Phytochemical analysis of *Hibiscus caesius* using high performance liquid chromatography coupled with mass spectrometry

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Abstract: Various species in genus *Hibiscus* are traditionally known for their therapeutic attributes. The present study focused on the phytochemical analysis of a rather unexplored species *Hibiscus caesius* (*H. caesius*), using high-pressure liquid chromatography coupled with mass spectrometry (HPLC-MS). The analysis revealed five major compounds in the aqueous extract, viz. vanillic acid, protocatechoic acid, quercetin, quercetin glucoside and apigenin, being reported for the first time in *H. caesius*. Literature suggests that these compounds have important pharmacological traits such as anti-cancer, anti-inflammatory, anti-bacterial and hepatoprotective etc. however, this requires further pharmacological investigations at *in vitro* and *in vivo* scale. The above study concluded the medicinal potential of *H. caesius.*

Keywords: *H. caesius,* Phytochemicals, medicinal value, HPLC-MS.

INTRODUCTION

Hibiscus sabdariffa commonly known as sorrel or roselle is extensively studied plant; literature bears a wealth of information about its phytochemistry and pharmacological effects. Anthocyanins, flavonoids and polyphenols are the major chemical constituents of flowers of *Hibiscus sabdariffa* (Lin *et al.,* 2007) contributing to its practical medicinal effects. The calyx drink is rich in ascorbic acid, carotene, calcium, riboflavin, iron and niacin which are nutritionally important (Yadong *et al.,* 2005). The petals are the potential resource of antioxidizing agents as anthocyanins and ascorbic acid (Prenesti *et al.,* 2007). Various biologically active compounds are reported in *H. tiliaceus* (Chen *et al.,* 2006), *H. rosasinensis* (Gauthaman *et al.,* 2006; Gilani *et al.,* 2005). *Hibiscus rosasinensis*, grown as ornamental plant is reported to have favorable effects in heart diseases especially in ischemic disease (Gauthaman *et al.,* 2006). However, *Hibiscus caesius* is a poorly studied species. The compounds isolated from *H. sabdariffa* could be a source of its therapeutic and pharmacological properties (Ali *et al.,* 2005). In the Indian Ayurvedic literature, different parts of this plant have been suggested as a cure for complaints for instance hypertension, pyrexia, liver disorders and as an antidote to poisoning chemicals (Agoreyo *et al.,* 2008). Various nerve and heart disorder, high blood pressure and calcified arteries have also been treated with this plant (Asolkar *et al.,* 1992). *Hibiscus* pigments reduce the blood viscosity thus decreasing frequent occurrence of liver inflammation, necrosis and leucocyte infiltration (Kong *et al.,* 2003).

Phytochemical investigation is vital to make appreciable use of medicinal plants. Current study was conducted to explore the phytochemistry of relatively unexplored species of *Hibiscus* using HPLC-MS and comparing it with a well-studied species *H. sabdariffa* which have several documented health benefits e.g., Hypoglycaemic (Agoreyo *et al.,* 2008; Sini *et al.,* 2011), Hypolipidaemic (Farombi and Ige 2007), hypocholestrodemic (Lin *et al.,* 2007), antioxidant (Akim *et al.,* 2011; Mohd-Esa *et al.,* 2010), antimicrobial (Fullerton *et al.,* 2011; Zhang *et al.,* 2011), antilithic (Laikangbam and Damayanti Devi 2012; Woottisin *et al.,* 2011), anticancerous (Lin *et al.,* 2012) and diuretic.

MATERIALS AND METHODS

Plant material and extraction

Two species of the genus *Hibiscus* (*H. caesius* and *H. sabdariffa*) were selected from botanical garden of Quaid-i-Azam University Islamabad and its taxonomic status were verified from Department of Plant Sciences, Quaid-e-Azam University, Islamabad, Pakistan. Three samples of each species were taken for the analysis.

The petals and sepals of the flowers were collected and shade dried for 10 days and ground to fine powder using mortar and pestle. 10 g powder was transferred to airtight container and kept at -20^{o}C before analysis. The weighed powdered samples were subjected to acid hydrolysis by adding 2M HCl (Wako Pure Chemical Industries Ltd., Osaka, Japan) and boiled for 3 hr at 100^{o}C. Acid is used to convert cellulose or starch to sugar and to separate the sugar moieties from aglycone part to analyze them

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separately. The crude extract was filtered using Whatman No.1 filter paper.

Sample preparation for UV spectroscopy

UV spectroscopy plays an essential role in identification of many plant constituents, and frequently used to screen crude plant extracts for the presence of phytochemicals. For colorless compounds, measurements were made in the range 200-400nm and for colored compounds at 200-700nm. The UV spectra of the isolated compounds were recorded on UV-Visible spectroscope. The analysis was done using Spectrophotometer model no. UV-1700 (E) 23 OCE, UV 1700 Pharmaspec, Shimadzu Corporation.

High-performance liquid chromatography (HPLC-MS)

The dried filter papers were dissolved in methanol and re-filtered with double filter paper and samples for UV spectroscopy were prepared by diluting with MeOH (Wako Pure Chemical Industries Ltd., Osaka, Japan). Solvent extraction with ethyl acetate (Wako Pure Chemical Industries Ltd., Osaka, Japan) was done for the separation of non-polar compounds and the polar compounds remained in the aqueous layer. The aqueous layer was then extracted with n-butanol (Wako Pure Chemical Industries Ltd., Osaka, Japan) for anthocyanidins. The n-butanol was evaporated using rotary evaporator at 38ºC. LC separation of the extracts were performed on an Agilent 1200 series LC system coupled with Chem station for LC, 3-D system Rev.B.01.03 [2004]. DAD detected peaks at three different wavelengths 254, 320 and 370nm. The volume of injected sample was 5 µL, and elution was performed at ambient temperature. The gradient program starting from 10% CH_3CN (eluent B, Wako Pure Chemical Industries Ltd., Osaka, Japan) organic solvent in 90% double distilled H_2O (eluent A) aqueous solvent. Compounds were separated on HPLC column (4.6 × 150mm stainless-steel column packed with Agilent Eclipse XRD C-18 (5 µm).

RESULTS

HPLC-MS Profile

The aqueous extract of *H. caesius* was analyzed by reverse phase HPLC. Using 60 min, 10-80% organic solvent CH_3CN. UV absorbance was observed at 254, 320 and 370 nm. The identification of compounds were inferred on the basis of comparing retention time (t_R), TIC, UV absorption spectrum and mass fragmentation pattern with reference literature (Marbey *et al.,* 1970). Graph was plotted between t_R and concentration of solvent B (CH_3CN). Different molecules were eluted at different t_R. During t_R 0-10 min., phenolic acid type moieties were eluted followed by various secondary metabolites glycosides and towards the end aglycones were eluted.

The compound appeared at t_R of 7.0min on HPLC chromatogram. This t_R corresponds to the eluent composition consisting of 10% CH_3CN in H_2O indicating its hydrophobic character and giving an evidence for the presence of phenolic acid type moiety. The DAD was observed at three different wavelengths i.e., 254, 320 and 380nm. The UV spectrum (fig. 1a) showed single band pattern at 260 nm. The λ_{max} value and also the shape of UV-visible spectrum indicated the presence of vanillic acid type moiety. The ESI-MS of compound displayed in positive mode $[M+H]^+$ (fig. 1b) showed the signal at TIC time 7.1 min. The molecular ion peak exhibited at m/z=527.1 a.m.u, which was also the base peak referring to most stabilized fragment formed by the addition of a molecule of glucose, an acetyl xylose molecule and an atom of sodium. The molecular mass was inferred to be 526 a.m.u and the compound was identified as vanillyl - 4 - [O-glucosyl] - O- acetylxyloside. The λ_{max} value and also the shape of UV-visible spectrum indicated the presence of vanillic acid type moiety when compared to literature (Yi *et al.,* 2000).

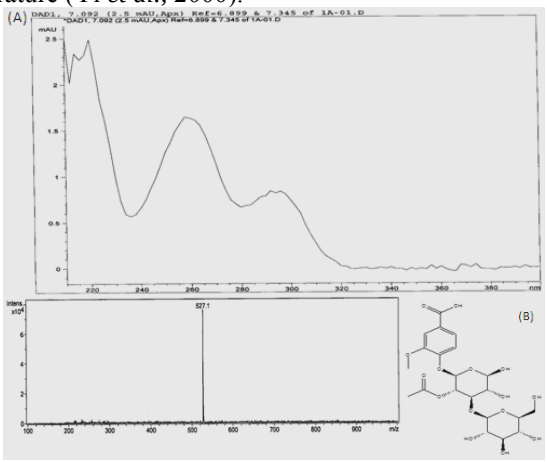

Fig. 1(a): DAD plot of vanillyl-4-[O-glucosyl]-O-acetylxyloside (b) Mass spectrum and structure of vanillyl-4-[O-glucosyl]-O-acetylxyloside.

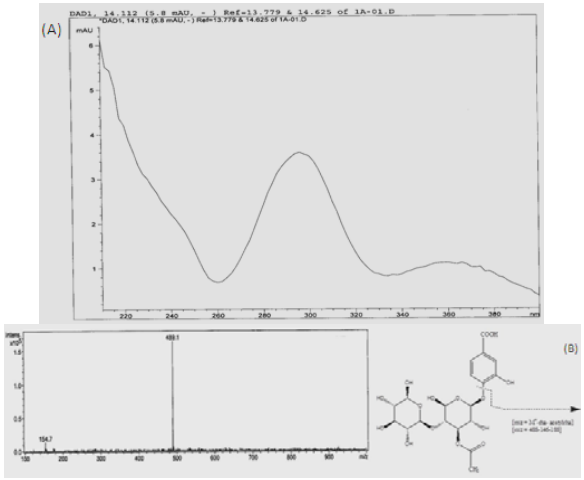

Fig. 2(a): DAD plot of protocatechoyl-4-[O-rhamnosyl]-O-acetylrhamnoside (b) Mass spectrum and structure of protocatechoyl-4-[O-rhamnosyl]-O-acetylrhamnoside.

The compound appeared at t_R14.11 min on HPLC chromatogram. This t_R corresponds to the eluent composition of 10% CH_3CN in H_2O indicating its hydrophobic character and giving an evidence for the presence of phenolic acid type moiety. The DAD showed single band pattern at λ_{max}295 nm (fig. 2a). The λ_{max} value and also the shape of the UV indicated the presence of protocatechuic acid type moiety. The molecular ion peak at m/z=489 displayed in positive ion mode $[M+H]^+$ (fig. 2b) at TIC time 14.2 min which was also the base peak referring to most stabilized fragment and the molecular mass was inferred to be 488 a.m.u. $[M+H]^+$. The fragment ion peak atm/z 155 $[M+H]^+$ appeared due to the loss of a rhamnose and an acetyl rhamnose moiety. The structure proposed on the basis of t_R, TIC time, DAD plot and mass fragmentation pattern was protocatechoyl-4-[O-rhamnosyl]-O-acetylrhamnoside. The λ_{max} value and also the shape of UV-visible spectrum indicated the presence of phenolic acid type moiety when compared to literature (Chen *et al.*, 2012).

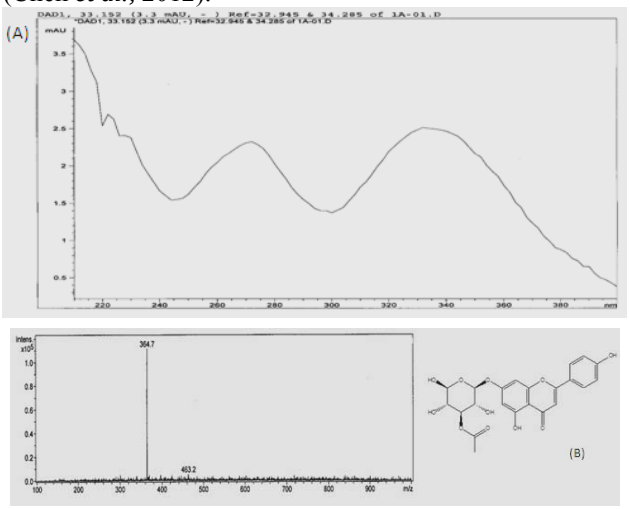

Fig. 3(a): DAD plot of apigenin-7-O-acetylxyloside (b) Mass spectrum and structure of apigenin-7-O-acetylxyloside.

The compound appeared at t_R 33.1 min. on HPLC chromatogram corresponding to the eluent composition of 33% CH_3CN in H_2O indicating its hydrophilic character and giving an evidence for the presence of flavone type moiety. The DAD observed at three different wavelengths i.e., 254, 320 and 370 nm showed double band pattern (fig. 3a) having peak II at 271 nm and peak I at 355 nm. The λ_{max} value and also the shape of UV spectrum are related to the apigenin type moiety. The ESI-MS of compound displayed in positive mode $[M+H]^+$ (fig. 3a) showed the signal at TIC time 33.2 min. The molecular ion peak appeared at m/z=463.2. The fragment ion peak which was also the base peak at m/z =364.7 appeared due to the loss of acetyl 1,3 X_o xylosyl residue along with a molecule of water where ionization occurred by Na^+ ion suggesting the presence of apeginine-7-O- acetylxyloside (fig. 3b). The λ_{max} value and also the shape of UV-visible spectrum indicated the presence of apigenin type moiety when compared to literature (Molyneux *et al.*, 1970).

The compound appeared at t_R of 34.072min on HPLC chromatogram. This t_R corresponds to the eluent composition of 34% CH_3CN in H_2O indicating its hydrophilic character and giving an evidence for the presence of flavonol type moiety. The DAD was observed at three different wavelengths i.e., 254, 320 and 370 nm. The UV spectrum (fig. 4a) having double band pattern showing band II at 255 nm and band I at 371 nm. The λ_{max} value and also the shape of UV spectrum indicated the presence of quercetin type moiety when compared to literature (Mabry *et al*., 1970). The ESI-MS of compound displayed in positive mode $[M+H]^+$ (fig. 4a) showed the signal at TIC time 34.4 min. The molecular ion peak appeared at m/z=465.2. The fragment ion peak which was also the base peak appeared at m/z =251.2 due to the loss of one glucose molecule along with one molecule of water and two carbon monoxide molecules [M+Na-glc-H_2O-2CO]. The molecular mass was inferred to be 464 a.m.u. indicating the presence of Quercetin-7-O-glucoside (fig 4b).

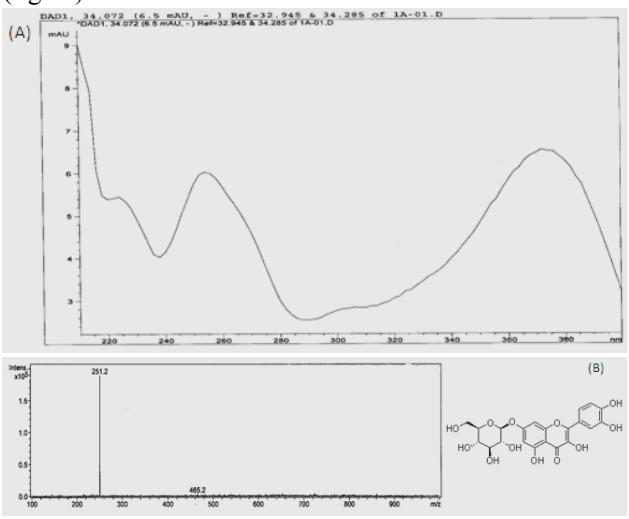

Fig. 4(a): DAD plot of quercetin-7-O-glucoside (b) Mass spectrum and structure of quercetin-7-O-glucoside.

The appearance of compound at t_R38.8 min corresponding to the eluent composition of 38% CH_3CN in H_2O indicated its hydrophilic character and giving an evidence for the presence of flavonol type moiety. The DAD was observed at three different wavelengths i.e., 254, 320 and 370 nm. The UV spectrum (fig. 5a) showed double band pattern having band II at 370 nm and band I at 255 nm. The λ_{max} values and also the shape of UV-visible spectrum indicated the presence of quercetin type moiety when compared to literature (Mabry *et al*., 1970). The molecular ion peak appeared at m/z=303.4 which is also the base peak displayed in positive mode $[M+H]^+$ (fig. 5b) showed the signal at TIC time 39.0 min. The molecular mass was inferred to be 302a.m.u, which indicated the presence of quercetin aglycone.

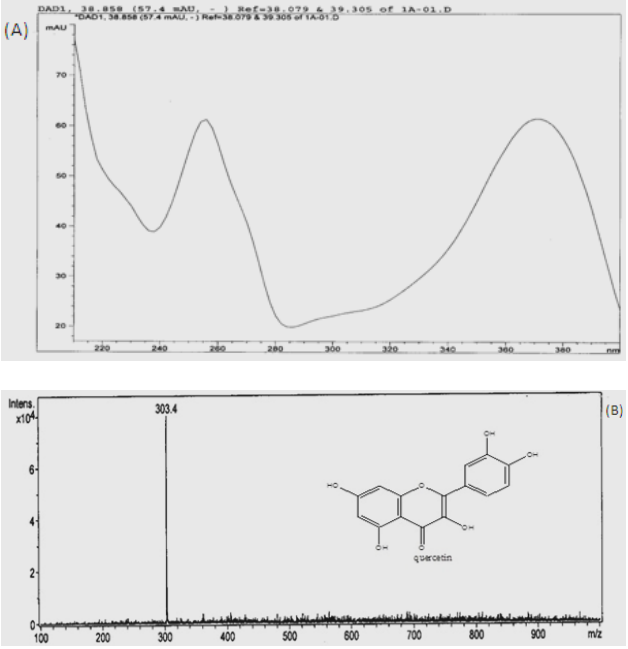

Fig. 5(a): DAD plot of Quercetinaglycone (b) Mass spectrum and structure of quercetinaglycone.

DISCUSSION

The compounds: Vanillic acid, protocatechoic acid, quercetin, quercetin glucoside, apigenin identified in the current study have previously been identified in the extracts of *H. sabdariffa* and found to be the cause of its pharmacological importance (Kong *et al.,* 2003; Lin *et al.,* 2012). Quercetin, quercetin glucoside and apigenin has been identified by HPLC-MS in *Hibiscus esculentus* and confirmed by NMR (Sini *et al.,* 2011). Apigenin has been found naturally in a number of plants (Lepley *et al.,* 1996) and is reported in literature to have pharmacological activities like anti-carcinogenic and anti-inflammatory (Birt *et al.,* 1997; Galati *et al.,* 1999). Phenolic acids have been found in many plant species playing important role like anti-inflammatory activity (Chiang *et al.,* 2003; Fernandez *et al.,* 1998). Protocatechoic acid has been previously reported in *Hibiscus sabdariffa* and found in medicinal herbs contributing to their antibacterial and anti-tumor activities (Chao and Yin 2009; Lin *et al.,* 2003). Vanillic acid has been found in *Hibiscus tiliaceus* and has shown anti-inflammatory properties (Narender *et al.,* 2009); also reported in other plants of medicinal value for example in *Amburana cearensis* (Leal *et al.,* 2011). Phytochemical screening and structural analysis identified anthocyanin responsible for anticancer, anti-inflammatory, hepatoprotective activities of *Hibiscus sabdariffa*. The activities of these compounds may be associated theoretically with the *H. caesius,* which may be further tested through pharmacognosy in the laboratory.

CONCLUSION

The current study focused on the phytochemical analysis of the unexplored species *H. caesius* concluding that the phytochemicals in *H. caesius* are of great pharmacological value, which may present a basis for the development of new drugs**.**

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