Detection of caffeic and chlorogenic acids from methanolic extract of *Annona squamosa* bark by LC-ESI-MS/MS

Hamzah Abdulrahman Salman¹, Senthilkumar Ramasamy¹, Bassam Shaker Mahmood²

¹Department of Microbiology, J.J. College of Arts and Science, Bharathidasan University, Pudukkottai, Tamil Nadu, India
²Biotechnology Division, Applied Science Department, University of Technology, Baghdad, Iraq

**ABSTRACT**

**Aim:** The aim of the present study was to determine the metabolite profile of *Annona squamosa* bark using high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

**Methods:** The plant material of *A. squamosa* bark was collected and processed during the month of February. The bark material was extracted by Soxhlet apparatus using methanol as a solvent. The metabolite profile of the plant extract was determined by using LC-ESI-MS/MS.

**Results:** Caffeic and chlorogenic acids were detected from the methanolic extract of *A. squamosa* bark. To the best of our knowledge, this is the first study to report the presence of caffeic and chlorogenic acids in *A. squamosa* bark.

**Conclusion:** The study suggested further investigations to be carried out to evaluate these compounds *in vitro* and *in vivo* to develop the pharmaceutical products.

**INTRODUCTION**

*Annona squamosa* belongs to the family Annonaceae, cultivated in India and other tropical countries. Commonly known as Custard apple in English, Sharifa in Hindi, Seema Atha in Tamil, Seetha Phala in Kannada, and Seetha Pandu in Telugu [1,2].

*A. squamosa* was traditionally used in medicine to treat epilepsy, constipation, diarrhea, hemorrhage, fever, dryness, and ulcers [3]. The extract of different parts of *A. squamosa* was reported to have anticancer, antioxidant, anti-inflammatory, and antimicrobial activity [4–7].

In the previous study, we tested the antibacterial activity of the methanolic extracts of *A. squamosa* and *A. reticulata* (leaves and bark) against *Streptococcus mutans* and *Streptococcus sobrinus*, and among the tested plant materials, *A. squamosa* bark showed antibacterial activity [8]. In this background, the present study was aimed to study the metabolite profile of *A. squamosa* bark using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

**MATERIALS AND METHODS**

**Collection and processing of test plant material**

The bark material of *A. squamosa* L was collected and authenticated by Dr. Vasundhara M, professor, Horticulture Department, UAS University, GKVK Bangalore, India. The bark was collected and processed during the month of February 2015. The plant material was cleaned and rinsed at least three times in sterile distilled water and dried in the hot air oven. The dried plant material was squashed by blender and then stored in an airtight bottle for further uses.

**Contact**

Hamzah Abdulrahman Salman  
hamza.alayash@gmail.com +91-9986604341  
Department of Microbiology, J.J. College of Arts and Science, Bharathidasan University, Pudukkottai, Tamil Nadu, India.

© EJManager. This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted, noncommercial use, distribution and reproduction in any medium, provided the work is properly cited.
Preparation of plant extract

The extraction of *A. squamosa* bark was performed by soxhlet apparatus. The soxhlet apparatus was filled with 600 ml of 100% v/v methanol (high-performance liquid chromatography [HPLC] grade) and 40 g of the dried material of *A. squamosa* bark. The extraction was carried out at a temperature of 25°C for 30 h. The extract was filtered using two sheets of Whatman paper. The rotary vacuum evaporator was used to concentrate the extract and the yield was transferred to a screw cap bottle and stored at 4°C for further uses [8].

LC-ESI/MS and data analysis

The photochemical from the methanolic extract of *A. squamosa* bark were analyzed by direct injection to auto-sampler in LC-ESI/MS. The LC-ESI/MS was performed using a Perkin Elmer Sciex API-3000 triple quadrupole mass spectrometer equipped with an Agilent 1100 series HPLC. A 150 × 3.9 mm C18, symmetry column (Waters, India) was used at a flow rate of 0.5 ml/min. Separation based on gradient chromatography was performed for the solvent extract of the samples by a mobile phase of solvent A: 0.01% formic acid in acetonitrile and solvent B: 0.01% of MilliQ water with a constant flow rate of 0.5 ml/min. The gradient program started with 100% A: 5 min, followed by 85% A: 10 min, 80% A: 20 min, 75% A: 25 min, 73% A: 27 min, 60% A: 30 min, 50% A: 35 min, 10% A: 40 min, 10% A, then returned to 100% A for 50 min and maintained for 60 min at 100% A. HPLC of the extract was measured by MS/MS. All the analyses were performed using the electrospray ionization in both positive ion and negative ion modes with the following settings: ion spray voltage 4200 V for positive, and −4200 V for negative; nebulizer gas (N₂) 7 units, curtain gas (N₂) 12 units, collision gas (N₂) 6 units, declustering potential (DP) between 50 and 80 V for positive and between −50 and −80 V for negative, focusing potential 300 V for positive and −300 V for negative, entrance potential 10 V for positive and −10 V for negative, collision energy (CE) 50 V for positive and −50 V for negative, and collision cell exit potential (CXP) 5 V for positive and −5 V for negative. The drying gas (N₂) was heated to 550°C and established at a flow rate of 6,000 cm³/min. The full scan data acquisition was executed by scanning from m/z 100 to 1,500 in profile mode with a cycle time of 2 second with a step size of m/z 0.1 and an interscan pause of 2 μs. The metabolites were identified by comparing the precursor and fragment ions m/z with METLIN database.

Results

In order to obtain metabolite profile of the methanolic extract of *A. squamosa* bark, an analytical method based on LC-ESI-MS/MS was used. The LC-ESI-MS/MS profile highlighted the existence of a large group of compounds related to the protonated molecular ions of various polyphenols. The m/z obtained in both positive mode (Fig. 1) and negative mode (Fig. 2) was subjected to METLIN metabolite search. The search for the respective positive or negative charges was depended on the ionization with an accuracy of 50 ppm tolerance to find the possible metabolites. Among the possible metabolites, caffeic acid and chlorogenic acid were found to be present in the methanolic extract of *A. squamosa* bark. The caffeic acid and chlorogenic acid were subjected to further fragmentation. The metabolites were confirmed by direct infusion method of MS/MS. LC/ESI-MS/MS spectra of caffeic acid (m/z 179.0) and chlorogenic acid (m/z 353.0) are shown in Figs. 3 and 4, respectively.

Discussion

Among the metabolites present in the methanolic extract of *A. squamosa* bark, caffeic acid (Fig. 3) and chlorogenic acid (Fig. 4) were detected. To the best of our knowledge, this report is the first to detect caffeic acid and chlorogenic acid in *A. squamosa* bark.

Caffeic acid with precursor ion of 179.0 was subjected to fragmentation in positive mode by varying the DP from −101 to −1 volt, fixed potential (FP) from −350 to −50 volts, CE from −130 to −5.0, CXP from −55 to 0. The MS/MS fragments of caffeic acid included 135.0, 134.0, 106.0, and 65.0 which was as reported earlier [9]. The chlorogenic acid was subjected to fragmentation in negative mode (precursor ion of 353.0) by varying the DP from −101 to −1 volt, FP from −350 to −50 volts, CE from −130 to −5.0, CXP from −55 to 0. The MS/MS fragments of chlorogenic acid included 191.5, 161.0, and 111.0 which was as previously reported [10–12].

*A. squamosa* bark was reported to contain anticariogenic activity against *S. mutans* and *S. sobrinus* [8]. Chlorogenic acid is a polyphenolic
Figure 1. LC/ESI-MS/MS spectra of positive mode of *A. squamosa* bark.

Figure 2. LC/ESI-MS/MS spectra of negative mode of *A. squamosa* bark.
Caffeic and chlorogenic acid from Annona Squamosa

A compound that forms an ester with caffeic acid with the 3-hydroxyl group of a quinic acid. It was demonstrated to have various health benefits including antiviral, antioxidant, antibacterial, antifungal, and other biological activities [13,14]. Furthermore, caffeic acid and chlorogenic acid were also found to be anticariogenic compounds [15,16]. Chlorogenic acid and caffeic acid are nonvolatile organic acids present in coffee, investigators reported the effectiveness of coffee extracts to reduce the adherence of *S. mutans* on the glass surface [17]. Caffeic acid was approved to inhibit the growth of *S. mutans* and *S. sobrinus* [18].

Moreover, Caffeic and chlorogenic acids reduce the ability to inhibit α-amylase and α-glucosidase activities and therefore lead to anti-diabetic effects [19]. Researchers also considered caffeic and chlorogenic acids as promising agents for treating human breast cancer, head and neck squamous, lung and cervical carcinoma cells [20–23].

**Conclusion**

The study concluded the presence of caffeic acid and chlorogenic acid in *A. squamosa* bark. The current investigation suggested that the combination of caffeic acid and chlorogenic acid may potentially increase the antibacterial activity of *A. squamosa* bark. Further investigations are warranted to understand the possibility of incorporation of these compounds into pharmaceuticals.

---

**Figure 3.** LC/ESI-MS/MS spectra of caffeic acid (*m/z* 179.0) fraction of *A. squamosa* bark.
References

[1] Morton JF. Sugar apple. In: Morton JF (ed) Fruits of Warm Climate. Miami: JF Morton; 1987.

[2] Bhardwaj A, Satpathy G, Gupta RK. Preliminary screening of nutraceutical potential of *Annona squamosa*, an underutilized exotic fruit of India and its use as a valuable source in functional foods. J Pharmacogn Phytochem 2014; 3:172–80.

[3] Vohora SB, Kumar I, Naqvi SA. Phytochemical, pharmacological, antibacterial and anti-ovulatory studies on *Annona squamosa*. Planta Med 1975; 28:97–100.

[4] Pardhasaradhi BV, Reddy M, Ali AM, Kumari AL, Khar A. Differential cytotoxic effects of Annona squamosa seed extracts on human tumour cell lines: role of reactive oxygen species and glutathione. J Biosci 2005; 30:237–44.

[5] Shirwaikar A, Rajendran K, Kumar CD. In vitro antioxidant studies of *Annona squamosa* Linn. leaves. Indian J Exp Biol 2004; 42:803–7.

[6] Rahman SM, Rashedul MI, Rahman S, Mosaiaib T, Ahmed R, Khatun F, et al. Antihyperglycemic studies with methanol extract of *Annona reticulata* L. (Annonaceae) and *Carissa carandas* L. (Apocynaceae) leaves in Swiss albino mice. Adv Nat Appl Sci 2011; 5:218–22.

[7] Kachhawa JB, Sharma N, Tyagi S, Sharma KK. Screening of stem bark methanol extract of *Annona squamosa* for antibacterial activity. Int J Curr Pharm Res 2012; 4:48–50.

[8] Salman HA, Senthilkumar R. Antibacterial activity of *Annona squamosa* L. and *Annona reticulata* L. against clinical isolates of mutans streptococci the causative agents of dental caries. Asian J Pharm Clin Res 2015; 8:152–5.

Figure 4. LC/ESI-MS/MS spectra of chlorogenic acid (m/z 353.0) fraction of *A. squamosa.*
Caffeic and chlorogenic acid from Annona Squamosa

[9] Li W, Sun Y, Liang W, Fitzloff JE, van Breemen RB. Identification of caffeic acid derivatives in Actea racemosa (Cimicifuga racemosa, black cohosh) by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2003; 17:978–82.

[10] Clifford MN, Johnston KL, Knight S, Kuhnert N. Hierarchical scheme for LC-MSn identification of chlorogenic acids. J Agric Food Chem 2003; 51:2900–11.

[11] Clifford MN, Knight S, Surucu B, Kuhnert N. Characterization by LC-MS(n) of four new classes of chlorogenic acids in green coffee beans: dimethoxyxycinnamoylquinic acids, diferuloylquinic acids, caffeoyl-dimethoxyxycinnamoylquinic acids, and feruloyl-dimethoxyxycinnamoylquinic acids. J Agric Food Chem 2006; 54:1957–69.

[12] Clifford MN, Marks S, Knight S, Kuhnert N. Characterization by LC-MS(n) of four new classes of p-coumaric acid-containing diacetyl chlorogenic acids in green coffee beans. J Agric Food Chem 2006; 54:4095–101.

[13] Jassim SA, Naji MA. Novel antiviral agents: a medicinal plant perspective. J Appl Microbiol 2003; 95:412–27.

[14] Karunanidhi A, Thomas R, van Belkum A, Neela V. In vitro antibacterial and antibiofilm activities of chlorogenic acid against clinical isolates of Stenotrophomonas maltophilia including the trimethoprim/sulfamethoxazole resistant strain. Biomed Res Int 2013; 2013:392058.

[15] Brandao F, Oliveira L, Landucci L, Koga-Ito C. Antimicrobial activity of coffee-based solutions and their effects on Streptococcus mutans adherence. Braz J Oral Sci 2007; 6:1274–7.

[16] Almeida AA, Naghetini CC, Santos VR, Antonio AG, Farah A, Gloria MB. Influence of natural coffee compounds, coffee extracts and increased levels of caffeine on the inhibition of Streptococcus mutans. Food Res Int 2012; 49:459–61.

[17] Sharma R, Reddy VL, Prashant GM, Ojha V, Kumar NP. Antimicrobial and anti-adherence activity of various combinations of coffee-chicory solutions on Streptococcus mutans: an in-vitro study. J Oral Maxillofac Pathol 2014; 18:201–6.

[18] Barrientos L, Herrera CL, Montenegro G, Ortega X, Veloz J, Alvear M, et al. Chemical and botanical characterization of Chilean propolis and biological activity on cariogenic bacteria Streptococcus mutans and Streptococcus sobrinus. Braz J Microbiol 2013; 44:577–85.

[19] Oboh G, Agunloye OM, Adefegha SA, Akinyemi AJ, Ademiluyi AO. Caffeic and chlorogenic acids inhibit key enzymes linked to type 2 diabetes (in vitro): a comparative study. J Basic Clin Physiol Pharmacol 2015; 26:165–70.

[20] Kabala-Dzik A, Rzepecka-Stojko A, Kubina R, Jastrzębska-Stojko Z, Stojko R, Wojtyszka RD, et al. Migration rate inhibition of breast cancer cells treated by caffeic acid and caffeic acid phenethyl ester: an In vitro comparison study. Nutrients 2017; 9:1144.

[21] Naso LG, Valcarcel M, Roura-Ferrer M, Kortazar D, Salado C, Lezama L, et al. Promising antioxidant and anticancer (human breast cancer) oxidovanadium (IV) complex of chlorogenic acid. Synthesis, characterization and spectroscopic examination on the transport mechanism with bovine serum albumin. J Inorg Biochem 2014; 135:86–99.

[22] Tyszka-Czochara M, Bukowska-Strakova K, Majka M. Metformin and caffeic acid regulate metabolic reprogramming in human cervical carcinoma SiHa/HTB-35 cells and augment anticancer activity of Cisplatin via cell cycle regulation. Food Chem Toxicol 2017; 106:260–72.

[23] Li W, Liu X, Zhang G, Zhang L. Mechanism of chlorogenic acid in apoptotic regulation through notch1 pathway in non-small cell lung carcinoma in animal level. Zhongguo Fei Ai Za Zhi 2017; 20:555–61.