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Essential oil of *Piper betle* L. leaves: Chemical composition, anti-acetylcholinesterase, anti-β-glucuronidase and cytotoxic properties

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ABSTRACT

The aroma and taste of different locally available varieties of *Piper betle* L. leaves vary due to their essential oil and other constituents. In this study, the essential oils were extracted from seven local varieties of *P. betle* L. leaves (Bangla, Bagerhati, Manikdanga, Meetha, Kalibangla, Chhaanchi and Ghanagete) with the aim to characterize the varieties on the basis of oil constituents and to study their enzyme inhibitory and cytotoxic properties. Forty five identified essential oil constituents were subjected to different multivariate statistical analyses e. g. PCA, PLS-DA ($R2 = 0.40694$; $Q2 = 0.31199$), OPLS-DA ($R2X = 0.154$; $R2Y = 0.742$; $Q2 = 0.664$) and sPLS-DA. The first three statistical models distinguished mainly Chhaanchi, Meetha, Manikdanga from the others. sPLS-DA model separated all the varieties. The biplot, VIP score plot and other loading plots obtained from the above mentioned models identified mainly the phenylpropanes and a few terpenes to be responsible for distinguishing the varieties chemically. The essential oils exhibited very good acetylcholinesterase inhibitory properties, suggesting their potential for enhancing memory function. The essential oils also inhibited β-glucuronidase, involved in excretion of xenobiotics and other materials from the body, and showed cytotoxic properties. The study suggested potential applicability of the *P. betle* L. leaf essential oils in medical and cosmetic sector.

1. Introduction

Essential oils are commercially important in perfumery, food and pharmaceutical industries because of their aroma. Bioactivities of different essential oils have been studied. Such oils have been screened for their antifungal, antimicrobial, and antiviral effects to a great extent. Essential oils and their components have also been reported to possess antioxidant, analgesic, digestive, anticarcinogenic, immunomodulatory, antiapoptotic, anti-angiogenic, semiochemical and other activities as reviewed recently (Koroch et al., 2007; Saad et al., 2013; Perricone et al., 2015).

The aromatic leaves of *Piper betle* L. (Piperaceae), commonly known as betel leaf, are consumed as masticatory as well as for its medicinal importance in South East Asian countries. The essential oil (EO) present in the leaves is also consumed during chewing. The aroma and taste of such leaves varies with local varieties (Karak et al., 2016). Volatile oil content of betel leaves varies with varieties from 0.15% to 0.2% (Saxena et al., 2014). A large number of agricultural families depend on cultivation of betel vine in rural areas of India, particularly the state West Bengal, for their livelihood. But a huge quantity of the unsold perishable leaves are wasted (Guha, 2006). In addition, the chewing habits of people are changing with the change of life style. As a result, consumption of *P. betle* by local people is gradually decreasing and consequently the farmers are worst affected. The wastage may be minimized by extracting unsold leaves for EOs which may have promising industrial future (Guha, 2006). Essential oil components were analyzed from betel leaves collected from South India (Jirovetz et al., 1999), North India (Saxena et al., 2014), Eastern India (Suryasnata et al., 2016), other parts of India (Kumar et al., 2007; Bajpai et al., 2010), Sri Lanka (Mohottalage et al., 2007), Philippine (Rimando et al., 1986), Thailand (Tawatsin et al., 2006). Different bioactivities of betel leaf oils e.g. antifungal (Dubey and Tripathi, 1987; Prakash et al., 2010; Basak and Guha, 2015), antimicrobial (Sugumaran et al., 2011; Saxena et al., 2014), larvicidal (Wardhana et al., 2007), insecticidal (Tawatsin et al., 2006; Mohottalage et al., 2007), phytotoxic (Dubey and Tripathi, 1987) and antioxidant (Prakash et al., 2010) activities have been reported. Nanoemulsion (oil-in-water) of betel leaf essential oil showed antibacterial activity against selected food pathogens (Roy and Guha, 2018). Betel leaf essential oil could be applied as food preservative (Basak, 2018). The aim of the present study was to compare the essential oil composition and bioactivities e.g. acetylcholinesterase, β-glucuronidase inhibitory and cytotoxic properties of different local

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varieties of *P. betle* leaves collected from West Bengal, a part of Eastern India.

2. Materials and methods

2.1. Plant material

Seven local varieties of betel leaves i.e., *Piper betle* L. var Bangla, Bagerhati, Manikdanga, Meetha, Kalibangla, Chhaanchi and Ghanagete were collected from betel baroujs located in specific areas of West Bengal, India, during late winter of 2014. Intermediary sized leaves were collected from more than 15 plants, for each variety considered here. The varieties were identified and validated. Voucher specimens [Accession no. 20018(CUH)/1,3,4,5,6,7 & 8] were deposited in the Herbarium of Department of Botany, University of Calcutta.

2.2. Chemicals and reagents

Acetylcholineesterase enzyme (Source: electric eel), β-glucuronidase (ex. bovine liver), 4-nitrophenyl-β-D-glucuronide, MTT 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide and alkane standard solution (C_8-C_{20}) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5, 5´ Dithiobis (2-nitrobenzoic acid) (DTNB), Acetyl thiocholine iodide, myristic acid methyl ester (Methyl myristate), HPLC grade water, anhydrous Na_2SO_4, DMSO and methanol were bought from Merck Specialities Private Limited (Mumbai, India). HPLC-grade n-Hexane (95%) was procured from Spectrochem Pvt. Ltd. (Mumbai, India). Eugenol, MEM, FBS, Antibiotic-antimycotic solution was bought from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

2.3. Sample preparation

Freshly procured betel leaves were cleaned and cut into small pieces. Essential oil from the leaves of each local variety was extracted by hydro distillation in double distilled water (2.5 L) using Clevenger type apparatus. The essential oils collected were dried over anhydrous sodium sulphate and stored at 4 °C in the dark. Leaves collected from at least 15 plants of each variety were separated into four portions (2 kg and 3 X 500 g) for distillation of oil and analysis of chemical composition. For bioactivity analysis, oil extracted from leaf (2 kg) was used.

2.4. GC/MS analysis

Profiling of the volatile oil constituents from the hydro distilled oil of 7 varieties of betel leaves was performed by GC–MS analysis. Essential oils were diluted with HPLC grade hexane (1: 200 V/V). GC–MS analysis was carried out using Agilent 7890 A GC [software driver version 4.01(054)] equipped with 5795C inert MS with Triple Axis Detector. HP-5MS capillary column [Agilent J & W; GC Columns (USA)] of dimensions 30 m x 0.25 mm x 0.25 μm was used. The analyses were done under the following oven temperature programme: injection at 60 °C (5 min), then temperature increase at 4 °C min to 220 °C and hold time 10 min (total 55 min run). The detector was operated on EI mode (70 V). The injection temperature was set at 230 °C; the MS transfer line at 280 °C; the ion source at 250 °C. Helium was used as the carrier gas with flow rate 1 ml / min (Carrier linear velocity 36.623 cm/sec). Samples (1 μl) were injected via split less mode onto the GC-Column. Mass spectra ranging from 30 to 500 *m/z* were recorded. Essential oil constituents were identified by comparing the fragmentation patterns of the mass spectra with entries of mass spectra library NIST 2008 (fit and retrofit values higher than 75%) and. Arithmetic indices (AI) with respect to n-alkanes (C_8 – C_{20}) were calculated for comparison, using the following formula: $AI(x) = 100\ P_z + 100\ [(RT(x) - RT(P_z))/\ RT(P_{z+1}) - RT(Pz))]$, where ‘x’ is the unknown compound; ‘RT’ means retention time; ‘P_z’ is the alkane before x; ‘P_{z+1}’ is the alkane after x (Adams, 2009). The relative amount of each constituent was expressed as percentage peak area, relative to the total peak area.

Table 1
Volatile oil constituents identified in seven local varieties of *P. betle* leaf.

	Oil constituents	AI^c	AI^r	Mode of identification
MONOTERPENES	α-Thujene	928	924	AI, MS
	α-Pinene	934	932	AI, MS
	Camphene	948	946	AI, MS
	Sabinene	971	969	AI, MS
	Myrcene	991	988	AI, MS
	α-Terpinene	1018	1014	AI, MS
	β-Phellandrene	1029	1025	AI, MS
	1,8-Cineole/Eucalyptol	1031	1026	AI, MS
	(E)-β-Ocimene	1052	1044	AI, MS
	γ-Terpinene	1060	1054	AI, MS
	Terpinolene	1086	1086	AI, MS
	Linalool	1108	1095	AI, MS
	Terpinen-4-ol	1179	1174	AI, MS
	α-Terpineol	1191	1186	AI, MS
SESQUITERPENES	δ-Elemene	1339	1335	AI, MS
	α-Copaene	1377	1374	AI, MS
	β-Elemene	1393	1389	AI, MS
	E-β-Caryophyllene	1421	1417	AI, MS
	β-Copaene	1431	1430	AI, MS
	γ-Elemene	1435	1434	AI, MS
	Aromadendrene	1441	1439	AI, MS
	α-Humulene	1455	1452	AI, MS
	γ-Muurolene	1478	1478	AI, MS
	Germacrene D	1483	1484	AI, MS
	β-Selinene	1488	1489	AI, MS
	α-Selinene	1497	1498	AI, MS
	Bicyclogermacrene	1498	1500	AI, MS
	α-Muurolene	1502	1500	AI, MS
	cis-β-Guaiene	1505	1492	AI, MS
	δ-Cadinene	1526	1522	AI, MS
	Palustrol	1568	1567	AI, MS
	Spathulenol	1580	1577	AI, MS
	Caryophyllene oxide	1583	1582	AI, MS
	Globulol	1585	1590	AI, MS
	Viridiflorol	1593	1592	AI, MS
	Cubenol	1642	1645	AI, MS
	α-Cadinol	1653	1652	AI, MS
PHENYLPROPANES	Estragole /Methyl chavicol	1200	1195	AI, MS
	Chavicol	1260	1247	AI, MS
	Anethole/Isoestragole	1286	–	MS
	Safrole	1290	1285	AI, MS
	Chavicol, acetate	1349	–	MS
	Eugenol	1368	1356	AI, MS
	Methyl eugenol	1407	1403	AI, MS
	Eugenol acetate	1532	1521	AI, MS

Index: AI^c = Calculated Arithmetic Index; AI^r = Reported Arithmetic Index; AI = Arithmetic Index; MS = Mass spectra.

2.5. Assay for acetylcholinesterase inhibition

Acetylcholinesterase (AChE) inhibition was measured following the method of Ellman et al. (1961) with a little modification as reported earlier from the laboratory (Acharya et al., 2016). In brief, oil (0.01 ml of different concentrations 0.5 - 0.8 mg/ml methanol), AChE (0.02 ml; 19.93 unit/ml buffer), buffer (1 ml, pH 8.0), 0.5 mM DTNB (0.01 ml), and 0.6 mM acetylthiocholine iodide solution (0.02 ml) were mixed. This reaction mixture was incubated at 37 °C for 20 min. Following incubation, optical density (OD) was measured immediately at 412 nm. Controls were devoid of test samples. The percentage of inhibition was calculated as: Inhibition (%) = [(Control OD – Sample OD)/ Control OD] × 100. One oil from each variety was analysed 4 times.

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