC (27.99±0.33 mg GAE/g) and TFC (9.00±0.20 mg QE/g) in *A. squamosa* and *A. reticulata*, respectively. TPC in *A. squamosa* was around 2.5 times higher than that in *A. reticulata* whereas TFC was found nearly similar in both the species (Table- 2). A TPC of 11.20 mg GAE/g of dry weight and TFC of 9.00 mg QE/g of dry weight were found in methanolic extract of *A. reticulata* peel (ARP) in the

present study whereas previous study reported lower phenolics (5.19 mg GAE/g) in acetone extract and flavonoid (4.34 mg QE/g) in aqueous (AQ) extract of ARP[13]. However, the TFC reported by them in AQ extract was two times higher than that noted in AQ extract of ARP in our case. Similar to our results maximum TPC was also reported in methanolic peel extract of *A. squamosa*[21]. A maximum TPC of 9.35 mg GAE/g of dry extract was reported in hydoxyethanol extract of CAB (custard apple bagasse) flour[22], which is three times lower than the amount obtained in methanolic extracts of *A. squamosa* peel (ASP) in present study. Similar to our results, several other researchers have also reported high TPC and TFC in the fruit peels[2, 8, 23, 24].

3.3. Antioxidant studies

3.3.1. DPPH Assay

DPPH assay has been the most widely used assay to evaluate antioxidant activity of plants. The results of the present study showed that the scavenging effect of sample (peel extract) and standard (BHT) on the DPPH radical increased sharply with the increasing concentration of sample in both the species of *Annona*. In both species highest percentage of free radical scavenging activity (RSA) was found in methanolic extract followed by hydroxymethanolic and aqueous. It was 95.49±0.18 % and 94.42±0.05 % in ASP and ARP, respectively which was much higher than that recorded for synthetic antioxidant (BHT), a positive control (77.57±0.38%) and that reported previously in 80% methanol extract of *A. squamosa* peel (67.1%)[12]. Our findings are in agreement with previous study that also found high free radical scavenging activity in peel of *A. cherimola*[23]. From a plot of concentration against percentage of inhibition, a linear regression analysis was performed to determine the IC_{50} value (the extract concentration that could scavenge 50% of the DPPH radicals). Lowest IC_{50} value (0.437 mg/ml) was reported in methanolic peel extract of *A. squamosa* with R^2=0.991 which indicated better radical scavenging activity than *A. reticulata* (IC_{50} =1.011 mg/ml). In the present study, methanolic extract showed great percentage of DPPH free radical scavenging activity around 95% in both the species of *Annona* (Table- 3).

3.3.2. ABTS Assay

ABTS is another important assay used to measure antioxidant activity. Ability of *A. squamosa* and *A. reticulata* peel extracts to scavenge the blue green colored $ABTS^+$ radical cation was measured relative to the radical scavenging ability of gallic acid. Around 96% inhibition of ABTS free radicals was shown by methanolic extract of both the species of *Annona* (Table- 3) indicating rapid scavenging activity. Similar to our results ABTS assay based high antioxidant activity in peels with TEAC values of 3.6 in *A. cherimola* ethanolic extract[23] and 121.3 in *A. squamosa* methanolic extract[21] has been reported earlier.

3.3.3. FRAP Assay

FRAP assay treats the antioxidants present in the sample as reductant in a redox linked colorimetric reaction[25]. FRAP assay is based on the ability to reduce ferric (III) to ferrous (II) ions. Out of all the solvent tested, methanolic peel extract showed highest FRAP values in both the species (Table- 3), with *A. squamosa* (43.44±0.19 mg AEAC/g) showing the value two times higher than that recorded for *A. reticulata* (24.95±0.27 mg AEAC/g). High reducing power with FRAP value of 52.8 µM Fe(II)/g in *A. cherimola* peel extract has been reported earlier[23].

High free radical scavenging activity as revealed by three assays could be attributed to presence of antioxidant compounds in the peel waste. In the present study, methanolic extract showed highest free radical scavenging activity based on all the three assays. In contrast to our findings different free radical scavenging activity assay showed varying results in the same set of extraction solvents[26, 27].

3.4. Correlation between TPC, TFC and antioxidant activity

To establish the relationship amongst TPC, TFC and antioxidant assays (DPPH, ABTS and FRAP) correlation analysis was performed (Table- 4). TPC and TFC showed positive correlation with not all but one or the other antioxidant activity assay. TFC showed correlation with DPPH in both the *Annona* species and with FRAP also in *A. reticulata* whereas TPC showed correlation with ABTS in ASP and with DPPH in ARP. Results of all three antioxidant assays revealed that the fruit peel waste possess free radical scavenging activity and there was a significant correlation ($p<0.05$) between DPPH and ABTS assay in ASP. These results indicated that in addition to phenol and flavonoid other antioxidant compounds might have contributed to free radical scavenging activity of peel extract. In some studies the correlation between TPC and antioxidant activity was absent, however majority of the studies show a positive correlation[28, 29].

3.5. GC-MS analysis

In order to identify the active principles and antioxidants in particular, GC-MS profiling of methanolic peel extract of *A. squamosa* and *A. reticulata* was carried out and the compounds were identified on the basis of retention times (RT) and mass-to-charge ratios (m/z). The result revealed the presence of various phytochemicals including phenols, flavonoids, terpenoids, tannins and alkaloids. The chromatogram obtained for methanolic peel extract showed a total of nineteen compounds in both the species (Table- 5; 6). The chromatograms revealed that the compounds with major retention peaks (7.97, 13.84, 21.93 and 22.61) in *A. squamosa* and (7.99, 13.87, 21.96 and 22.64) in *A. reticulata* are 4H-Pyran-4-one, spathulenol, 1-Napthalenemethanol decahydro-5-(5-hydroxy-3-methyl-3-pentenyl)-1,4a-dimethyl-6-methylene and β-Pimaric acid, respectively (Fig. 1, 2). 4H-Pyran-4-one with peak area of 7.92% in ASP and 12.13% in ARP has been reported to possess antimicrobial, anti-inflammatory[30], anti-proliferative and pro-apoptotic activity through inactivation of NF-κB in human colon cancer cells[31], antioxidant and antidiabetic activity[32]. Spathulenol peak area was 14.65% and 9.53% in ASP and ARP, respectively. It is an oxygenated sesquiterpenes having aromadendran-type structure. Sesquiterpenes are rare compounds[33]. Spathulenol has been reported to show immunomodulatory[34], antioxidant, antiproliferative, anti-inflammatory and antimycobacterial activities[35, 36]. Spathulenol has also been reported to be a major volatile component of essential oils in leaves of *Psidium guineense* and *Cinnamomum osmophloeum*[35, 37]. Previous studies also reported the presence of spathulenol in *A. muricata* leaves (1.01%) and *Syncarpia glomulifera* leaves (0.96%)[38, 39] however, the content was lower as compared to that obtained in the peel extract in the present study. The peak area of 1-Napthalenemethanol, decahydro-5-(5-hydroxy-3-methyl-3-pentenyl)-1,4a-dimethyl-6-methylene was 7.62% and 4.37% in ASP and ARP, respectively. This compound possessed insecticidal and larvicidal activity[40, 41]. β-Pimaric acid with a peak area of 24.32% and 18.58% in ASP and ARP, respectively has been reported to show antibacterial and anti-atherosclerotic activity[42, 43, 44].

In the present study, other bioactive compounds identified were n-Hexadecanoic acid (6.05%), 9, 12-Octadecadienoic acid (5.03%), n-Heptacosane (6.0%) and 1H-Napthol[2,1-b]pyran-8(4aH)-one (5.03%) in ASP (Table- 5) and 1H- Napthol[2,1-b]pyran-7-carboxylic acid, 3-ethenyldodecahydro-3,4a,7, 10a- tetramethyl -methyl ester (14.17%), 1,2-Benzenediol (8.33%), 9,12-Octadecadienoic acid (ZZ)- (7.83%) and 4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-(6.03%) in ARP (Table- 6). Most of these compounds are heterocyclic and such compounds are known to contribute significantly to the antioxidant and anticancer properties.

4. CONCLUSION

Methanol proved to be the best solvent in terms of extraction yield, total phenol, flavonoid content and antioxidant activity in peel of both the species of *Anoona*. *A. squamosa* peels were richer in phenolics and showed greater free radical scavenging ability as compared to *A. reticulata.* GC-MS based metabolite profiling revealed the presence of two main bioactive compounds β-Pimaric acid and Spathulenol in peel of both the species. However, the percentages of these compounds were higher in ASP than ARP. The results indicate *Annona* fruit peel waste to be a potential natural resource for bioactive compounds. Further research is required to isolate and characterize spathulenol and β-Pimaric acid from fruit peels waste and tests their bioactivity.

5. CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Table 1: Percentage extraction yield of *A. squamosa* and *A. reticulta* peel in different solvent systems.

Sample	**Colour of extract**	**Yield in different solvent systems (%)**	
		M	AQ:M (1:1)
		AQ	
A. squamosa peel	Dark browm	28.80	27.28
		20.34	
A. reticulata peel	Light brown	18.40	17.42
		9.72	

Methanol (M), Aqueous:Methanol (AQ:M) and Aqueous (AQ).

Table 2: Total phenolic Content (TPC) and total flavonoid Content (TFC) in peel extract of *A. squamosa* and *A. reticulata*.

Analysis	***A. squamosa* peel (ASP)**			***A. reticulata* peel (ARP)**		
	M	AQ:M	AQ	M	AQ:M	AQ
TPC	27.99 ± 0.33^{a}	15.17 ± 0.52^{b}	9.00 ± 0.0^{d}	11.2 ± 0.20^{c}	6.44 ± 0.09^{e}	1.43 ± 0.11^{f}
TFC	8.88 ± 0.38^{b}	5.83 ± 0.28^{d}	1.73 ± 0.23^{f}	9.00 ± 0.20^{a}	6.28 ± 0.09^{c}	2.53 ± 0.11^{e}

Values are average of three replicates ± standard deviation. Means followed by different letters in a row are significantly different ($p<0.05$), DMRT.

*TPC value expressed as mg GAE/g of dried material. **TFC value expressed as mg QE/g of dried material.

Table 3: Antioxidant activity of *A. squamosa* and *A. reticulata* peel extract.

Assay	***A. squamosa* peel (ASP)**			***A. reticulata* peel (ARP)**		
	M	AQ:M	AQ	M	AQ:M	AQ
DPPH RSA (%)	95.49 ± 0.18^{a}	80.63 ± 0.07^{d}	45.74 ± 0.38^{f}	94.42 ± 0.05^{b}	90.08 ± 0.07^{c}	51.52 ± 0.09^{e}
IC_{50} (mg/ml)	0.437	0.573	1.106	1.011	1.252	2.932

ABTS I(%)	96.46±0.37[a]	89.92±0.31[c]	61.79±0.40[d]	96.66±0.40[a]	94.19±0.23[b]	52.38±0.54[e]
FRAP (mgAEAC/g)	43.44±0.19[a]	19.41±0.14[c]	7.80±0.00[e]	24.95±0.11[b]	13.50±0.11[d]	6.34±0.05[f]

Control	
BHT	77.57±0.38
IC_{50}(mg/ml)	0.705
Gallic acid I (%)	94.68±0.19

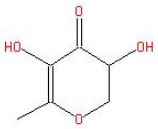
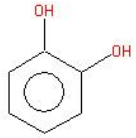
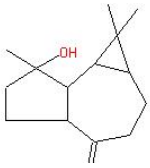
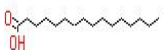
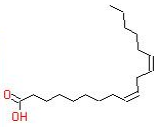
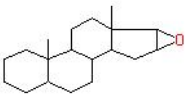
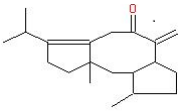
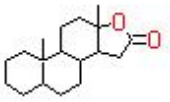
Values are average of three replicates ± standard deviation. Means followed by different letter in a row are significantly different ($p<0.05$), DMRT.
DPPH radical scavenging activity (RSA) expressed in percentage (%), IC_{50} value expressed in mg/ml and BHT, positive control.
ABTS antioxidant activity expressed in inhibition percentage (%) and gallic acid used as control.
FRAP result expressed as mg Ascorbic acid Equivalent Antioxoidant Capacity/g of dried material.

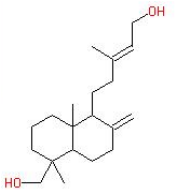
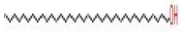
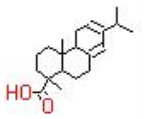
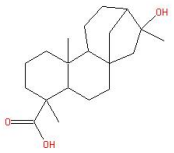
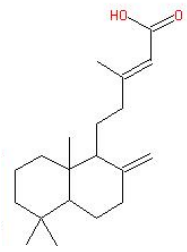
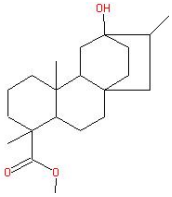
Table 4: Correlation (r) between total phenolic content (TPC), total flavonoid content (TFC) and different antioxidant capacity parameters (DPPH, ABTS and FRAP) of methanolic peel extract of *A. squamosa* (ASP) and *A. reticulata* (ARP).

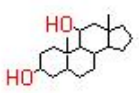
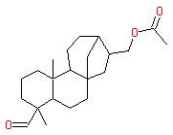
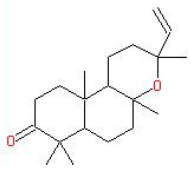
	TPC	TFC	DPPH	ABTS	FRAP
ASP					
TPC	1	-0.104	-0.156	0.030	-0.416
TFC	-0.104	1	0.405	0.407	-0.479
DPPH	-0.156	0.405	1	0.709*	0.051
ABTS	0.030	0.407	0.709*	1	-0.137
FRAP	-0.416	-0.479	0.051	-0.137	1
ARP					
TPC	1	0.224	0.355	-.063	-0.095
TFC	0.224	1	0.231	-0.667*	0.555
DPPH	0.355	0.231	1	-0.296	0.560
ABTS	-0.063	-0.667*	-0.296	1	-0.612
FRAP	-0.095	0.555	0.560	-0.612	1

* Correlation is significant at the $p< 0.05$ level (2-tailed).

Table 5: List of bioactive compounds detected in methanolic peel extract of *A. squamosa* by GC-MS analysis.

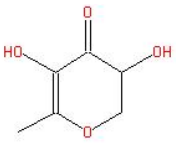
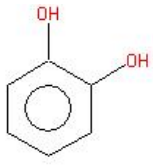
No.	Name of Compound	M.F.	M.Wt. (g/mol)	R.T. (min)	Peak Area (%)	Expected structure	Biological activity*
1.	4H-Pyran-4-one, 2,3-dihydro- 3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.12	7.97	7.92		Antiproliferative, pro-apoptotic, antioxidant, antidiabetic, antimicrobial, anti-inflammatory
2.	1,2-Benzenediol	$C_6H_6O_2$	110.11	9.09	3.21		Antimycobacterial, antimicrobial
3.	(-)-spathulenol, 1H-cyclopropa[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-	$C_{15}H_{24}O$	220.35	13.84	14.65		Antioxidant, Antiproliferative, Immunomodulatory
4.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	17.78	6.05		Antioxidant, Antidiabetic,Hypo-cholesterolemic, Nematicide, Pesticide, Anti-Androgenic
5.	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280.44	19.47	5.03		Anti-neosporal
6.	5α,14β-Androstane,16α,17α-epoxy	$C_{19}H_{30}O$	274.44	20.34	1.34		Antioxidant, antitumor
7.	Cyclopenta[a,d]cyclooocten-5-one	$C_{20}H_{30}O$	286.45	21.04	2.21		Antioxidant
8.	17-Oxaandrostan-16-one,(5α)-	$C_{18}H_{28}O_2$	276.41	21.49	1.46		Antioxidant, antitumor
9.	1-	$C_{20}H_{34}O_2$	306.48	21.93	7.62		Insecticidal,

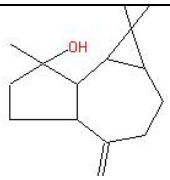
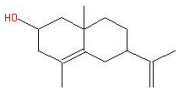
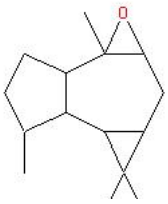
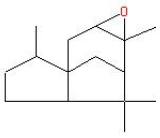
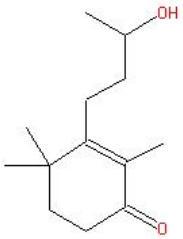
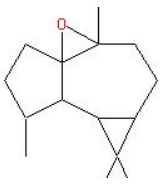
	Napthalenemethanol, decahydro-5-(5-hydroxy-3-methyl-3-pentenyl)-1,4a-dimethyl-6-methylene-,[1S-[1α,4aα,5α(E),8aβ]]-						Larvicidal
10.	1-Heptatriacotanol	$C_{37}H_{76}O$	536.99	22.29	1.14		Anti-Hypercholesterolemic
11.	β-Pimaric acid	$C_{20}H_{30}O_2$	302.45	22.61	24.32		Antibacterial, anti-atherosclerotic
12.	Kauran-18-oic acid,16-hydroxy-(4α)-	$C_{20}H_{32}O_3$	320	22.92	2.43		Antioxidant, cytotoxic
13.	2-Pentenoic acid, 5-(decahydro-5,5,8a-trimethyl-2-methylene-1-napthalenyl)-3-methyl-,[1S-[1α(E),4aβ,8aα]]-	$C_{20}H_{32}O_2$	304.46	23.77	1.52		Analgesic, antitumor
14.	2H-Pyran,2-(7-heptadecynyloxy) tetrahydro-	$C_{22}H_{40}O_2$	336.55	24.39	2.18		Antidote, hematopoietic
15.	Dihydro-isosteviol methyl ester	$C_{21}H_{34}O_3$	334	25.16	1.45		Antioxidant, antidiabetic

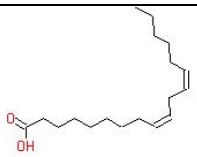
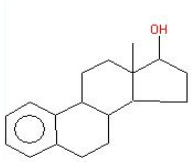
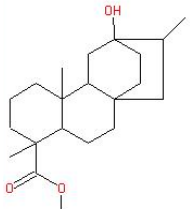
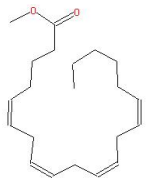
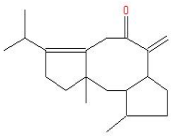
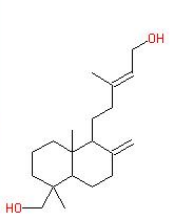
16.	Androstane-3,11-diol, (3β, 5α-11β)-	$C_{19}H_{32}O_2$	292.45	25.34	2.84		Antioxidant
17.	Kauran-18-al, 17-(acetyloxy)-,(4β)-	$C_{22}H_{34}O_3$	346.50	26.67	2.67		Antidote, prevent alopecia, alzheimerigenic
18.	n-Heptacosane	$C_{27}H_{56}$	380.73	28.78	6.00		Antitumor, increase NK cell activity, inhibit production of TNF
19.	1H-Napthol[2,1-b]pyran-8(4aH)-one, 3-ethenyldecahydro-3,4a,7,7,10a-pentamethyl-	$C_{20}H_{32}O_2$	304.46	28.96	5.03		Prevent abcess, abortifacient

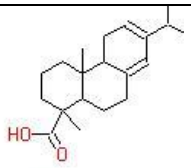
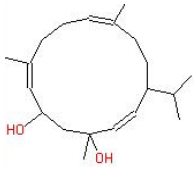
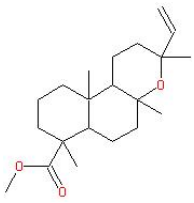
M.F. – Molecular formula, M.Wt.- Molecular weight, R.T.- Retention time
*Biological Activity source: Dr. Duke's Phytochemical and Ethnobotanical Database, ScienceDirect and WorldCat.org, Springer link [online database].

Table 6: List of bioactive compounds detected in methanolic peel extract of *A. reticulata* by GC-MS analysis.

No.	Name of Compound	M.F.	M.Wt. (g/mol)	R.T. (min)	Peak Area (%)	Expected structure	Biological activity*
1.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144.12	7.99	12.13		Antiproliferative, pro-apoptotic, antioxidant, antidiabetic, antimicrobial, anti-inflammatory
2.	1,2-Benzenediol	$C_6H_6O_2$	110	9.14	8.33		Antimycobacterial, antimicrobial

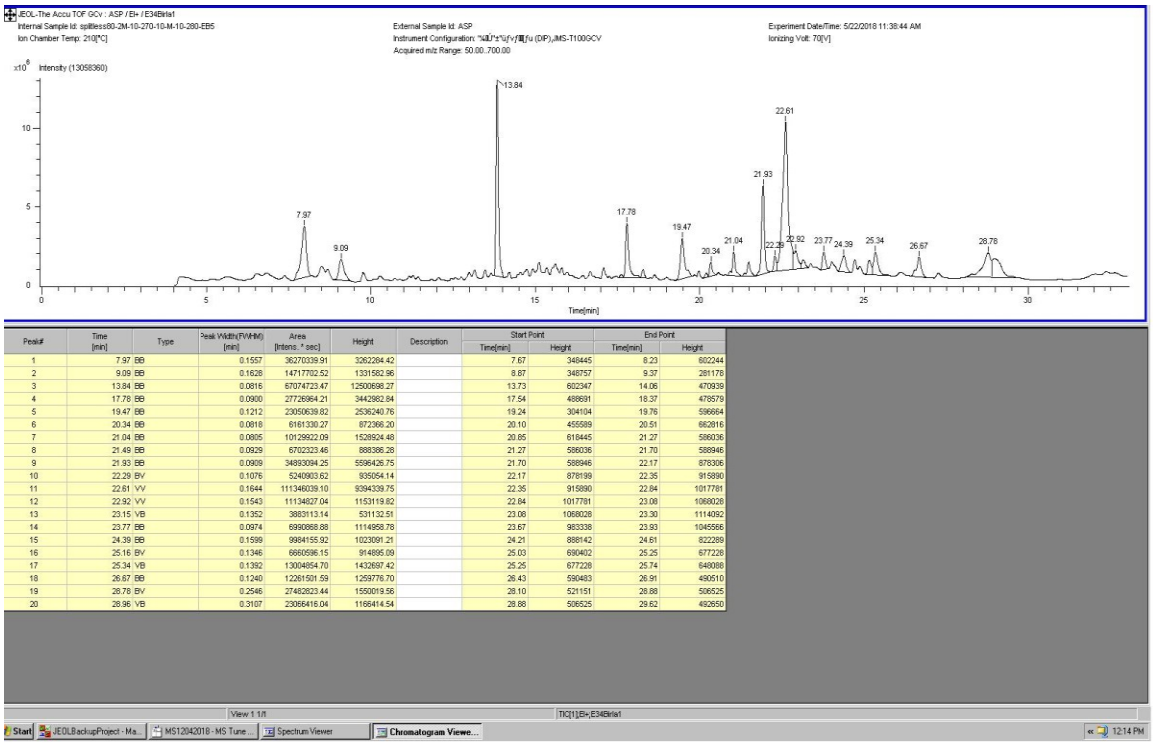
3.	(-)-Spathulenol, 1H-cyclopropa[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-	$C_{15}H_{24}O$	220.35	13.87	9.53		Antioxidant, Antiproliferative, Immunomodulatory
4.	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-napthalen-2-ol	$C_{15}H_{24}O$	220	15.16	4.17		Antifungal, antitumor
5.	Isoaromadendrene epoxide	$C_{15}H_{24}O$	220.35	17.11	2.49		Cytotoxic, Gastroprotective
6.	Diepi-α-cedrene epoxide	$C_{15}H_{24}O$	220	17.67	0.37		Antibacterial, Antimicrobial, antioxidant, anti-inflammatory
7.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	17.82	4.90		Antioxidant, Antidiabetic,Hypo-cholesterolemic, Nematicide, Pesticide, Anti-Androgenic
8.	2-Cyclohexen-1-one, 3-(3-hydroxybutyl)-2,4,4-trimethyl-	$C_{13}H_{22}O_2$	210.31	18.16	0.37		Antioxidant, antifungal
9.	Ledene oxide -(II)	$C_{15}H_{24}O$	220.35	18.30	0.19		Nematicidal, antioxidant, antimicrobial

10.	9,12-Octadecadienoic acid (ZZ)-	$C_{18}H_{32}O_2$	280.44	19.52	7.83		Acidifier, antitumor
11.	Estra-1,3,5(10)-trien-17β-ol	$C_{18}H_{24}O$	256.38	19.88	0.48		Estrogenic activity, Antitumor
12.	Dihydro-isosteviol methyl ester	$C_{21}H_{34}O_3$	334.49	20.01	0.51		Antioxidant, antidiabetic
13.	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)-	$C_{21}H_{34}O_2$	318	20.37	1.32		Anti-arthritic, anti-coronary, anti-inflammatory
14.	Cyclopenta[a,d] cycloocten-5-one	$C_{20}H_{30}O$	286.45	21.07	3.22		Antioxidant
15.	1-Napthlenemethanol, decahydro-5-(5-hydroxy-3-methyl-3-pentanyl)-1,4a-dimethyl-6-methylene-,[1S-[1α,4aα,5α(E),8aβ]]-	$C_{20}H_{34}O_2$	306.48	21.96	4.37		Insecticidal, Larvicidal
16.	1-Heptatriacotanol	$C_{37}H_{76}O$	536.99	22.33	0.90		Anti-Hypercholesterolemic

17.	β-Pimaric acid	$C_{20}H_{30}O_2$	302.45	22.64	18.58		Antibacterial, anti-atherosclerotic
18.	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-	$C_{20}H_{34}O_2$	306.48	23.21	6.03		Antioxidant, anti-inflammatory, anticancerous
19.	1H- Napthol[2,1-b]pyran-7-carboxylic acid, 3-ethenyldodecahydro-3,4a,7, 10a-tetramethyl -methyl ester	$C_{21}H_{34}O_3$	334	27.64	14.17		Prevent abcess, abortifacient

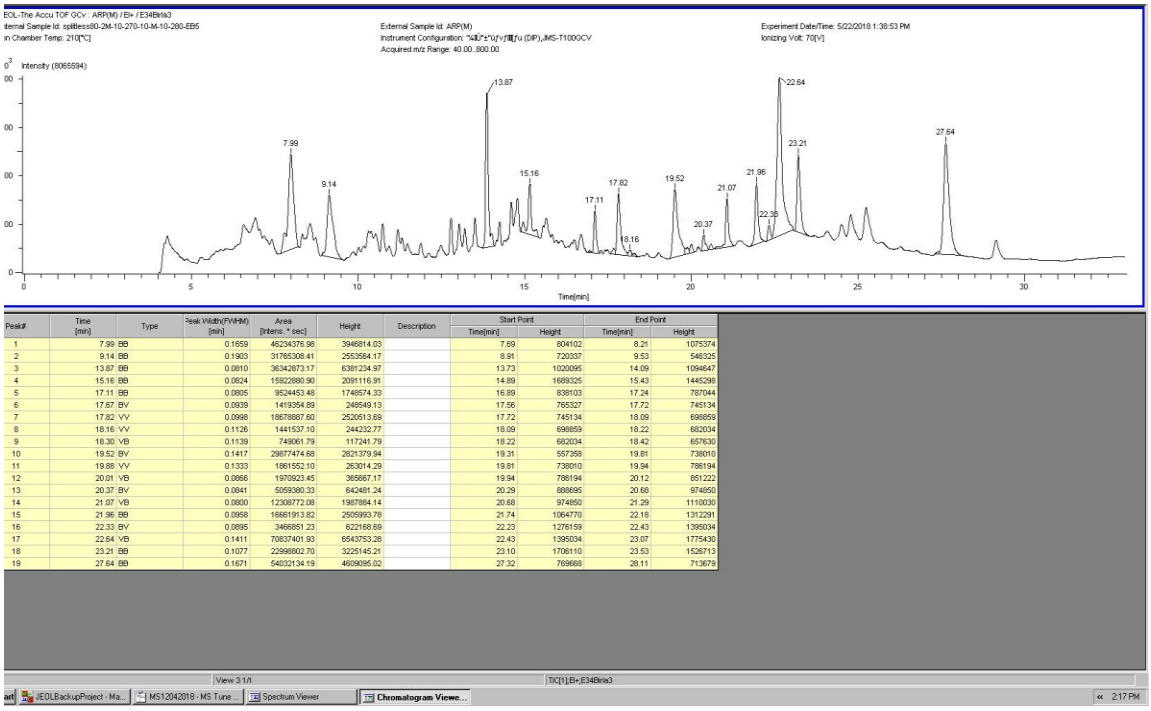
M.F. – Molecular formula, M. Wt.- Molecular weight, R.T.- Retention time

*****Biological Activity source: Dr. Duke's Phytochemical and Ethnobotanical Database, Science Direct and WorldCat.org, Springer link [online database].



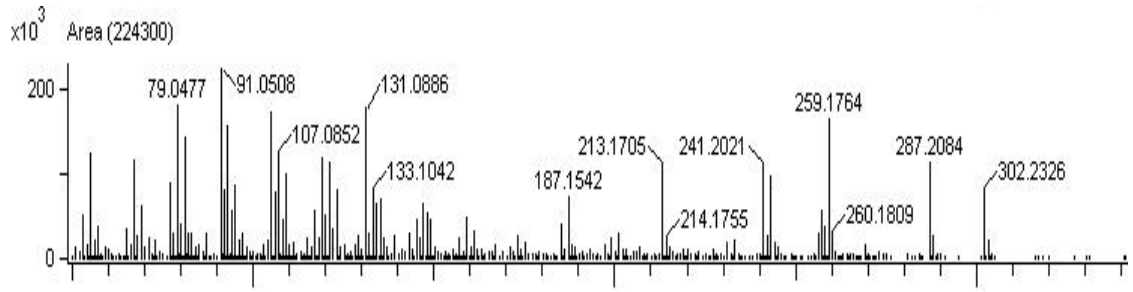
Peak#	Time [min]	Type	Peak Width(FWHM) [min]	Area [Intens.*sec.]	Height	Description	Start Point Time[min]	Start Point Height	End Point Time[min]	End Point Height
1	7.97	BB	0.1557	36270339.91	3262284.42		7.67	348445	8.23	802244
2	9.09	BB	0.1628	14717702.52	1331582.96		8.87	348757	9.37	281178
3	13.84	BB	0.0816	67074723.47	12500698.27		13.73	602347	14.06	470939
4	17.78	BB	0.0900	27726964.21	3442982.84		17.54	488691	18.37	478579
5	19.47	BB	0.1212	23050639.82	2536240.76		19.24	304104	19.76	596664
6	20.34	BB	0.0818	6161330.27	872366.20		20.10	455589	20.51	662816
7	21.04	BB	0.0805	10129922.09	1528924.48		20.85	618445	21.27	586036
8	21.49	BB	0.0929	6702323.46	888386.28		21.27	586036	21.70	588946
9	21.93	BB	0.0909	34893094.25	5596426.75		21.70	588946	22.17	878306
10	22.29	BV	0.1076	5240903.62	935054.14		22.17	878199	22.35	915890
11	22.61	VV	0.1644	111346039.10	9394339.75		22.35	915890	22.84	1017781
12	22.92	VV	0.1543	11134027.04	1153119.82		22.84	1017781	23.08	1068026
13	23.15	VB	0.1352	3883113.14	531132.51		23.08	1068026	23.30	1114092
14	23.77	BB	0.0974	6990868.88	1114958.78		23.67	983338	23.93	1045566
15	24.39	BB	0.1599	9984155.92	1023091.21		24.21	888142	24.61	822289
16	25.16	BV	0.1346	6660596.15	914895.09		25.03	690402	25.25	677228
17	25.34	VB	0.1392	13004854.70	1432697.42		25.25	677228	25.74	648088
18	26.67	BB	0.1240	12261501.59	1259776.70		26.43	590483	26.91	490510
19	28.78	BV	0.2546	27482823.44	1550019.56		28.10	521151	28.88	506525
20	28.96	VB	0.3107	23066416.04	1166414.54		28.88	506525	29.62	492650

Fig. 1: GC-MS chromatogram of methanolic extract of *A. squamosa* peel (ASP).

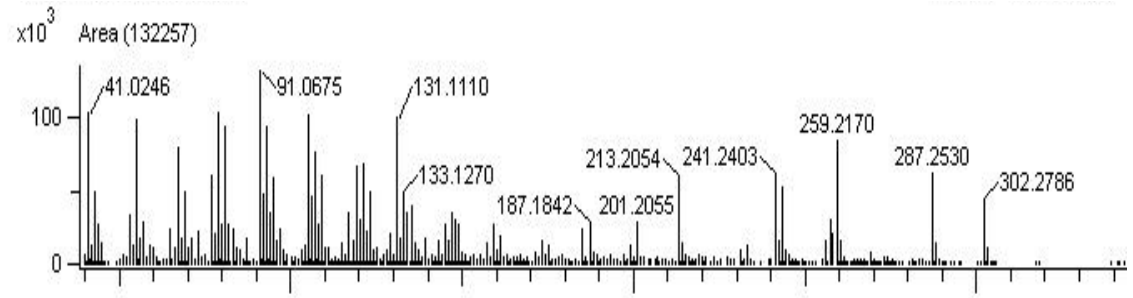


Peak#	Time [min]	Type	Peak Width(FWHM) [min]	Area [Intens. * sec]	Height	Description	Start Point Time[min]	Start Point Height	End Point Time[min]	End Point Height
1	7.99	BB	0.1659	46234376.98	3946814.03		7.69	804102	8.21	1075374
2	9.14	BB	0.1903	31765308.41	2553584.17		8.91	720337	9.53	546325
3	13.87	BB	0.0810	36342873.17	6381234.97		13.73	1020095	14.09	1094647
4	15.16	BB	0.0824	15922890.90	2091116.91		14.89	1689325	15.43	1445298
5	17.11	BB	0.0805	9524453.48	1748574.33		16.89	838103	17.24	787044
6	17.67	BV	0.0939	1419354.89	246549.13		17.56	765327	17.72	745134
7	17.82	VV	0.0998	18678887.60	2520513.69		17.72	745134	18.09	698859
8	18.16	VV	0.1126	1441537.10	244232.77		18.09	698859	18.22	682034
9	18.30	VB	0.1139	749061.79	117241.79		18.22	682034	18.42	657630
10	19.52	BV	0.1417	29877474.68	2821379.94		19.31	557358	19.81	738010
11	19.88	VV	0.1333	1861552.10	263014.29		19.81	738010	19.94	786194
12	20.01	VB	0.0886	1970923.45	365667.17		19.94	786194	20.12	851222
13	20.37	BV	0.0841	5059380.33	642481.24		20.29	889695	20.68	974850
14	21.07	VB	0.0800	12308772.08	1987884.14		20.68	974850	21.29	1110030
15	21.96	BB	0.0958	16661913.82	2505993.78		21.74	1064770	22.18	1312291
16	22.33	BV	0.0895	3466851.23	622168.69		22.23	1276159	22.43	1395034
17	22.64	VB	0.1411	70837401.93	6543753.28		22.43	1395034	23.07	1775430
18	23.21	BB	0.1077	22998802.70	3225145.21		23.10	1706110	23.53	1526713
19	27.64	BB	0.1671	54032134.19	4609095.02		27.32	769668	28.11	713679

Fig. 2: GC-MS chromatogram of methanolic extract of *A. reticulata* peel (ARP).



(a)



(b)

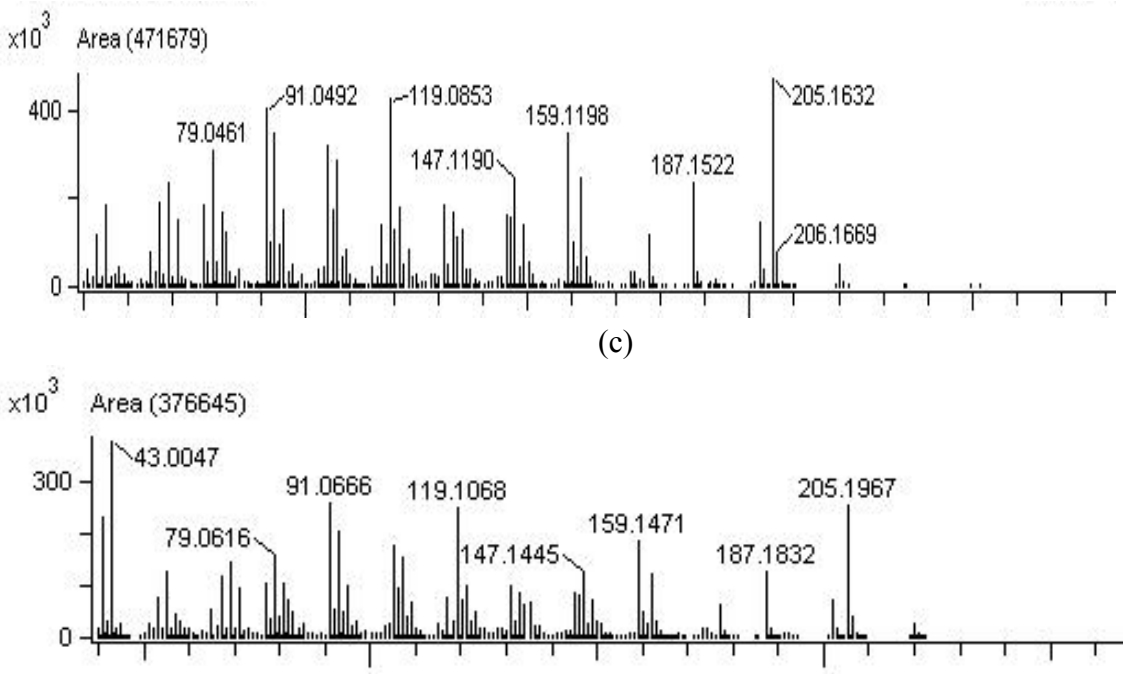


Fig. 3: Mass spectrum of (a, b) peak found at R.T.- 22.61 in ASP and peak found at R.T.- 22.64 in ARP, identified as β-Pimaric acid (c, d) peak found at R.T.- 13.84 in ASP and peak found at R.T.- 13.87 in ARP, identified as Spathulenol.