Reverse Phase Liquid Chromatography Coupled with Quadra pole- Time of Flight Mass Spectrometry for the Characterisation of Phenolics from *Acacia catechu* (L.f.) Willd.

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**Abstract**

A rapid method was developed for the identification of phenolics from *Acacia Catechu*. The analysis of phenolic compounds was carried out by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to electrospray ionization-time-of-flight mass spectrometry (ESI-Q-TOF-MS). The accurate mass data for the molecular ions were processed using the software Mass Hunter work station (Agilent Technologies), which provided a list of possible elemental formulas by using the Molecular featured extraction editor. The subsequent structure characterization was carried out by a tandem mass spectrometric method. Fragmentation behavior of phenolic compounds was investigated using ion trap mass spectrometry in negative mode. The total fragmentation of the compound ion leading to other fragments was corroborated by MS–MS. Five phenolic compounds have been identified from the methanolic extract of Acacia Catechu.

**Keywords:** Acacia, HPLC, LC-ESI-MS.

**Introduction**

Mass spectrometry is one of the most sensitive methods of molecular analysis and yields information on the molecular weight as well as on the structure of the analytes. Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) has been recognized as a powerful analytical tool with its high sensitivity, short run time and less use of toxic organic solvents used as mobile phase compared to reversed phase stand alone HPLC coupled with Diode-Array Detector[1]. LC coupled to mass spectrometry (LC/MS) is better suited for the analysis of nonvolatile polar compounds in their natural form. Accurate mass measurement is the important feature of Q-TOF MS/MS Which is useful to differentiate compounds with same nominal mass but dissimilar exact masses. Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress[2], which plays a role in heart diseases, neurodegenerative diseases, cancer and in the aging process[3]. This concept is supported by increasing evidence indicating that oxidative damage plays a role in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this, thus lowering the risk of disease[4].

Antioxidants are substances that when present in low concentrations, compared to those of an oxidisable substrate significantly delay or prevent oxidation of that substance [5]. Acacia is the second largest genus in the Leguminosae family, comprising more than 1200 species worldwide, with members found in almost all habitats. Out of the 1200 Acacia species, approximately 800 are found in Australia, 130 in Africa, 20 in India [6]. Although the genus *Acacia* is quite large and widespread in the warm sub-arid and arid parts of the world, relatively little is known about the chemistry of most species. *Acacia* can provide the nutrients and therapeutic ingredients to prevent, mitigate or treat many diseases. This plant contains variety of bioactive components such as phenolic acids, alkaloids, terpenes, tannins and flavonoids[7-11], which are responsible for numerous biological and pharmacological properties like hypoglycemic, anti-inflammatory, anti-bacterial, anti-platelet aggregatory, anti-hypertensive, analgesic, anti-cancer, and antiatherosclerotic due to their strong antioxidant and free radical scavenging activities[12]. Phenolics are largest group of phytochemicals and accounts for most of the antioxidant activity in plants or plant products[13].

The reverse-phase high-performance liquid chromatography (RP-HPLC) coupled to tandem mass spectrometry have become the best method for separation, identification and characterisation of compounds from natural products. The combination of high-performance liquid chromatography and mass spectrometry (LC/MS) has had a significant impact on drug development over the past decade. Continual improvements in LC/MS interface technologies combined with powerful features for structure analysis, qualitative and quantitative, have resulted in a widened scope of application [14].

The molecular masses of phenolic acids and flavonoids were assigned by electrospray ionization mass spectrometry. The subsequent structure characterization was carried out by a tandem mass spectrometric method. Fragmentation behavior of phenolic
compounds was investigated using ion trap mass spectrometry in negative mode. The fragmentation rule in mass spectrum offers the ability to identify the related unknown compounds. The MS/MS and UV data together with HPLC retention time of phenolic acids and flavonoids allowed structural characterization of these compounds.

Materials and Methods

Sample Preparation

50 grams of dried bark powder of *Acacia catechu* was extracted in 80% methanol for 48 hrs in a soxhlet apparatus. After filtration, the filtrate was concentrated to dryness by rotary evaporator at 48°C, then weighed and diluted to 20 ml with 80% MeOH. The solution was washed with 30 ml petroleum ether (2 x 15 ml) to remove lipids. Twenty ml of absolute ethanol (2 x 10 ml) was used to precipitate protein from the solution. After centrifugation at 10000 rpm for 20 min, the supernatant was concentrated by rotary evaporator at 48°C.

HPLC Analysis

HPLC profiling was done using a Shimadzu High Performance Liquid Chromatographic system equipped with LC-10ATVP pump, SPD M10AVP Photo Diode Array Detector in combination with CLASS-VP 6.12 SP5 integration software. The mobile phase used for the separation was HPLC grade 0.1% ortho phosphoric (A) acid and methanol(B) in a time programming 0-10 10% A, 10-20 30% A, 20-30 50% A, 30-40 60% A and 40-50 70% A. The column used was C18 – ODS (Octadecylsilane), Lichrospher RP 18e (5 μm) (Merck) with a Phenomenex guard column (4mm x 2 mm i.d 5μm). The sample was injected using a 20 µl loop (Rheodyne Rohnet Park, CA, USA). The flow rate was maintained to 0.75 ml/min.

LC–MS/MS instrumentation and conditions

LC-ESI-MS analysis was conducted on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 with Extend-C18 column of 1.8 μm, 2.1 x 50 mm equipped with an ESI interface operating in negative and positive ion mode. After several attempts for the optimal separation of the compounds using different mobile phases and also different gradients, the two mobile phases chosen consisted in A: water (0.05% formic acid) and B: acetonitrile. Gradient elution was performed with water/0.05% formic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.3 ml/ min. An increasing linear gradient (v/v) of solvent B was used [t (min), % A]: 0, 94; 3, 83.5; 5, 82.5; 8, 81.5; 10, 0; 12, 95. The injection volume in the HPLC system was 20 µl and the temperature of the column was set at 30°C. TOF-MS transfer parameters were optimized by direct infusion with Tuning mix (Agilent Technologies). The other optimum values of the ESI-MS parameters were drying gas heater temperature, 300°C; drying gas flow, 5 L/min; nebulizing gas pressure, 30 psi. The mass fragmentation was carried out in automated MS/MS mode.

The accurate mass data for the molecular ions were processed using the software Mass Hunter work station (Agilent Technologies), which provided a list of possible elemental formulas by using the Molecular featured extraction editor. The Generate Molecular featured extraction provides the mass matching percentage, space matching percentage, abundance percentage, as well as a sophisticated comparison of the theoretical with the measured isotope pattern for increased confidence in the suggested molecular formula.

Results and Discussion

The accuracy of mass data generated by Q-TOF-MS together with the fragmentation pattern of the full scan run of MS/MS analysis have been a useful tool to tentatively characterize the compounds. Fig. 2 shows the HPLC –PDA chromatograms obtained using the optimum gradient elution program. Major peaks were found at Rt 4.427, 9.021, 12.928, 14.197, 20.853 and 23.349. The same extract was subjected to LC-ESI-MS analysis in negative mode. The molecular feature algorithm of Agilent mass hunter software showed the negative ion mass of the compounds. In the Total Ion Chromatogram (TIC) a mass 169.04 was found at Rt 0.917, on further MS/MS analysis it showed a mass of 125.019 which was identified as Gallic acid on the basis of mass fragmentation pattern. The ion with m/z [M-H] 315 yielded a fragment with m/z 153.18 on ms/ms analysis, using the algorithm programmed in Agilent mass hunter software it showed a tentative molecular formula as C13H15O9 and was tentatively identified as Protocatechuic-acid-4-
glucoside. The ion found at 4.02 min, m/z 447 presented a fragment at m/z 301 (M−1)−, consistent with quercetin derivative fragmentation patterns, so the rhamnoside was proposed as structure. It showed the ion at m/z 301 on targeted ms/ms analysis, (fig: 3) corresponds to the loss of the sugar moiety. From the mass fragmentation pattern the compound was tentatively identified as quercetin 3-rhamnoside. LC–MS/MS methods were used to characterize the quercetin derivatives with ms/ms fragmentation pattern[15]. The mass spectrum obtained at Rt 5.16 with m/z 477.3 presented fragments 301 and 178 with a tentative molecular formula C21H17O13 and it was identified as Quercetin 3-glucuronide. Epicatechin was easily identified with the help of molecular featured extraction of Agilent mass hunter software and matching with the Metlin database mass library.

Figure 2. HPLC–PDA Chromatogram of Acacia catechu
Figure: 3: LC ESI Mass Spectrum of Identified compounds

Table 1 Identification of Phenolics from *Acacia catechu*

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<th>m/z experimental</th>
<th>MS/MS</th>
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<tr>
<td>0.917</td>
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<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;13&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Epicatechin</td>
</tr>
</tbody>
</table>

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