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Original article

Characterization and evaluation of bioactive polyphenolic constituents from *Zanthoxylum armatum* DC., a traditionally used plant

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ABSTRACT

Zanthoxylum armatum or Timoor has been used in different traditional system of medicine due to its aromatic properties and also in the treatment of cancer, diarrhea and cholera. In the present investigation, four chemically distinct compounds namely Tambulin (6), Prudomestin (7), Ombuin (8) and 3, 4, 5, 3′, 4′, 5′-hexahydroxydiphenyl ether (9) have been isolated and quantified from the fruits. To explore the biological activities, we have further studied the antiproliferative, antimicrobial and antioxidant efficacy. Tambulin which was also found in maximum amount (0.125%) in fruits revealed significant antiproliferative activity (IC_{50} 37.96 ± 0.36 to 48.7 ± 0.21 μg/mL) against breast, liver, colon and skin cancer cell lines corroborated by resilient binding interaction with SDH (−6.76 Kcal/mol) and inhibition constant (Ki: 11.02 μM). Hexane and ethyl acetate fraction exhibited moderate antibacterial efficacy (MIC: 250–1000 μg/mL) against selected pathogenic microbes while Ombuin displayed broad spectrum antibacterial effect with MIC ranges from 125 to 500 μg/mL. Total phenolic content (5.27 ± 0.06 to 46.12 ± 0.40 mg/g of gallic acid equivalents), total flavonoids content (6.05 ± 0.24 to 14.46 ± 0.73 mg/g of quercetin equivalents), ferric reducing power (42.35 ± 0.85 to 62.52 ± 0.66 mg/g of ferrous sulfate equivalents) and percent free radical scavenging activity (59.56 ± 0.38 to 64.85 ± 1.78) were also estimated. Our findings infer that Tambulin exhibited significant antiproliferative activity whereas Ombuin was found to display broad spectrum antibacterial activity which adds one more positive attribute to its traditional usage.

1. Introduction

Fruits and vegetables are very rich source of biologically active constituents which not only provide health benefits but also being the source of nutrients and are the integral part of the human and/or animal diet [1]. Earlier reports have confirmed that the phenolics and anthocyanins present in fruits contribute to its antioxidant and protective effect against chronic and degenerative diseases [2,3]. Phenolics compound and anthocyanin occur naturally in plants as secondary metabolites, are primarily concerted in fruits, leaves, seeds and flowers [4–6], and provides protection against various predators and/or environmental stress [7]. In addition, phenolics and related compounds are common ingredients of spices, jams, ice cream and cake icing, which make them worthy in the development of functional foods [8].

Zanthoxylum armatum (DC.) is an aromatic plant of family Rutaceae popularly recognized as Indian thorny ash, or toothache tree, timur or timoor. Colloquially, it is famous as Nepali Dhania and Chinese coriander extensively used in the Indian system of medicine [9]. It is a commonly used spice in Chinese, Indian, Nepali, Sichuan and Tibetan cuisine. Biscuits, chicken chilli, chowmein, momo, sweetened cakes and thupka are some of the food platter where timoor is used as an active ingredient for flavor and aroma [10,11]. The fruits of *Zanthoxylum* genus possessed peculiar aroma resembling lemon fragrance which make it useful in the preparation of variety of dishes and drink to give natural

Abbreviations: SDH, succinate dehydrogenase; MIC, minimum inhibitory concentration; MTT dye, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified essential eagle medium; FBS, fetal bovine serum; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; TLC, thin layer chromatography; GAE, gallic acid equivalent; PDB, protein data bank; CFU, colony forming unit; FRAP, ferric reducing antioxidant power; TAC, total antioxidant capacity; FSE, ferrous sulphate equivalent; AAE, ascorbic acid equivalent; EtOAc, ethyl acetate.

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flavor. It also produces post-soothing effect due to its numbness properties [12]. Traditionally, fruits pickles of *Z. armatum* are used in Nepal to combat common illness such as fever, tonsillitis, and cold besides enhancing the taste of food, whereas powdered fruits are used in Pakistan to treat common indigestion [9]. The Bhotiya tribal community in Uttaranchal, India utilizes timoor fruit recurrently in the form of condiments, spices and also as local medicine. Soups prepared from fruits (also known as Hag) are consumed by this community during winter to keep themselves warm. An incredibly popular dish 'Dunkcha' is also a trendy food item made from timoor fruits [13]. People of Cameroon use dry powder of *Zanthoxylum heitzii* fruits as a spice to prepare two local dishes 'Nkui' and 'Nahpoh' [14,15], in a study they evaluated the acceptability and food potential of *Z. alatum* (Roxb.) and observed that scores were significantly higher for foods (Chilli paneer, Punjabi chole and Besansev) containing timoor powder (400–500 mg) as compared to foods which lack them. In addition to its usage in various food commodities, timoor has also been considerably used in various traditional system of medicine for the treatment of stomach pain, cancer, cholera, metabolic disorders, respiratory disease, diarrhea, headache, heptoses and toothache, also effective as vasodilator and as a cardio protective, analgesic, anti-inflammatory and carminative [16–18]. The fruits and seeds of *Z. armatum* found its utility in the treatment of rheumatism and skin problem, muscle cramp, varicose veins and varicose ulcers [19,20]. Previous phytochemical investigations of *Z. armatum* have afforded lignans, alkaloids, long chain hydroxy acids and monoterpenetriol compounds [21]. Phenolic compound reported from *Zanthoxylum* species having anticancer activity [22–25]. Anti-inflammatory, antifungal and antioxidant activities of fruits and stem bark extracts have also been reported earlier [26–28]. Since polyphenolic and flavonoid group of compounds are known to reduce the occurrence of cancer by their scavenging and antioxidant properties and also prevent from various infections [29,30], there is always a need of natural products to be used as a functional food (e.g. spice) which counteract the ill effect of carcinogens and avert us from infections.

Hence forth, in this study an attempt has been made to explore the antiproliferative, antibacterial and antioxidant activities of *Z. armatum* fruits extract, fractions and its four isolated compounds. The different extracts, fractions of fruits were obtained by liquid-liquid partition extraction method and pure compounds were isolated, identified and characterized using spectroscopic and NMR techniques.

2. Materials and methods

2.1. Chemical and instruments

All the solvents used in the experiments were purchased from E-Merck Ltd, India. Cell culture media DMEM (Dulbecco's modified essential eagle medium) and FBS (fetal bovine serum) were bought from Gibco, India. MTT dye (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) antibiotic/antimycotic (Ab/Am), Trypsin, Phosphate buffer saline (PBS), Aluminium chloride, 2, 2-Diphenyl-1-picrylhydrazyl,Sodium/Potassium phosphate, Sodium acetate, Folin's reagent, Quercetin, Ascorbic acid and β-sitosterol were bought from Sigma-Aldrich, India. Sodium bicarbonate, Ammonium molybdate and Microbial culture media (Nutrient agar and broth), Gallic acid, Tris-[hydroxymethyl] aminomethane, 2, 4, 6-Tripyridyl-*s*-Triazine, sulphanilamide, Sodium nitropruside, *N*-(1-Naphthyl) ethylenediamine, *m*-phosphoric acid Sodium carbonate, Potassium ferricyanide, Ferric chloride and Ferrous sulphate were obtained from Himedia Ltd., India. Digital melting point apparatus from Sonar India was used to determine melting points, whereas Rudolf autopol model polarimeter for measurement of the optical rotations. Pre-coated TLC plates of thickness 0.25 mm, silica gel of 70–230 mesh ASTM and LiChroprep RP-18 (40–63 μm) were procured from Merck (Germany). Ultraviolet-visible spectroscopy with TU-1800_{PC} UV–vis spectrophotometer and Spectra max plus 384 with Soft max pro v5.3 software, Molecular Devices, USA were used. ^{1}H and ^{13}C NMR spectra were recorded in Bruker DRX-300 model spectrometer (300 and 75 MHz, respectively). FAB-MS data were recorded on a JEOL SX-102 spectrometer and Electrospray ionization mass (ESI) in direct mass analysis of HPLC-PDA-MS spectrometer and HR ESI MS on an agilent 6520 QTOF (ESI-HRMS). Infrared spectroscopy was recorded on an FT-IR spectrophotometer Shimadzu 8201 PC (4000–400 cm^{-1}). Thin layer chromatography was performed on pre-coated silica gel 60 F_{254}, plates (Merck). Visualization of the TLC spots was performed using 5% H_2SO_4 in ethanol spray reagent.

2.2. Plant materials and extract preparation

The fruits of *Z. armatum* were purchased from the local market of Lucknow, Uttar Pradesh, India during March 2014. It was authenticated in the Department of Botany and Pharmacognosy and a voucher specimen (ZA/F/14) was deposited in the herbarium of the CSIR-CIMAP, Lucknow, India. Dried fruits of *Z. armatum* (14.5 kg) in powdered form were extracted with methanol (70 L) by refluxing 8 h for three days and concentrated *in vacuo* to obtained a semi-solid brown liquid to yield (2.9 kg) of an extract, which was suspended in water and extracted with hexane, ethyl acetate and *n*-butanol, successively, to produce 1.5 kg, 300 g, 258 g and 603 g extracts, respectively. All the experiments related to biological activity evaluation were performed three times in replicates and data presented are average ± standard deviations.

2.3. Estimation of total phenolic and flavonoids content

The polyphenolic content in extracts, fractions and isolated compounds of *Z. armatum* fruits were quantified spectrophotometrically by estimation of phenolic and flavonoids content [31,32] in a concentration-dependent manner (10–100 μg/mL). Total phenolics content were determined by Folin's reagent as described by Singleton and Rossi with slight modifications [33]. For the estimation of phenolic content, *Z. armatum* extracts, fractions and isolated compounds were added to Folin's reagent and sodium carbonate, then absorbance was read at 765 nm after incubation at 37 °C for 90 min. Results of total phenolic content were expressed as gallic acid equivalents (GAE: mg/g of dried extract/fraction). Total flavonoids content were estimated by the colorimetric method [32–34]. In brief, fruits extracts, fractions and isolated compounds were mixed with 150 μL of methanol, 10 μL of aluminium chloride and 10 μL Potassium acetate followed by the addition of 280 μL of distilled water and optical density was recorded at 415 nm against a reagent blank, results were expressed in terms of quercetin equivalent (QE: mg/g of dried extract/fraction).

2.4. Isolation of compounds

The hexane fraction (1.5 kg) was subjected to normal-phase column chromatography over silica gel column (60–120 mesh, 7 kg, 180 cm × 5.5 cm) and yielded 55 fractions (each fraction (1 L) with the following eluants: fractions 1–5 in hexane, fractions 6–10 in hexane: EtOAc (9:1), fractions 11–15 in hexane:EtOAc (8:2), fractions 16–20 in hexane:EtOAc (7:3), fractions 21–25 in hexane: EtOAc (6:4), fractions 26–30 in hexane:EtOAc (1:1), fractions 31–35 in hexane:EtOAc (4:6), fractions 36–40 in hexane:EtOAc (3:7), fractions 41–45 in hexane:EtOAc (2:8), fractions 46–50 in hexane: EtOAc (1:9), fractions 51-–55 in EtOAc. Fractions 46–50 (31.19 g)