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Leaf essential oil composition, antimicrobial and cytotoxic activities of *Cleistocalyx operculatus* from Hetauda, Nepal

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Abstract
The present study investigated the chemical composition and bioactivity of the leaf essential oil from *Cleistocalyx operculatus* (Roxb.) Merr. & L.M. Perry from Nepal. In this work, leaf essential oil of *C. operculatus* was obtained by hydrodistillation and analyzed by GC-MS. The major component of the leaf oil was myrcene (69.7%), followed by (*E*)-β-ocimene (12.24%), (*Z*)-β-ocimene (4.79%), and linalool (4.08%), with other minor constituents. Antimicrobial activities were determined using the microbroth dilution technique, and *in-vitro* cytotoxic activity against MCF-7, MDA-MB-231 and 5637 cells was tested using the MTT assay. *C. operculatus* oil has shown no antimicrobial or cytotoxic activity.

Keywords: *Cleistocalyx operculatus*, GC-MS, essential oil composition, myrcene.

1. Introduction

Cleistocalyx operculatus (Roxb.) Merr. & L.M. Perry (Myrtaceae) is a well-known evergreen perennial tree. It is also known as *Cleistocalyx cerasoides* (Roxb.) I.M. Turner, *Syzygium nervosum* DC, *S. operculatum* (Roxb.) Nied, and *Eugenia operculata* Roxb [1]. It is widely distributed in India, Nepal, Indonesia, Malaysia, Myanmar, Sri Lanka, Thailand, Vietnam, and Australia. In Nepal, it is commonly known as "kyaman". The common English name is "sugar root" [2]. The average tree can reach up to 15 m high and 8-12 m wide. The trunk is smooth, covered with thin brown or greyish brown, flaky bark [1]. Leaves are simple, opposite, elliptical to obovate, leathery, coarsely veined, measuring 8-10 cm long and 4.5-7 cm wide [3]. When crushed, leaves emit a mango-like odor. Flowers arise on lateral branches, in 6-12 cm long panicles, collecting the flowers in sessile terminal heads as a compound cyme. Pale yellow flowers are enclosed in the calyx [1, 4, 5]. Each inflorescence has an average of 30-40 flowers. There are numerous stamens and one pistil. Fruit is a fleshy berry, ovoid, purple to black when ripe [6]. *C. operculatus* blossoms in March and April and the seeds ripen in May and June.

For many years, leaves and bark of *C. operculatus* have been used as a tea to treat stomachache and gastrointestinal disorders as well as an antisepsis for dermatophytic disorders [6]. The bark decoction is used in taking bath against sunstroke and to wash wounds. A paste of bark and leaves mixed with *Glycyrrhiza glabra* is used to treat pneumonia [2]. Previous reports showed that the bark of *C. operculatus* contains oleanane-type triterpenes with antidermatophytic activity [7], and the buds contain flavonoids and triterpene acids [8]. The essential oil from the buds of *C. operculatus* from Vietnam has previously shown antimicrobial activity against several bacterial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA) [9] and *Xanthomonas campestris* [10]. The components of leaf essential oil of *C. operculatus* from Vietnam have been reported [11]. However, the constituents of plant essential oils can differ from one place to another. The chemical composition and yield of essential oils are influenced by provenance, weather, soil conditions, time of harvest and the drying technique [12]. Over time, these factors can encourage the biosynthesis of certain terpenes and stop the synthesis of others which subsequently can change the chemical profile of plant essential oils [13, 14]. To the best of our knowledge, the chemical composition of leaf essential oil of *C. operculatus* from Hetauda, Nepal has not been studied yet, nor have its antimicrobial and cytotoxic activities been investigated.

2. Materials and Methods
2.1 Plant Material
The leaves of *Cleistocalyx operculatus* were collected from Makwanpur district, Hetauda, Nepal (27°25′N 85°02′E, 550 m asl) in August 2014. The plant was collected and identified by Kiran K. Pokharel, and a voucher specimen (M2013.01) has been deposited with the Department of Forest Research and Survey, Ministry of Forest Research and Soil Conversation, Babarmahal, Kathmandu. The plant material was air-dried for several days. A sample of 100 g was hydrodistilled using a Likens-Nickerson apparatus with continuous extraction using chloroform for 4 h to give 0.4 mL of yellow essential oil with pleasant odor, which was stored at 4 ºC until analysis.

2.2 Gas Chromatographic – Mass Spectral Analysis
The essential oil of *C. operculatus* was analyzed by GC-MS using an Agilent 6890 GC with Agilent 5973 mass selective detector, an HP-5ms fused silica capillary column and an Agilent Chem Station data system as previously described [15]. Identification of the oil components was based on their retention indices (RI) and by comparison of their mass spectral fragmentation patterns with those reported in the literature [16].

2.3 Antimicrobial Screening
The essential oil of *C. operculatus* was screened for antimicrobial activity against *Bacillus cereus* (ATCC No. 14579), *Staphylococcus aureus* (ATCC No. 29213), *Aspergillus niger* (ATCC No. 16888) and *Candida albicans* (ATCC No.10231). The minimum inhibitory concentration (MIC) was determined using the microbroth dilution technique as previously reported [17].

2.4 Cytotoxicity Screening
MCF-7 human breast adenocarcinoma cells (ATCC No. HTB-22) and MDA-MB-231 cells (ATCC No. HTB-26) were grown in RPMI 1640 supplemented with 10% Fetal bovine serum (FBS), 30 mM HEPES, $NaHCO_3$, and Penicillin-Streptomycin. 5637 human bladder cancer cells (ATCC No. HTB-9) were grown in RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 2.5 g/L glucose, 30 mM HEPES, $NaHCO_3$, and Penicillin-Streptomycin. *In-vitro* cytotoxic activity of *C. operculatus* oil on MCF-7, MDA-MB231 and 5637 cells was performed using the 96-well MTT assay as previously reported [18].

3. Results and Discussion
Monoterpenes predominated in *C. operculatus* essential oil composition as shown in Table 1. The essential oil is mainly composed of myrcene (69.7%), followed by (*E*)-β-ocimene (12.24%), (*Z*)-β-ocimene (4.79%), and linalool (4.08%), with other minor constituents (3% or less of each). The essential oil composition and percentages in the current study are significantly different from the previously published report from Vietnam. The major components of leaf essential oil of *C. operculatus* of Vietnamese origin were reported to be (*Z*)-β-ocimene (32.1%), myrcene (24.6%), and (*E*)-caryophyllene (14.5%) and (*E*)-β-ocimene (9.4%) [11]. This difference might be due to several factors including age, vegetative cycle stage, climate, season, soil composition and edaphic factors [19]. Also, the essential oil composition of *C. operculatus* is different from the leaf essential oil of *Syzygium aromaticum* and *S. cumini*. *S. aromaticum* had eugenol (94.4%) as the main constituent followed by (*E*)-caryophyllene (2.9%) [20] While the main oil constitutes in *S. cumini* were α-pinene (17.53%), α-terpineol (16.67 %) and *allo*-ocimene (13.55%) [21].

Table 1: Chemical composition of *Cleistocalyx operculatus* leaf oil from Nepal.

RI[a]	Compound	% ± SD[b]
934	α-Thujene	0.53 ± 0.03
992	Myrcene	69.70 ± 1.02
1003	α-Phellandrene	0.69 ± 0.02
1023	*p*-Cymene	2.71 ± 0.06
1027	β-Phellandrene	1.20 ± 0.05
1037	(*Z*)-β-Ocimene	4.79 ± 0.06
1047	(*E*)-β-Ocimene	12.24 ± 0.07
1057	γ-Terpinene	0.82 ± 0.04
1086	Terpinolene	0.26 ± 0.03
1098	Linalool	4.08 ± 0.20
1174	Terpinen-4-ol	tr[c]
1416	(*E*)-Caryophyllene	1.11 ± 0.17
1562	(*E*)-Nerolidol	1.57 ± 0.27
1521	Caryophyllene oxide	0.23 ± 0.09
1620	(*Z*)-Asarone	0.05 ± 0.08
	Compounds Identified	15 (99.72%)

[a] RI determined with respect to a homologous series of *n*-alkanes on an HP-5ms column.
[b] Average of three measurements ± standard deviations.
[c] tr = "trace" (< 0.05%).

The most abundant constituent was myrcene (7-methyl-3-methylene-1, 6-octadiene), a well-known monoterpene. It was also reported in the essential oils of several plant species such as *Myrcia* spp. [22], *Porophyllum ruderale* [23], fresh mango fruit [24], *Cannabis sativa* [25], *Pimenta racemosa* [26], *Citrus aurantium* [27], *Humulus lupulus* [28], *Verbena* spp., and *Cymbopogon citratus* [29]. Due to its muscle-relaxing, anti-depressant, anti-inflammatory [23], anti-tumor and analgesic effects [30], myrcene is used in the treatment of spasms, insomnia, and pain. It was also used to minimize epileptic seizures [31] and to prevent peptic ulcer disease [32]. In addition, myrcene was found to affect the integrity and permeability of the cell membrane [33]. Myrcene is also very important in perfumery because of its pleasant odor, which is described as earthy, clove-like, and fruity. It is used as an intermediate in the commercial production of various compounds like geraniol, nerol, linalool, myrcenol, citral, citronellal, hydroxycitronellal, menthol, and ionones [34]. Based on our results, the mature leaves of *C. operculatus* from Nepal can be considered as a good source for commercial isolation of natural myrcene.

The essential oil of *C. operculatus* was screened for potential antimicrobial activity (Table 2). The results showed that there was no antibacterial activity against *Bacillus cereus* (MIC = 1250 μg/mL) or *Staphylococcus aureus* (MIC = 625 μg/mL) and no antifungal activity against *Aspergillus niger* (MIC = 625 μg/mL) and *Candida albicans* (MIC = 2500 μg/mL). The essential oil was screened for *in-vitro* cytotoxic activity against MCF-7, MDA-MB-231 and 5637 cells but showed no significant activity (0% kill at 100 μg/mL).

Table 2: Antimicrobial activity (MIC, μg/mL) of *Cleistocalyx operculatus* leaf oil from Nepal.

B. cereus	*S. aureus*	*A. niger*	*C. albicans*
1250	625	625	2500

4. Conclusions
The essential oil of *Cleistocalyx operculatus* from Nepal was analyzed by GC-MS and was found to be a good source of myrcene (69.7%) followed by (*E*)-β-ocimene (12.24%), (*Z*)-β-ocimene (4.79%), and linalool (4.08%). *C. operculatus*

essential oil was screened for antimicrobial activity and cytotoxic activity but was found to be inactive.

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