Industrial Crops & Products 118 (2018) 246–258

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Chemical composition, genetic diversity, antibacterial, antifungal and antioxidant activities of camphor-basil (*Ocimum kilimandscharicum* Guerke)

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ARTICLE INFO

Keywords:
Ocimum kilimandscharicum
Essential oil
Chemotype
ISSR marker
Bioactivity
Safety evaluation

ABSTRACT

This study investigates the variations in the chemical, genetic, and biological activity (antibacterial, antifungal, and antioxidant) profiles of thirteen populations of *Ocimum kilimandscharicum* Guerke (OK1-OK13) from India. Correlations between chemical composition and the biological activities of *O. kilimandscharicum* were assessed. The essential oils (EOs) of different populations, extracted by hydrodistillation process were characterised by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC–MS). The antimicrobial activities of the EOs were evaluated by disc diffusion and micro dilution broth assays. The antioxidant activity of the EOs was determined by ferric reducing antioxidant power (FRAP) assay. The essential oil (EO) content varied significantly among the studied populations (0.15–0.93%). The EO analysis, and subsequent cluster and principal component analyses classified the populations in three distinct chemotypes, namely camphor (52.0–57.2%), linalool (65.2–91.0%), and phenylpropanoid/sesquiterpene. Amplification of genomic DNA using 20 inter simple sequence repeat (ISSR) primers yielded a total of 224 loci, out of which 210 loci were polymorphic in nature, representing 93.75% polymorphism. Pearson coefficient correlation ($r = 0.32$) suggesting low correlation between the distances obtained by molecular markers and EO compositions. The EO of population OK12 showed significant activity against *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Staphylococcus aureus*. However, EOs of populations OK11 and OK13 showed significant activity against *Candida albicans* (clinical isolate) and *Candida albicans* (ATCC), respectively. The EO of OK13 exhibited significant antioxidant activity. *In-vitro* safety evaluation study revealed that the EOs of most of the populations showed no significant toxicity against peritoneal macrophages cells. It is concluded that chemical and genetic profiles of *O. kilimandscharicum* varied considerably and these variations determined changes in its biological activities. The observations of this study could pave the way to optimize the use of *O. kilimandscharicum* populations in relation with its biological properties.

1. Introduction

The secondary metabolite profile of the medicinal and aromatic plants varies substantially due to inter and intraspecific variations. The occurrence of such diversity offers an excellent opportunity for need base screening and subsequent utilization of plant genetic resources in pharmaceutical, flavor and fragrance industries. However, lack of scientific knowledge on such variability often limits the optimal use of plant species (Suppakul et al., 2003). The genus *Ocimum* L. (Lamiaceae), collectively called basil, comprises several economically important annual and perennial herbs and shrubs native to the tropical and subtropical regions of Asia, Africa and Central South America (Labra et al., 2004). The members of the genus *Ocimum* are characterised by a substantial morphological and chemical variability. The ease of cross pollination leads to a large number of subspecies, varieties and forms, which differ in both, EO composition and morphological characters (Krishnan, 1981; Simon et al., 1990). The EO of *Ocimum* spp. has been used in the flavoring of confectionary, food products and nonalcoholic beverages. The EOs have also been found a wide application in perfumery as well as in oral hygiene (Suppakul et al., 2003). The extract and EOs are reported to possess diverse biological activities (Hussain et al., 2008). *Ocimum kilimandscharicum* Gurke, commonly known as Kapoor Tulsi in Hindi and Camphor Basil in English, is an economically important aromatic perennial under-shrub having simple

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https://doi.org/10.1016/j.indcrop.2018.03.050
Received 28 December 2017; Received in revised form 17 February 2018; Accepted 23 March 2018

ovate-oblong leaves. In East Africa, the leaves have been traditionally used for the treatment of cold, cough, abdominal pain, measle, and diarrhea (Obeng-Ofori et al., 1998). The plant possessed carminative, stimulant, antipyretic, antifungal, antibacterial, insect repellent, and antioxidant properties (Hakkim et al., 2008; Joshi, 2013; Kokwaro, 1976).

The EO composition of *Ocimum* spp. has been extensively investigated from different parts of the world. So far more than 200 chemical components have been identified in the EO of different *Ocimum* spp. (Hassanpouraghdam et al., 2010). Based on the EO composition, *Ocimum* spp. exist in various chemotypic forms, viz. methyl chavicol, linalool, eugenol, methyl eugenol, geraniol, methyl cinnamate, citral, bergamotene, α-cubebene, germacrene D, *β*-elemene, 1,8-cineole, α-cadinol, and limonene (Simon et al., 1990; Verma et al., 2013). Moreover, it has been observed that the EO constituents in *Ocimum* biosynthesized by two distinct biochemical pathways, shikimic acid (phenylpropanoids) and cytosolic mevalonic acid pathway (terpenes) (Lawrence, 1988).

Genetic resource characterization through various molecular markers is of great importance for protection and sustainable utilization of important medicinal and aromatic plants (Kumar et al., 2014). For assessment of genetic variation in plants, molecular markers has shown superiority over other markers system, as molecular markers are neutral, not related to age and tissue type, and not influenced by the environmental conditions (Marshall, 1997). Inter simple sequence repeat (ISSR) markers are dominant genetic markers, which use a single primer that amplifies the regions between adjacent and inversely oriented simple sequence repeats (Zietkiewicz et al., 1994).

Studies concerning inter and intraspecific variability of *Ocimum* taxa, particularly of *O. basilicum* and *O. tenuiflorum* have been reported. A remarkable chemical and morphological variation due to the possibility of inter- and intra-specific hybridization and the occurrence of numerous cultivars and chemotypes within species has been documented (Grayer et al., 1996; Labra et al., 2004; Lawrence, 1988; Malav et al., 2015). However, to the best of our knowledge, studies linking the chemical and genetic characters to the biological activities are meager on *O. kilimandscharicum* from India. Therefore, the present research was undertaken with the main objective to investigate the chemical and genetic diversity, and assess the antibacterial, antifungal, antioxidant and cytotoxic activities of thirteen populations of *O. kilimandscharicum* grown in the foothills of north India.

2. Materials and methods

2.1. Plant materials

Thirteen populations of *O. kilimandscharicum* (five collections from different locations of Uttarakhand: OK1, OK2, OK3, OK4, and OK13 and eight open pollinated seed progenies of OK4, namely OK5, OK6, OK7, OK8, OK9, OK10, OK11, and OK12) were vegetatively propagated using soft stem cuttings at the experimental farm of CSIR-Central Institute of Medicinal and Aromatic Plants, Research Centre Pantnagar, Uttarakhand. Twenty days old rooted stem cuttings of each population were transplanted in the well prepared field using a plot size of 3.0 × 3.0 m at 60 × 60 cm plant spacing in mid February 2017. The populations were raised using uniform agricultural inputs. The experiment was performed in a randomized block design with thirteen treatments as population in three replicates. The morphological data were recorded for 25 characters (13 qualitative and 12 quantitative characters) in the end of April, at full bloom stage (75 days after transplanting). The experimental site is located between latitude 29° N and longitude 79.38° E, and at an altitude of 243 m above mean sea level, experiencing the subtropical, humid climate. The experimental oil was mollisol with neutral in reaction (pH 7.1).

2.2. Isolation and GC and GC–MS analysis

The extraction of the EOs from fresh herbage was carried out by hydrodistillation in a Clevenger's type apparatus for 3 h. The EOs obtained were dried over anhydrous sodium sulphate and kept in the refrigerator until further analysis. GC was performed for quantification of the EO constituents, using Nucon Gas Chromatograph (5765) equipped with DB-5 (30 m × 0.25 mm internal diameter, 0.25 µm film thickness) fused silica capillary column and flame ionization detector (FID). Hydrogen was used as carrier gas at 1.0 mL min^{-1}. Temperature programming was done from 60 to 230 °C at 3 °C min^{-1}. The injector and detector temperatures were 220 °C and 230 °C, respectively. The injection volume was 0.03 µL neat with a split ratio of 1:40. GC–MS, performed for identification of the EO constituents, was done using a Clarus 680 GC interfaced with a Clarus SQ 8C mass spectrometer of PerkinElmer fitted with Elite-5 MS fused-silica capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The oven temperature program was from 60 to 240 °C, at 3 °C min^{-1}, and programmed to 270 °C at 5 °C min^{-1}. Injector temperature was 250 °C; transfer line and source temperatures were 250 °C; injection size 0.03 µL neat; split ratio 1:50; carrier gas He at 1.0 mL min^{-1}; ionization energy 70 eV; mass scan range 40–500 amu. Identification of the EO constituents was achieved on the basis of retention index (RI), MS Library search (NIST and WILEY), and by comparing RI and mass spectral data with the literature (Adams, 2007).

2.3. DNA isolation and ISSR-PCR amplification

Fresh leaves were collected from the experimental farm and kept in –80 °C till further use. Total genomic DNA of *O. kilimandscharicum* populations was isolated using the protocol described earlier (Khanuja et al., 1999). Isolated genomic DNA was quantified spectrophotometrically (Nano Drop ND-1000) by quantifying absorbance at 260 nm and its integrity was checked by visualizing under UV light after agarose gel electrophoresis on 0.8% agarose gel. Stock DNA was diluted to make a working solution (25 ng $µl^{-1}$). Routine PCR reactions were conducted with a PCR mixture containing buffer 1X, Mg^{2+} 2.5 mm, dNTPs 0.2 mM, Taq DNA polymerase 1 U (TaKaRa), primer (UBC set No. 9) 0.5 µM and water to a final volume of 20 µL. Amplifications were carried out using a thermal cycler (Applied Biosystems) using the following parameters: initial denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 48–60 °C for 1 min, extension at 72 °C for 2 min; final extension at 72 °C for 7 min. PCR products were electrophoresed on 1.2% agarose gel alongside a 2-Log DNA ladder and documented by means of the Bio-Rad universal hood II gel documentation system (Bio-Rad laboratories Inc.).

2.4. Antibacterial assays

The antibacterial activity of the EOs was determined against six pathogenic strains, namely *Mycobacterium smegmatis* (UDSC-MC^2 155), *Enterococcus faecalis* (MTCC-439), *Escherichia coli* (MTCC-723), *Streptococcus mutans* (MTCC-890), *Staphylococcus epidermidis* (MTCC-435), and *Staphylococcus aureus* (MTCC-96) by disc diffusion assay and micro dilution broth assay as per CLSI guidelines (CLSI, 2006; CLSI, 2012). Inoculum of the test bacteria was prepared equivalent to McFarland Standard 0.5 (1×10^6 CFU mL^{-1}). Uniform bacterial lawns were made using 100 µL inoculums on a Mueller Hinton agar plate. Sterile discs (6.0 mm) from Himedia labs were used in the study. 8 µL of the test sample was placed over the disc and discs were placed over seeded plates. The plates were incubated at 37 °C for 24 h. Activity was measured in terms of zone of inhibition (ZOI, mm). The net zone of inhibition was determined by subtracting the disc diameter (6.0 mm) from the total zone of inhibition shown by the test disc in terms of clear zone around the disc. The minimum inhibitory concentration of the oil samples was determined by micro dilution broth assay using 96 'U'