Research Paper

Phytochemical Analysis of *Acacia auriculiformis* Pericarp through Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

BEAUTY SAHA AND S. K. SAHA*

Department of Zoology, Fish Biology Research Unit, Siksha Bhavana (Institute of Science), Visva-Bharati (A Central University), Santiniketan, West Bengal 731235, India

***Acacia auriculiformis*, widely used for afforestation, produces large amount of fruits which has no specific use other than as fuel for cooking by the ethnic people. The aim of our study is to determine chemical constituents with their definite proportion from different solvent extracts of *Acacia auriculiformis* pericarp at different maturity stages. Qualitative phytochemical analyses of three different solvents extracts *viz.* methanolic, 80 % ethanolic and aqueous of nine different maturity stages pericarps were screened to identify the presence of secondary metabolites. Quantitative analyses were performed through standard biochemical procedures to obtain the amount of the secondary metabolites. Finally, liquid chromatography-electrospray ionization-tandem mass spectrometry analysis was performed to identify different fractions of the major phytochemical groups. The biochemical assay of the preparation helped to identify saponin, phenols, tannin, flavonoid, alkaloid, steroid, terpenoids, glycosides, cardiac glycosides and reducing sugar as the major groups. Methanolic extract of the 9th maturity stage pericarp contains the highest amount of saponin and a fairly good amount of phenol, flavonoids, tannin and proanthocynidin. With the help of liquid chromatography-electrospray ionization-tandem mass spectrometry analyses 7 triterpenoid saponins (acaciasides) with their relative percentages, 22 flavonoids, 8 tannins and 1 phenolic rich fraction-galloyl glucose were detected. Among the 7 triterpenoid saponins, acaciasides A and B are worked out as the major components. A detail toxicological study may help to establish it as a substitute of conventionally used piscicides as well for other pharmaceutical purposes.**

Key words: *Acacia auriculiformis* pericarp, liquid chromatography-electrospray ionization-tandem mass spectrometry peaks, triterpenoid saponin, tannin, flavonoid

Acacia auriculiformis (*A. auriculiformis*), commonly called Northern black wattle and in Bengali as akashmoni, has been used widely in afforestation for its rapid growth rate. It produces large amount of fruits which have no particular utility other than as fuel by the ethnic people and that too causing coughing to the users. So, there is an availability of the fruit in plenty from a small area at a time. Research on chemical component of the fruit is still in its infancy; only 7 compounds have been identified till date[1-3]. Limited general pharmacological activities of the fruit ingredients have been established[4-8]. However, it is neither used in medicinal purpose at industrial scale nor explored for other usages. Though the whole plant with fruit is famous as one of the folk medicines in its natural habitat Australia, there is a dearth of information about detailed phytochemical analysis of the fruit. It is a general feature that chemical composition and percentage (%) content of the ingredients of a plant part varies with the plant's habitats, maturity stages and extraction procedures[9-11]. At full maturity, the pods are split, the seeds are dispersed and the pericarps are shed off from the plant. It becomes easier to collect the fallen fruit parts lying underneath the plant. Keeping all these considerations in mind a comprehensive study has been apprehended for phytochemical analyses of *Acacia* pericarp to explore the possibility of its utilization. The present study is thus aimed to analyse the phytochemical contents of *A. auriculiformis* pericarp at different maturity stages.

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***Address for correspondence**
E-mail: sahaskvb@gmail.com

MATERIALS AND METHODS

Collection of plant material:

A. auriculiformis (A. Cunn. ex Benth.) pericarps of 9 different maturity stages were collected at every 15 d intervals, ranging from small and green to ripen. The samples were collected from Sonajhuri forest (23º41′36′′N 87º40′33′′E), a part of Ballavpur wildlife sanctuary adjacent to Santiniketan, Bolpur, West Bengal, India. The collected pericarps were cleaned, sundried and pulverized separately in powdered form and sieved through uniform mesh no. 40.

Preparation of *Acacia* pericarp extracts:

Solvent extractions of 9 different stages of pericarp were done with petroleum ether in 60°-80° followed with Chloroform ($CHCl_3$). The aqueous extract were done soaking the pericarp powder in distilled water and subsequently filtered through Whatman filter paper No.1 and evaporated through rotary evaporator. Both alcoholic (methanolic and 80 % ethanolic) extractions were done in soxhlet apparatus at 40°-50° for 4 h and then evaporated through rotary evaporator.

Qualitative analyses of phytochemicals:

All the solvent extracts from 9 different stages of pericarp were screened to identify the presence of secondary metabolites *viz*. saponin, phenols, tannin, flavonoid, alkaloid, steroid, terpenoids, glycosides, cardiac glycosides, reducing sugar and starch using standard assay methods[12-15] for phytochemicals.

Quantitative determination of phytochemicals:

Total Saponin Content (TSC): A volume of 1 ml methanolic extract (1 mg/ml) was added to same volume 8 % vanillin (w/v) and incubated in ice-water bath. After adding 8 ml 77 % (v/v) sulphuric acid, the test tube was placed in hot water bath at 60° for 10 min. After cooled down in ice-water bath for 5 min, absorbance was measured at 544 nm by UV-visible spectrophotometer (Beckman Coulter DU730). Diosgenin standard curve was used. TSC was expressed as mg Diosgenin Equivalents (DE) per gram (g) of extract[16].

Total Phenolic Content (TPC): A volume of 1 ml methanolic extract (1 mg/ml) was taken with 9 ml distilled water. Then 1 ml of Folin and phenol reagent was added and mixed thoroughly. After 5 min incubation, 10 ml 7 % Sodium carbonate (Na_2CO_3) solution was added and made the volume up to 25 ml with distilled water. After incubation for 90 min absorbance was recorded at 750 nm. TPC were calculated using gallic acid as standard and expressed as mg Gallic Acid Equivalent (GAE) per g of extract[17].

Total Tanin Content (TTC): Total tannin was determined with small modification of Folin-Ciocalteu method. A volume of 0.1 ml methanolic extract (1 mg/ml) was taken with 7.5 ml distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent. Thereafter, 1 ml Na_2CO_3 solution (35 %) was added and adjusted the volume to 10 ml with distilled water. After incubation for 30 min, absorbance was measured at 725 nm. TTC were calculated using tannic acid as standard and expressed as Tannic Acid Equivalent (TAE) per g of extract[18,19].

Total Flavonoid Content (TFC): Aluminium chloride colorimetric assay has been used to quantify TFC. A volume of 1 ml methanolic extract (1 mg/ml) was taken with 4 ml distilled water and 0.3 ml 5 % Sodium nitrite ($NaNO_2$). After 5 min incubation, 0.3 ml 10 % aluminium chloride was added. Thereafter, 2 ml of 1 M Sodium hydroxide (NaOH) was added and total volume was made 10 ml with distilled water. Absorbance was taken at 510 nm. TFC was calculated using quercetin as standard and expressed as mg of Quercetin Equivalents (QE) per g of extract[20].

Proanthocynidin Content (PC): Total condensed tannin was evaluated by slightly modified method of Tounsi *et al.*[21]. In brief, 50 µl of the methanolic solution (1 mg/ml) was mixed with 2 ml of 4 % methanol vanillin solution and 450 µl concentrated sulphuric acid. After 15 min, absorbance was read at 527 nm and results were expressed as mg CE/g of extract using catechin methanol as standard solution.

Total Alkaloid Content (TAC): A volume of 1 ml water extract (1 mg/ml) was mixed with same amount 2 N Hydrochloric acid (HCl) and filtered. Then transferred to separatory funnel and washed three times with 10 ml $CHCl_3$. pH was adjusted to neutral with 0.1 N NaOH. After that 5 ml of Bromocresol Green (BCG) solution and 5 ml of phosphate buffer were added to the solution and shaken. The formed complex was then extracted with 1, 2, 3 and 4 ml $CHCl_3$ by vigorous shaking. Diluted in 10 ml with $CHCl_3$ and absorbance was determined at 470 nm. TAC was expressed as mg of Atropine Equivalent (AE)/g of extract using atropine as standard[22].

Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry (LC-ESI-MS/MS) analysis:

An untargeted LC-ESI-MS/High-Performance Liquid Chromatography (HPLC)-MS analysis was performed to identify the saponin fractions and other chemical components. A comparison of relative % of the saponin fractions was also subsequently done from the MS data. This analysis was performed on Waters ACQUITY® TQD equipped with Sunfire C_{18} column of 250×4.6 mm, 5 µm. 'Xevo TQ-S micro' quadrupole ion trap mass spectrometer fitted with high performance spray dual-orthogonal Atmospheric Pressure Ionization (API) sources. Ion transfer optics with Ultraperformance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) sensitivity was with automated Multiple Reaction Monitoring (MRM) sensitivity (1 pg reserpine S/N 2000:1). The column was held at 95 % solvent A (0.1 % acetic acid in water) and 5 % solvent B (0.1 % acetic acid in acetonitrile). The column temperature was fixed at 35° at a constant flow rate of 0.4 ml/min. The injection volume was 20 µl. The MS analysis was performed using ESI in the negative and positive ion MS and MS/MS modes in the range of 150–2000 m/z with acquisition speed: 10 scan/s, polarity switching (ES+/ES-): 20 ms and ESI mode switching: 20 ms.

Statistical analysis:

Statistical analyses were done through Analysis of Variance (ANOVA) test with repeated measurements (n=5) using Tukey method in Minitab 17 software.

RESULTS AND DISCUSSION

A. auriculiformis fruit needs 135 d on an average from formation to become fully matured and dried up *in vivo*. The physical characteristic of the fruit have been recorded at every 15 d intervals of maturity period as shown in Table 1. Analysis for 11 major chemical groups resulted in confirmative presence of 10 groups *viz.* saponin, phenols, tannin, flavonoid, alkaloid, steroid, terpenoids, glycosides, cardiac glycosides and reducing sugar in all stages of pericarps as shown in Table 2. Among the solvent extracts the methanol extract has been resulted the highest yield as shown in Table 1. Based on this result only the methanolic extract has been selected for further quantitative analyses. The standard curves for biochemical assays are depicted in the fig. 1. Although the pericarp extracts from all the maturity stages are having similar chemical components, their concentration varied considerably as shown in Table 3. While the chemicals such as phenol, flavonoids, tannin, proanthocynidin and alkaloid were found maximum in 1st stage pericarps, 2nd highest amounts were obtained in the 9th maturity stage. Saponin concentration of the extracts was estimated >39 % in all the maturity stages with slightly higher in the stage nine. The phenolics ranks 2nd highest in content. Although the first maturity stage contains good amount of secondary metabolites, it estimates a less biomass content in comparison to that of the 9th maturity stage pericarps. For this reason the methanolic extract of 9th maturity stage pericarp was selected for LC-ESI-MS analysis.

TABLE 1: SOLVENT EXTRACTIONS OF *A. auriculiformis* PERICARP OF DIFFERENT MATURITY STAGES

Different maturity stages of fruit	Small description of fruit pericarp	Distilled water extract (%)	80 % Ethanolic extract (%)	Methanolic extract (%)
1st stage	Green, small, leafy, without seed	16.73	18.35	23.6
2nd stage	Green longer pericarp but leafy without seed	20.27	22.88	28.6
3rd stage	Pericarp thicker and longer without seed	23.32	26.33	32.9
4th stage	Long and thick pericarp, very small green colour seed appeared	21.27	24	30
5th stage	Pericarp larger with small mid dilation, with green small seed	26	29.27	36.66
6th stage	Green thick with the matured green seed	24.57	27.66	34.66
7th stage	Reddish green pericarp with green seed,	21.93	24.68	30.93
8th stage	Semi dried dark brown pericarp with brownish seed	25.06	28.2	35.33
9th stage (fully matured)	Fully dried dark brown pericarp with separated pods and exposing seeds attached through funicle with pods	29.85	33.6	42.1

TABLE 2: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS IN SOLVENT EXTRACTS OF *A. auriculiformis* PERICARP PREPARATION

Phytochemicals	Methanolic extract of nine stage pericarp	80 % Ethanolic extract of nine stage pericarp	Distilled water extract of 9th stage pericarp
Saponin	+ve	+ve	+ve
Phenolic content	+ve	+ve	+ve
Tanin	+ve	+ve	+ve
Flavonoid	+ve	+ve	+ve
Alkaloid	-ve	+ve	+ve
Steroid	+ve	+ve	+ve
Terpenoids	+ve	+ve	+ve
Glycosides	+ve	+ve	+ve
Cardiac glycosides	+ve	+ve	+ve
Reducing sugar	+ve	+ve	+ve

Note: (+ve): Presence of the phytochemical group and (-ve): Absence of the phytochemical group

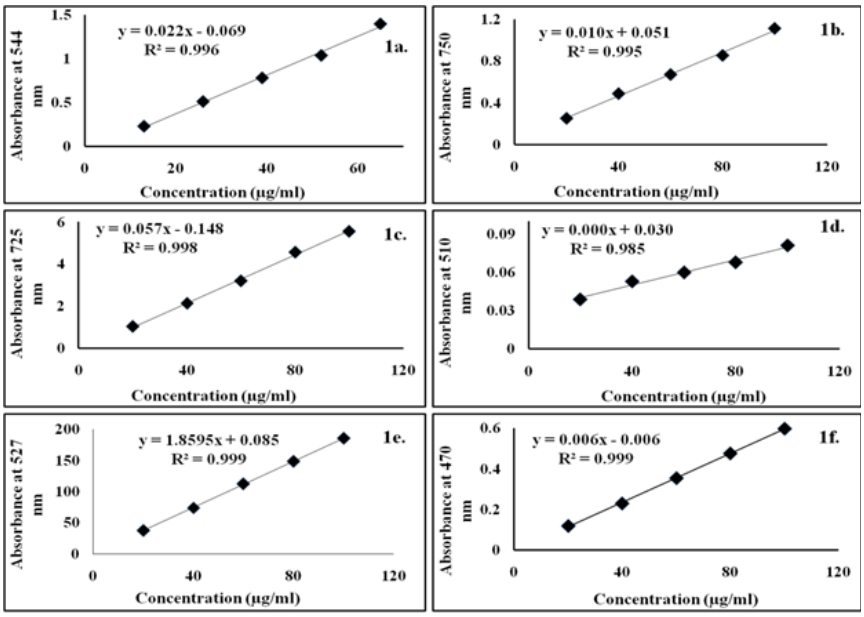

Fig. 1: Calibration curve for quantitative analysis of phytochemicals, (a): Calibration curve of standard diosgenin for determination of TSC; (b): Calibration curve of standard gallic acid for determination of TPC; (c): Calibration curve of standard tannic acid for determination of total tannin content (d): Calibration curve of standard quercetin for determination of TFC; (e): Calibration curve of standard methanolic catechin for determination of total proanthocyanidin content and (f) Calibration curve of standard atropine for determination of TAC

TABLE 3: QUANTITATIVE ANALYSIS OF SOME SECONDARY METABOLITES IN METHANOLIC AND WATER EXTRACT FROM DIFFERENT MATURITY STAGES PERICARP OF *A. auriculiformis*

Different maturity stages of fruit	TSC (mg of DE/g methanolic extract)	TPC (mg of GAE/g methanolic extract)	TFC (mg of QE/g methanolic extract)	Total tannin content (mg of TAE/g methanolic extract)	PC (mg of catechin equivalent/g methanolic extract)	TAC (mg of AE/g water extract)
1st stage	390.2824±0.029	219.179245±0.2331	26.98±0.004	20.45256±0.051	0.414377±0.0002	14.078± 0.039
2nd stage	390.46759±0.012	208.235849±0.196	21.82±0.0038	19.94021±0.042	0.307898±0.00013	13.124± 0.021
3rd stage	390.60648±0.025	200.00943±0.312	18.9±0.0290	18.90798±0.039	0.29768±0.00021	12.253± 0.052
4th stage	390.3287±0.061	197.3113208±0.411	16.72±0.0036	15.91729±0.057	0.139575±0.0003	12.0325± 0.084
5th stage	380.72685±0.032	167.3113208±0.156	10.81±0.0047	12.95704±0.07	0.034709±0.00034	11.0685± 0.077
6th stage	390.14352±0.033	169.00943±0.185	10.88±0.0031	15.22091±0.028	0.042776±0.00051	12.71± 0.069
7th stage	390.18981±0.046	171.198113±0.325	11.68±0.0032	16.52449±0.045	0.150624±0.00049	12.971± 0.056
8th stage	400.11667±0.035	176.179245±0.423	13.18±0.00279	17.16971±0.033	0.230433±0.00047	12.66± 0.029
9th stage (fully matured)	410.22685±0.065	213.933962±0.3521	24.92±0.00511	19.314±0.059	0.399268±0.00035	13.72± 0.042

Note: Values are mean±standard error of the mean, n=5

In the previous studies fourteen flavonoids (1-14) have been reported from heartwood[23-25] and another flavonoid quercetin from bark[26] and seedpod[27] of *A. auriculiformis*. Seven other flavonoids (16-22) have been previously identified from different species of *Acacia*[25,28] but not in *A. auriculiformis*. In our study, all those 22 flavonoids have been detected from the pericarp itself of *A. auriculiformis* through LC-ESI-MS analysis (fig. 2, fig. 3, fig. 4, fig. 5 and Table 4). The three flavonoids isoteracacidin I, isoteracacidin II and teracacidin which have been detected in accordance with their retention time have exhibited same peak having same m/z value. Thus these three flavonoids are considered as isomers. In corroboration with the earlier report[25] present study also detected teracacidin and melacacidin (a 3,4-diol like teracacidin) in dimeric forms $[2M-H]^-$ ion in negative ESI spectra. The peak XII has been identified as flavonoid but the specific characteristic of this compound is still not known.

Tannins of eight different components have been detected first time in the pericarp extract through negative ion ESI-MS spectra (fig. 2, fig. 3, fig.5 and Table 4). In Ultraviolet (UV) chromatogram, epigallocatechin has shown earlier retention time than that of galloepicatechin. So these are established as isomers. In LC-UV chromatogram procyanidin dimer-II has an earlier retention time, followed by procyanidin dimer-III and procyanidin dimer-I has longer retention time. These three fractions are also considered as isomers. In UV chromatogram catechin has an earlier retention time than that of epicatechin. These are exhibiting isomeric feature. There is no earlier report of tannins from *A. auriculiformis* but reported from other species of *Acacia*[28].

This is for the 1st time, one phenolic rich fraction-gallyl glucose has also been detected in *A. auriculiformis* (fig. 3 and fig. 5) (Table 4). In other *Acacia* species however, it had been reported earlier[28].

Proanthocyanin like compounds have been identified as an amalgamation of broad peak on UV chromatogram but no ESI-MS spectra of those has been detected because of their molecular weights beyond the range of 2000 m/z. A small % of proanthocyanidin has been quantified in the methanolic extract (Table 3). To get it in ESI-MS, depolymerization of the compounds is required. Those compounds, not reported earlier from *A. auriculiformis,* are needed to analyse by Nuclear Magnetic Resonance (NMR) spectroscopy for further detailing.

One triterpenoid saponin (acaciaside) (7) which was previously reported from seed[29] has now been identified from the pericarp of *A. auriculiformis* (fig. 3) (Table 4). Acaciamine and five other saponins (1-6) have also been detected in pericarp, were previously detected by Fast Atom Bombardment-MS (FAB-MS)[1-3]. However, in the present study relative % of those compounds has been estimated to come to the inference that among the seven triterpenoid saponin, acaciaside A and acaciaside B are the major components as shown in Table 5.

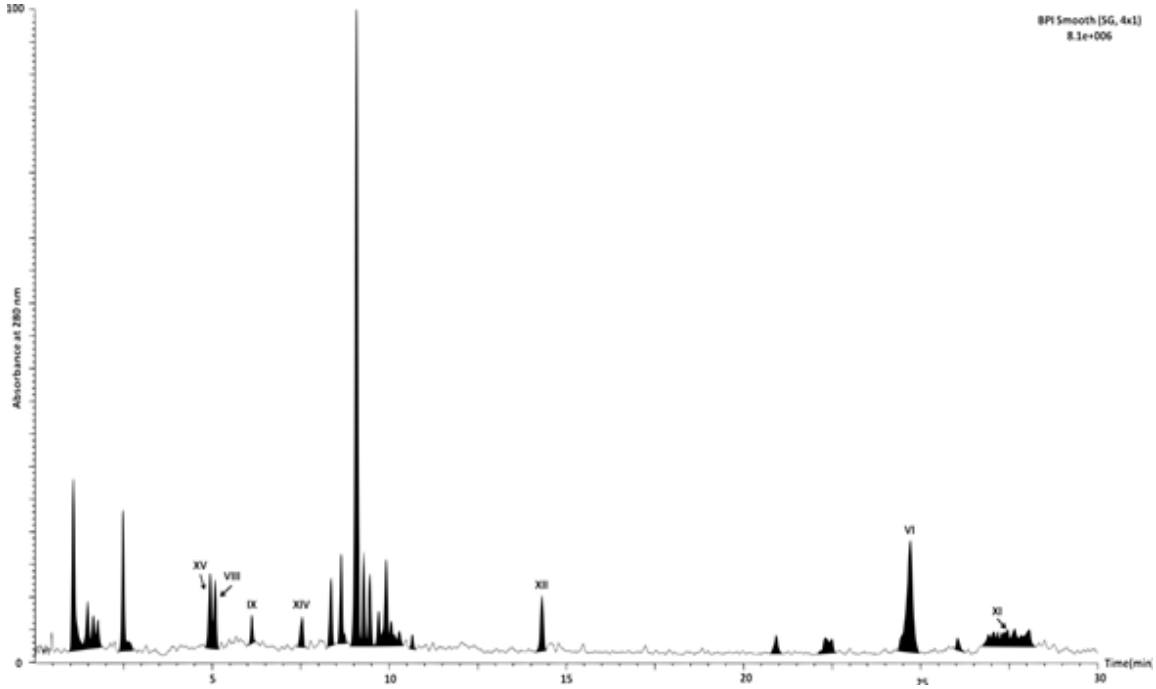

Fig. 2: LC-UV chromatogram at 8.1e+006 (280 nm) of methanolic extract from 9th maturity stage pericarp of *A. auriculiformis*

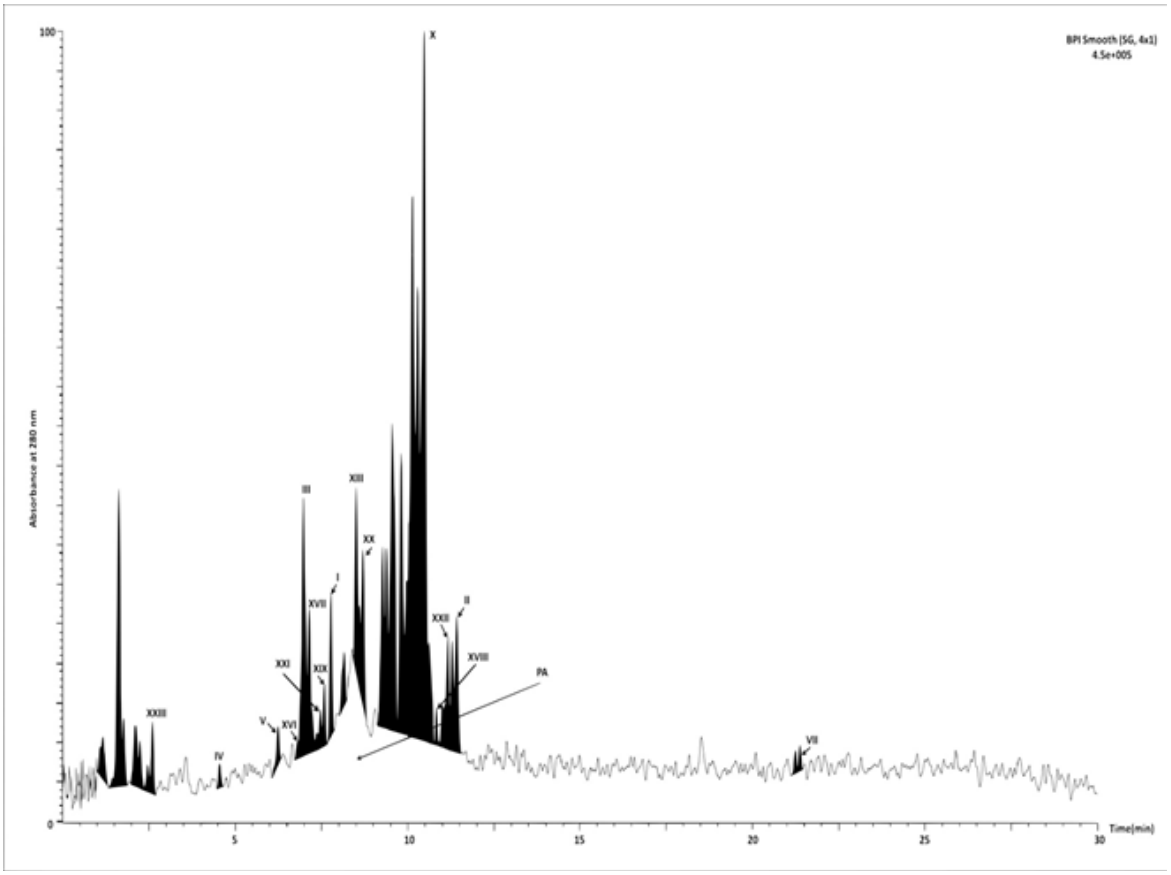

Fig. 3: LC-UV chromatogram at 4.5e+005 (280 nm) of methanolic extract from 9th maturity stage pericarp of *A. auriculiformis*

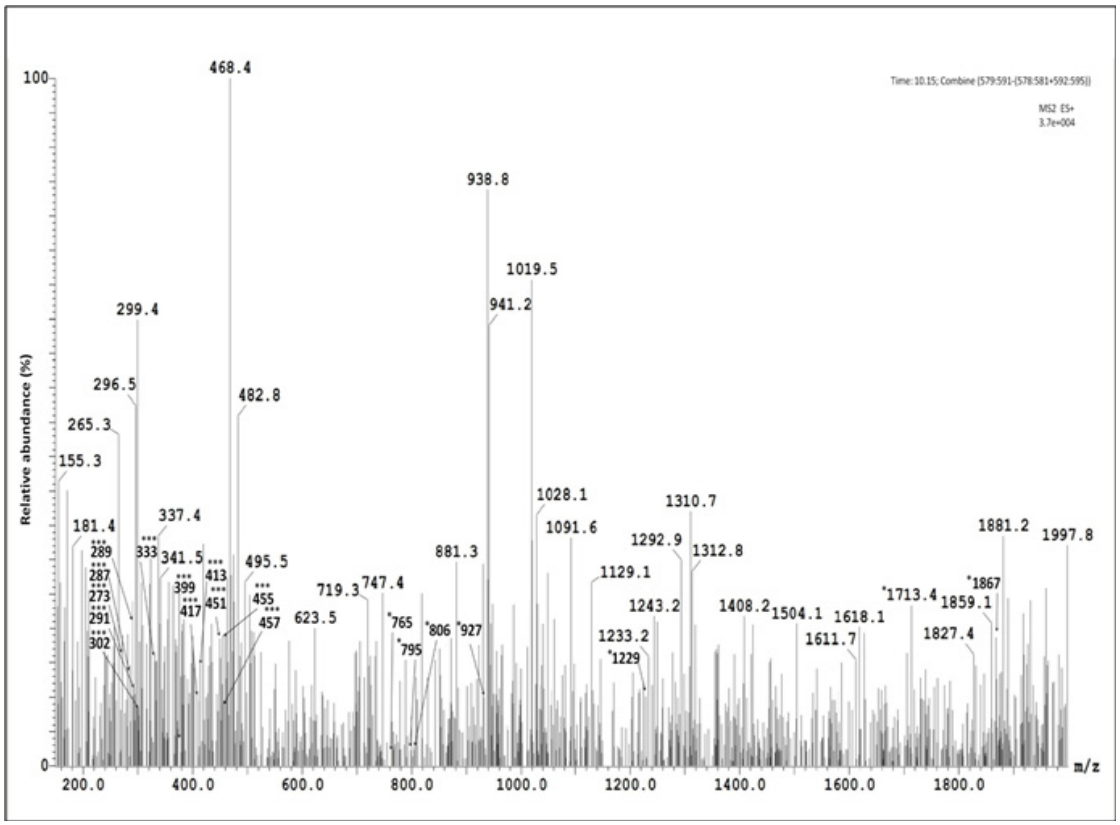

Fig. 4: Positive ion ESI spectrum of methanolic extract from 9th maturity stage pericarp of *A. auriculiformis* (m/z 150 to 2000 m/z range)
Note: (*) Positive ion ESI-MS spectra of saponins; (**) positive ion ESI-MS spectra of tannins and (***) positive ion ESI-MS spectra of flavonoids

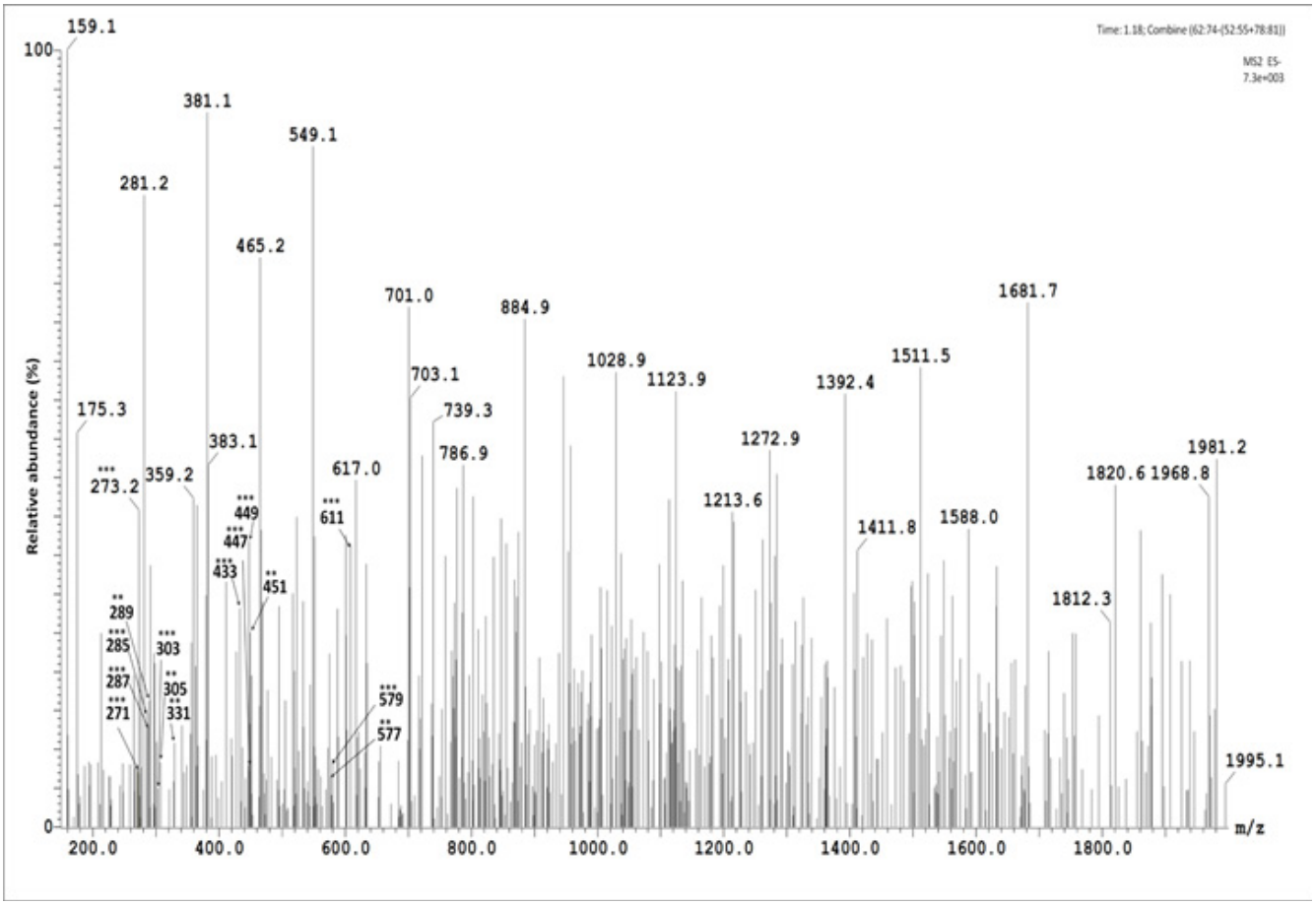

Fig. 5: Negative ion ESI spectrum of methanolic extract from 9th maturity stage pericarp of *A. auriculiformis* (m/z 150 to 2000 m/z range)
Note: (*) Negative ion ESI-MS spectra of saponins; (**) negative ion ESI-MS spectra of tannins and (***) negative ion ESI-MS spectra of flavonoids

TABLE 4: LIST OF COMPOUNDS DETECTED IN METHANOLIC EXTRACT OF 9th MATURITY STAGE PERICARPS FROM *A. auriculiformis* THROUGH LC-ESI-MS/MS ANALYSIS

SL. No	Identified compounds from pericarp of *A. auriculiformis*	Molecular Weight (MW)	Peak in LC-UV chromatogram	Ion in ESI-MS spectra	m/z value	References
Saponins						
1	Acaciaside A	1712		$[M+H]^+$	1713	[2]
2	Acaciaside B	1844		$[M+Na]^+$	1867	[2]
3	Acaciaside C	1206		$[M+Na]^+$	1239	[1]
4	Proacaciaside-I	794		$[M+H]^+$	795	[3]
5	Proacaciaside-II	764		$[M+H]^+$	765	[3]
6	Acaciamine	805		$[M+H]^+$	806	[3]
7	Acaciaside	926		$[M+H]^+$	927	[29]
Flavonoids						
1	2,3-trans-3,4',7,8-tetrahydroxyflavanone	288	I	$[M-H]^-$ $[M+H]^+$	287 289	[14]
2	4',7,8- trihydroxyflavanone	272	II	$[M-H]^-$ $[M+H]^+$ $[2M-H]^-$	271 273	[14]
3	Teracacidin	290	III, IV, V	$[M-H2O+H]^+$ $[M+H]^+$	579	[12] [14]
4	Isoteracacidin-I				273	
5	Isoteracacidin-II				291	
6	α-spinasterol ($C_{29}H_{48}O$)	412	VI	$[M+H]^+$	413	[13]
7	Auriculoside ($C_{22}H_{26}O_{10}$)	450		$[M+H]^+$	451	[13]
8	4,7,8-trihydroxy-2,3-trans-dihydroflavonol ($C_{15}H_{12}O_6$)	288		$[M+H]^+$	289	[12]

9	4,7,8-trihydroxyflavonol ($C_{15}H_{10}O_6$)	286		$[M+H]^+$	287	[12]
10	4,7,8-trimethoxy-2,3-cis-flavan-3,4-trans-diol ($C_{18}H_{20}O_6$)	332		$[M+H]^+$	333	[12]
11	3,4-trans-diacetoxy-4,7,8-trimethoxy-2,3-cis-flavan ($C_{22}H_{24}O_8$)	416		$[M+H]^+$	417	[12]
12	3,4,7,8-tetraacetoxy-2,3-trans-dihydroflavonol ($C_{23}H_{20}O_{10}$)	456		$[M+H]^+$	457	[12]
13	4,7,8-triacetoxyflavanone ($C_{21}H_{18}O_8$)	398		$[M+H]^+$	399	[12]
14	3,4,7,8-tetraacetoxyflavone ($C_{23}H_{18}O_{10}$)	454		$[M+H]^+$	455	[12]
15	Quercetin	301	XIII	$[M+H]^+$	302	[15] [16]
16	Kaempferol	286	XIV	$[M-H]^-$	285	[17]
17	2,3,4,4-tetrahydroxychalcone ($C_{15}H_{12}O_5$)	272	VII	$[M+H]^+$	273	[17]
18	Melacacidin	306	VIII	$[2M-H]^-$	611	[14]
19	Taxifolin	304	IX	$[M-H]^-$	303	[14] [17]
20	Flavonoid hexoside	448	X	$[M-H]^-$	447	[17]
21	Flavonoid glycoside	434	XI	$[M-H]^-$	433	[17]
22	Flavonoid	450	XII	$[M-H]^-$	449	[17]
Flavonoids						
1	Catechin hexoside	452	XV	$[M-H]^-$	451	[17]
2	Epigallocatechin	306	XVI, XVII	$[M-H]^-$	305	[17]
3	Galloepicatechin					
4	Procyanidin dimer I	578	XVIII, XIX, XX	$[M-H]^-$	577	[17]
5	Procyanidin dimer II					
6	Procyanidin dimer III					
7	Catechin	290	XXI, XXII	$[M-H]^-$	289	[17]
8	Epicatechin					
Phenolic rich fraction						
1	Gallyl glucose	332	XXIII	$[M-H]^-$	331	[17]

TABLE 5: RELATIVE CONCENTRATION OF SAPONIN FRACTIONS ACCORDING TO LC-ESI-MS/MS CHROMATOGRAM OF METHANOLIC EXTRACT FROM 9th MATURITY STAGE PERICARP OF *A. auriculiformis*

Triterpenoid saponins	m/z value	Relative concentrations (%)
Acaciaside A	1712	29.27
Acaciaside B	1844	21.95
Acaciaside	926	12.195
Acaciaside C	1206	12.195
Acaciamine	805	12.195
Proacaciaside-I	794	9.756
Proacaciaside-II	764	2.439

Other than pharmacological applications[4-8,30], in aquaculture saponin and tannin containing plant products are used as piscicides for prestocking pond management[31]. Since *Acacia* pericarp contains good amount of saponin and tannin, it has been recommended very recently[32] as a better a substitute of the conventionally used piscicide Mohua (*Bassia latifolia*) oil cake which also contains saponin as the principal ingredient[32].

It can be concluded that highest amount of saponin (41 %) with other phytochemicals are present in the methanolic extract of the 9th stage pericarps. LC-ESI-MS/MS analyses revealed 7 triterpenoid saponins, 22 flavonoids, 8 tannins and 1 phenolic rich compound-gallyl glucose in this preparation. Among these components acaciaside A is present in maximum concentration, followed by acaciaside B.

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Conflict of interests:

The authors declared no conflicts of interest.

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