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Quantification of steroidal alkaloids in *Buxus papillosa* using electrospray ionization liquid chromatography–triple quadrupole mass spectrometry

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ABSTRACT

Buxus papillosa is one of the most extensively studied species of the genus *Buxus* known to possess steroidal alkaloids which can be used for assessing the various pharmacological activities of this plant. This paper describes the liquid chromatography–electrospray ionization triple quadrupole mass spectrometry (LC–ESI-QQQ-MS) method for the quantification of six steroidal alkaloids as chemical markers in the extracts of leaves, roots and stems of *B. papillosa*. Quantitative MS/MS analysis was carried out by optimization of the most sensitive transition for each analyte. This has yielded detection and quantification limits of 0.486–8.08 ng/mL and 1.473–24.268 ng/mL, respectively for all analytes. Linearity of response was also achieved and the regression coefficient found to be >0.99 for all analyzed compounds. The newly developed MRM (Multiple Reaction Monitoring) method showed excellent sensitivity for the quantification of steroidal alkaloids within 15 min run time. This paper describes the application of LC–QQQ-MS technique for steroidal alkaloids analysis in plant samples.

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1. Introduction

Buxus alkaloids represent a unique class of compounds possessing a triterpenoidal skeleton in which one or two nitrogen atoms are incorporated as side chains. About 100 species of genus *Buxus* are present in Africa, America, Asia and Europe [1]. The genus *Buxus* is among the richest source of steroidal alkaloids and more than 200 new steroidal alkaloids have already been reported [2].

Herbal formulations based *Buxus* species are widely used in Ayuverdic, Greco-Arabic and Chinese medicines in Asian countries due to their impressive biological activities. Extracts of genus *Buxus* have been used in the indigenous systems of medicine for the treatment of various disorders, such as malaria, rheumatism and skin infections. They also showed anticholinesterase [3], anti-HIV [4], immunosuppressive, and cytotoxicity activities [5,6]. Most of the biological activities of *Buxus* species are largely due to the steroidal alkaloids present therein. *Buxus papillosa* is widely distributed in Khyber Pakhtunkhwa (KPK) province of Pakistan. It is a well studied species of the genus *Buxus* with over 50 triterpenoidal and steroidal alkaloids. Many of the alkaloids are known for the various pharmacological activities, such as acetylcholinesterase and butyrylcholinesterase inhibitory activity [7]. *Buxus* alkaloids, also called alkamine, have nitrogen atoms, either at C-3 or C-20 or at both positions. On the basis of their basic skeleton, *Buxus* alkaloids can be divided into the following groups: (1) derivatives of 9b,19-cyclo-4,4,14a-trimethyl-5a-pregnane: having a basic skeleton with a cyclopropane ring between C-9 and C-19. (2) derivatives of 9(10–19)*abeo*,14a-trimethyl-5a-pregnane, resulting from the opening of the 9β-19-cyclopropane ring system.

Numerous studies on quantitative and qualitative analysis of different steroidal alkaloids have been published. The identification of glycosides of solasodine steroidal alkaloid have been reported by using techniques such as thin layer chromatography (TLC) [8], capillary electrophoresis mass spectrometry (CE-MS) [9] and high-performance liquid chromatography (HPLC) [10]. An HPTLC method was developed for the determination of steroidal glycoalkaloids in *Solanum xanthocarpum* [11]. Similarly, method was developed for the simultaneous identification and analysis of *Veratrum* alkaloids [12]. Steroidal glycoalkaloids (SGAs) extracted from tomato leaves and berries (*Lycopersicon esculentum*), have been identified by using optimized reversed-phase LC with electrospray ionization (ESI) and ion trap mass spectrometry (IT-MS) [13]. An HPLC–MS method for characterizing cevanine-type, veratramine-type, jervine-type and secosolanidine-type alkaloids in *Fritillaria* species has been developed [14,15]. Electrospray ionization multi-stage mass spectrometry was used to study the fragmentation of protoverine-type, germine type and zygadenin-type alkaloids, isolated from the Chinese herb, *Veratrum nigrum* L. [16]. However, only one report on the quantification of steroidal alkaloids through LC–ESI-TOF-MS method is found in

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literature used for quantification of steroidal alkaloids from *Fritillaria* species [17].

In continuation of our studies on the application of electrospray ionization mass spectrometry and high throughput dereplication strategy development [18–22], we describe here the development and validation of an LC–ESI-QQQ-MS/MS method for the quantification of steroidal alkaloids in *B. papillosa* with better limit of detection in short analysis time. This is the first report on the quantification of steroidal alkaloids in any plant using ESI-QQQ-MS.

2. Experimental

2.1. Chemicals and reagents

Chemicals and solvents were of analytical reagent and HPLC grades, respectively, and purchased from Aldrich–Sigma (USA). Deionized water (Milli-Q), used throughout the study, was obtained from Millipore Milli Q Plus System (Bedford, USA). The steroidal alkaloid standards, cyclomicrobuxine (**1**), cyclomicrobuxinine (**2**), *E*-buxenone (**3**), *N*-benzoylbuxahyrcanine (**4**), buxidine (**5**), buxandrine (**6**), and *N-Iso*butyroylbuxahyrcanine (internal standard, I.S.) (Fig. 1) were obtained from the Molecular Bank facility of the Dr. Panjwani Center for Molecular Medicine and Drug Research (International Center for Chemical and Biological Sciences), University of Karachi. The isolation and characterization details of the analyzed steroidal alkaloids have been reported previously [23,24].

2.2. Standard solution and calibration standards

Stock standard solutions were prepared in methanol at a final concentration of 1 mg/mL. These solutions were stored at 4 °C. Working solutions, used for LC–QQQ-MS analysis, were obtained by diluting the stock solutions with acetonitrile (with concentrations between 0.1 μg/mL and 19 μg/mL). Stock solution (260 μg/mL) of internal standard, I.S. (*N-Iso*butyroylbuxahyrcanine) was prepared in methanol and stored at 4 °C until use, and the final concentration of the internal standard was 0.5 μg/mL in the calibration standards range of 0.1–1 μg/mL and 12 μg/mL in the calibration range of 3–19 μg/mL, respectively.

2.3. Sample preparation

The plant *B. papillosa* (leaves, stems & roots) was collected from the Swat, Pakistan (Voucher # 9718). The plant material was identified by Mr. Mehboob ur Rehman, Plant taxonomist, the Department of Botany, Government Postgraduate Jahanzeb College, Swat. The dried leaves, roots and stems of *B. papillosa* were separately powdered to a homogeneous size, and sieved through a 400 μm mesh, followed by drying at 60 °C in the oven for 2 h. The dried leaves, roots and stem powder (500 mg) was pre-alkalized with 5 mL ammonia solution (33%) for 1 h, and immersed in 12 mL methanol overnight, then ultrasonicated for 1 h. After being filtered, the extract was concentrated to dryness in vacuum at 45 °C. 10 mL methanol was added in the residue and ultrasonicated for 5 min. Two working solutions for two different concentrations of internal standard were prepared by taking 100 μL of this reconstituted residue solution, and both were made up to 1 mL with acetonitrile containing a final concentration of 0.5 and 12 μg/mL internal standard respectively. The resultant solutions were centrifuged at 12,000 rpm for 10 min, the supernatants were filtered through Millipore filter (0.45 μm), and transferred to an autosampler vial for LC–QQQ-MS analysis.

2.4. LC–QQQ-MS analysis

Liquid chromatography–electrospray ionization triple quadrupole mass spectrometry (LC–ESI-QQQ-MS) in positive ionization

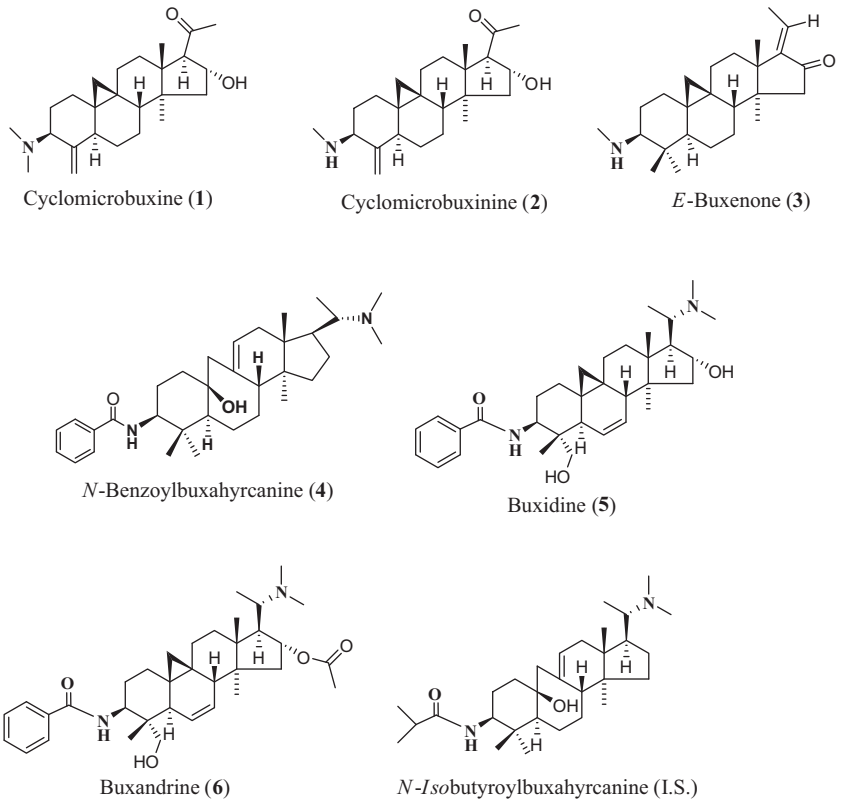

Fig. 1. The structures of *Buxus* steroidal alkaloids.

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