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Article

Assessment of Chemical Composition and Therapeutic Activities of *Clausena excavata* Burm.: An Important Medicinal Plant of Eastern Ghats of India

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Abstract: *Clausena excavata* Burm. is an important medicinal plant belonging to the family Rutaceae. It is well known for its ethnomedicinal uses and therapeutic activities. This plant has been used in different traditional systems of medicine for the treatment of various diseases worldwide. In the present study, the chemical composition of essential oil and phytochemical contents of methanol extract of *C. excavata* leaf were performed. Further, the therapeutic activities of essential oil and methanol extract of *C. excavata* leaf were evaluated. The chemical composition of essential oil was analyzed by Gas Chromatography/Mass Spectrometry (GC/MS). Myristicin (33.92%), terpinolene (21.29%), and sabinene (13.65%) were found to be the major compounds in leaf essential oil. Similarly, the phytochemical composition of the methanol extract of *C. excavata* leaf was analyzed using standard procedures. The phenolic content (165.23 mg GAE/g DW) was found to be the major phytoconstituents followed by alkaloids (89.30 mg/g DW) in leaf methanol extract. Furthermore, the antioxidant, antidiabetic, anticancer, and antimicrobial activities of the essential oil and methanol extract were evaluated. *C. excavata* essential oil showed more antioxidant activity (DPPH IC_{50} 15.92 µg/mL; ABTS IC_{50} 41.85 µg/mL; FRAP IC_{50} 39.9 µg/mL) compared to leaf methanol extract. Similarly, essential oil exhibited higher antidiabetic activity (in terms of α-amylase inhibitory capacity) (IC_{50} 97.50 µg/mL) compared to methanol extract and lower antidiabetic activity in comparison to the standard (IC_{50} 71.9 µg/mL). The anticancer activity of essential oil was found to be more effective against the PC-3 cell line (IC_{50} 39.1 µg/mL) while leaf methanol extract was more effective against OVCAR-3 (IC_{50} 47.5 µg/mL), MCF-7

(IC_{50} 48.1 µg/mL), and h-1299 (IC_{50} 45.9 µg/mL). *C. excavata* essential oil exhibited lower antimicrobial activity compared to leaf methanol extract. In summary, this study on the characterization of phytoconstituents and pharmacological activities of *C. excavata* leaf essential oil could be helpful in exploiting its therapeutic potential sustainably as well as the discovery of new lead molecules.

Keywords: Anticancer activity; antidiabetic activity; antimicrobial activity; antioxidant activity; *Clausena excavata*; essential oil.

Introduction

The *Clausena* genus of the family Rutaceae includes around 27 species and is widely distributed in Africa, India, Taiwan, Australia, New Guinea, and the Pacific Islands [1-3]. *Clausena* spp. has been used in Indian, Chinese, and Thai traditional systems of medicine for the treatment of numerous diseases worldwide and is also a source of drugs for the modern medicine system [4,5]. The plant extract of this genus, including *Clausena anisata, C. lansium, C. harmandiana, C. heptaphylla, C. suffruticosta, C. dentata, C. wallichii, C. lenis, C. indica,* and *C. anisumolens* have shown potential antibacterial activity [6-9]. At the same time species including *C. anisata, C. lansium, C. harmandiana, C. heptaphylla, C. suffruticosta, C. dentata, C. emarginata, C. dunniana,* and *C. indica* have antifungal activities [10-12]. A few reports have also been documented regarding the antiprotozoal activity of this genus, such as *C. anisata* and *C. guilaminii* [13-16]. Furthermore, *Clausena* spp. also possesses anticancer, antiinflammatory, hepatoprotective, immunomodulatory, and neuroprotective activities [5]. Aforesaid pharmacological activities of these plants are due to the presence of certain pharmacologically important bioactive compounds, including carbazole alkaloids, coumarins, osthol, mukonal, heptazoline, clausine, clauszoline, and glycosinine [17]. Important essential oils are also present in a few plants belonging to this genus, including *C. dunniana, C. emarginata, C. heptaphylla,* and *C. lansium,* [18,19]. The major chemical components of essential oil differ from one species to another. For example, Estrgole (99.8%) in *C. dunniana*; α-Zingiberene (32.7%), Myristicin (15.7%), and ar-Curcumene (5.7%) in *C. emarginata*; *trans*-Anethole (88.59%) in *C. heptaphylla*; α-Santalol (31.7%), α-Santalene (19.5%), β-Caryophyllene (8.3%), Nerolidol (6.0%), α-Bergamotene (5.0%) in *C. lansium* essential oil [18,19]. The essential oil also possesses various pharmacological activities, such as antimicrobial, anticancer, antidiabetic, anti-inflammatory, neuroprotective, antioxidant, and antiprotozoal [3,4,19].

Among the various species of the genus *Clausena*, the species *C. excavata* is an evergreen perennial shrub. It is commonly known as Pink Lime-Berry, Cama, Cemama, Cemamar, Cerek Hitam [3]. This plant is extensively distributed in a number of countries, including India, Myanmar, China, Taiwan, Thailand, Malaysia, Singapore, Phillippines, Indonesia, and Brunei [4,20]. In India, it is found in Odisha, Andhra Pradesh, Kerala, Tamil Nadu, Mizoram, and Manipur [21,22] (https://sites.google.com/site/efloraofindia/species/m---z/r/rutaceae/clausena/clausena-excavata).

C. excavata has been used in folk medicine for the treatment of various diseases including hepatitis, enteritis, asthma, dysentery, cold, malaria, cough, headache, rhinitis, and gastrointestinal problems [18,23,24]. The plant is also well known for its detoxification properties [4]. Root decoction of this plant is used for the treatment of bowel complaints and colic pain [25], while leaf decoction is given to women after childbirth to get rid of pain [4]. Further, dried root powder is used for the treatment of decayed teeth [26]. This plant has been documented to possess several pharmacological activities including anti-inflammatory, antimicrobial, anti-nociceptive, immunomodulatory, antioxidant, analgesic, anti-cancer, antimalarial, antiplatelet, and insecticidal [3,4,19]. These pharmacological activities are attributed to the presence of various secondary metabolites, including carbazole alkaloids, coumarins, and limonoids and essential oil, which are rich in β-caryophyllene, elixene, β-cubebene, aromadendrene, γ-muurolene, spathulenol, methoxyeugenol, safrole, terpinolene, and 3-carene [18,20]. Till date only few studies has been carried out on the phytochemical analysis of different plant part extracts of *C. excavata* and their clinical aspects. Similarly, reports on the

chemical composition of *C. excavata* essential oil and its pharmacological activities are scanty. The composition of secondary metabolites as well as essential oil of same plant species in different part of the world showed variation due to their genetic makeup, geographical location, age, growth conditions as well as climatic conditions [27]. In this context, the chemical composition of essential oil of *C. excavata* of Indian region and its therapeutic activities study is yet to be explored. Thus, the present study was envisaged for the analysis of the chemical composition of leaf essential oil and its pharmacological activities vis-à-vis methanol leaf extract of *C. excavata*.

Materials and methods

Collection of plant materials

The fresh twigs (20-30 cm) with leaves of *C. excavata* were collected from Jeypore ghati (18º50′17.48″N lat., 82º36′53.55″E long., and 6081 ft alt), Koraput district, Odisha, India in January 2021. The identity of this plant was confirmed by Dr. C. Kalidass, Senior Scientist, Taxonomy and Conservation Division, Regional Plant Resource Centre (RPRC), Bhubaneswar, Odisha, India. The voucher specimen was deposited at the Herbarium of RPRC, Bhubaneswar, Odisha, India.

Essential oil extraction

Leaves were washed properly under running tap water followed by distilled water, sliced into small segments, and used for extraction of essential oil. The essential oil was extracted from the leaves sample using a 2 L capacity Clevenger's apparatus following the standard protocol described by Guenther [28]. Following extraction, the essential oil was collected and treated with Na_2SO_4 to reduce its moisture content. The essential oil was then kept in the refrigerator at 4 °C for further use.

GC/MS analysis of essential oil

The chemical constituent of *C. excavata* leaf essential oil was analyzed by Gas Chromatography/ Mass Spectroscopy (GC/MS). About 1 µL of dilute essential oil (oil: hexane; 1:10) was used for this study. A 7980A gas chromatography system (Agilent Technologies, USA) in conjunction with an HP5-MS column (dimension 30 m × 320 µm × 0.25 µm) was used to identify the ingredients of essential oil. The program conditions are as follows: a two-minute initial hold at 40°C, a gradual increase in temperature from 40°C to 125°C with an increment of 4°C/min, a four-min hold at 125°C, an increase in temperature to 250°C with an increment of 3 °C/min, a three-min hold at 250°C, and a final increase in temperature to 310°C with an increment of 10°C/min. At a transfer line and ion source temperature of 250°C, a quadrupole temperature of 150°C, an ionization potential of 70 eV, and a scan range of 50 to 550 atomic mass units (amu), MS was performed. The carrier gas was helium with a split ratio of 10:1 and a flow rate of 1.5 mL/min. The MSD Chem-station F.01.01.2317 software (Agilent Technologies, USA) was used to process the data. Retention Index (RI) was calculated using straight chain n-alkanes series C_8-C_{32} run on HP5-MS column under the same GC conditions as that of the essential oil. Compound identification was carried out using the NIST 2011/2017 mass spectral library and further confirmed by comparing their retention indices (RI) value obtained from the experiment with published literature using the same column.

Phytochemical analysis

C. excavata leaf samples were washed properly under running tap water followed by distilled water. The leaves were cut into small pieces and dehydrated under a tray drier at 40°C to get a constant weight. The dried leaf samples were crushed, and stored in an airtight container at 4°C for phytochemical studies and extract preparation. The quantitative phytochemical analysis of leaves in terms of tannins, saponins, phenolic, alkaloids, and flavonoids was estimated using the standard procedure described by Behera *et al*.[29] and Sutar *et al.*[30].

Extract preparation

For the study of therapeutic activities (i.e., antioxidant, antidiabetic, anticancer, and anti-microbial) of *C. excavata,* the leaf powder was taken for extract preparation in a methanol solvent system using the standard protocol

described by Naik *et al*.[31]. Briefly, the leaf powder sample and solvent (1:10) were used for the preparation of the extract. The Advanced Micro Wave Digestion System (Milestone, Italy) was used to prepare the extracts. The conditions used for extraction were 40°C for 1 h with a ramping time of 15 min. After completion of the extraction process, the extracts were collected, filtered, dried till achieved a constant weight, and finally stored in the refrigerator at 4ºC for therapeutic activities study.

Antioxidant assay

DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging activity

The free radical scavenging activity of *C. excavata* leaf essential oil and methanol extract was assessed by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay following the protocol as reported earlier by Behera *et al* [27]. Six different concentrations (10-100 µg/mL) of essential oils and extract samples were prepared by diluting them in methanol. Then 1 mL of each concentration of the samples (essential oil and methanol extract) was properly mixed with 1 mL of DPPH solution (0.15 mM in methanol). About 1 mL of DPPH solution was properly mixed with 1 mL methanol and considered as a control. These mixture samples were incubated at room temperature for 30 min in the dark. The absorbance was measured at 517 nm. In this study, ascorbic acid and α-tocopherol were used as positive control and methanol was used as blank. DPPH scavenging activity of *C. excavata* leaf essential oil and methanol extract was calculated using the following equation:

DPPH scavenging activity (%) = [(control absorbance – sample absorbance) / (control absorbance)] × 100

ABTS [2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] scavenging activity

Free radical scavenging activity of *C. excavata* leaf essential oil and methanol extract was also analyzed using 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\cdot+}$) decolorization assay following the standard protocol reported by Begum *et al*.[32]. About 17.2 mg of ABTS was dissolved in 5 mL water and $ABTS^{\cdot+}$ radical cations were generated after mixing the stock solution with 3.3 mg $K_2S_2O_8$. The solution was incubated at room temperature for 16 h in the dark. Then 1 mL of $ABTS^{\cdot+}$ solution was diluted with 60 mL distilled water to attain an absorbance of 1 ± 0.2 units at 732 nm. Diluted $ABTS^{\cdot+}$ solution (2.5 mL) mixed with 50 µL of sample and left at room temperature for 10 min. Then the absorbance was measured at 732 nm. About 50 µL of methanol mixed with 2.5 mL diluted $ABTS^{\cdot+}$ solution instead of the extract was used as a reference control and ascorbic acid and α-tocopherol were used as positive control. ABTS scavenging activity of *C. excavata* leaf essential oil and methanol extract was calculated using the following equation:

ABTS scavenging activity (%) = [(control absorbance – sample absorbance) / (control absorbance)] × 100

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) assay was carried out using the standard protocol described by Berker *et al*.[33]. Different concentrations of the sample (10-100 µg/mL) were prepared by diluting it with methanol. About 200 µL of tested samples were mixed with 1.8 mL FRAP reagent and then incubated in a water bath at 37°C for 10 min. The FRAP reagent contains 20 mM of 2, 4, 6-tri(2-pyridial)-S-triazine (TPTZ) solution, 20 mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer with pH 3.6. After incubation, the absorption was measured immediately at 595 nm. The above mixture without the plant extract was considered as a control.

Antidiabetic activity assay

The antidiabetic activity of *C. excavata* leaf essential oil and methanol extract was evaluated in terms of α-amylase inhibitory activities using the standard protocol described by Xiao *et al*.[34] with minor modification. Five different concentrations (0.1-0.8 mg/mL) of samples (both essential oil and methanol extract separately) and standard solutions were prepared for this experiment. The reaction mixture was prepared

by adding 100 µL of sample mixed with 900 µL of 10 mM sodium phosphate buffer (pH 6.9) and 6 mM NaCl having α-amylase solution 0.5 mg/mL. Distilled water was used as blank and acarbose was used as standard. The reaction mixture was left at 37ºC for 10 min. The hydrolysis reaction was initiated by adding 500 µL of a 1% starch solution in 20 mM sodium phosphate buffer (pH 6.9) with 6 mM NaCl in each tube and incubated at 37ºC for 10 min. Then 1 mL of 10% HCl, was added, followed by 300 µL of iodine solution. Finally, the reaction mixture was diluted by adding 10 mL of distilled water and absorbance was measured at 620 nm. α-amylase inhibitory activity of *C. excavata* leaf essential oil and methanol extract was calculated using the following equation:

$$I\ (\%) = (1\text{-}Abs_s\text{-}Abs_b\ /\ Absc^+ - Absc^-) \times 100$$

where, I(%):- the inhibition percentage; Absc+:- 100% enzyme activity with only solvent and the enzyme; Absc:- 0% enzyme activity with solvent in the absence of the enzyme; Abs_s:- test sample with α-amylase; Abs_b:- a blank a test sample without α-amylase.

Anticancer activity

Cell viability assay

The anticancer activity of essential oil and methanol extract of *C. excavata* leaf was determined in terms of cell viability assay and apoptosis assay. In this study, four different cancer cell lines, such as metastatic prostate cancer cell line (PC-3), ovarian cancer cell line (OVCAR-3), lung cancer cell line (h-1299), and breast cancer cell (MCF-7) were used to estimate the antiproliferative activity of *C. excavate* leaf essential oil and methanol extract. These cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. The cell viability assay was carried out following the standard procedure described earlier by Meher *et al*.[35] and Behera *et al*.[27] using different concentrations (10-100 µg/mL) of essential oil and leaf methanol extract of *C. excavata*.

Apoptosis assay by using Acridine Orange-Ethidium Bromide (AO/EtBr) staining

PC-3 cancer cells were cultured in culture plates and treated with leaf essential oil and methanol extract of *C. excavata* at IC_{50} concentration for 72 h to evaluate apoptotic cells. After 72 h incubation, coverslips were fixed in cold methanol and washed with phosphate-buffered saline (PBS). Subsequently, it was stained with Acridine orange and Ethidium bromide (AO/EtBr) stain and mounted on slides. The apoptotic cells were characterized using fluorescence microscopy (Nikon Eclipse Ts2R-FL) based on morphological changes in comparison to untreated cells as control.

Antimicrobial activity

To check the antimicrobial activity of essential oil and methanol extract, derived from the leaf sample of *C. excavate*, seven different strains of bacteria were employed. Of the seven bacterial strains, three were Gram +ve [*Bacillus subtilis* (MTCC 1133), *Streptococcus mutans* (MTCC 497), and *Streptococcus pyogenes* (MTCC 1926)] and four were Gram -ve bacterial strains [*Escherichia coli* (MTCC 40), *Salmonella typhii* (MTCC 1252), *Shigella flexneri* (MTCC 1457), and *Vibrio cholerae* (MTCC 0139)]. The bacterial strains used in this study were procured from MTCC, IMTECH, Chandigarh, India. The pure culture of all bacterial strains was maintained on a Nutrient Agar medium in a refrigerator at 4ºC. These bacterial strains were sub-cultured at regular intervals to sustain their viability. The antimicrobial activity study was performed using the agar well diffusion method following protocol as reported by Moharana *et al*.[36]. In this experiment, about 10 µL/well (5 mg/mL) of leaf essential oil, 5 mg/well of leaf methanol extract, and 10 µg/well of kanamycin were used separately in each Petri dish. After the treatment of individual bacterial strains in Petri dishes with essential oil, methanol extract as well as kanamycin, as mentioned above, the Petri dishes were incubated at 37ºC for 24 h. Finally, the diameter of the zone of inhibition was recorded. The negative control in this experiment consisted of Petri dishes that lacked both samples and antibiotics and the petri dishes containing kanamycin were considered as a positive control.

Data analysis

In this study, all experiments were repeated three times. Phytochemical analysis, and antioxidant, anticancer, & antimicrobial activity data were analysed using Analysis of Variance for a Completely Randomized Design and Duncan's new multiple range test [37] identified the significant difference among the mean values. Essential oil composition and antidiabetic activity data were represented in mean with standard deviation (SD).

Results and discussion

Chemical composition of essential oil

The essential oil of *C. excavata* leaf was extracted by hydro-distillation process. The yield of essential oil was found to be 1.3% (v/w), which was yellow in colour. The chemical composition of essential oil was analyzed by GC/MS and compounds were identified through NIST 2011/2017 mass spectral library. A total of thirty-seven compounds were identified in this study, corresponding to 92.88% of the total essential oil area percentage (**Table 1**). While Cheng *et al*.[20] identified a total of fourteen compounds with an area percentage of 99.22% of reported compounds in *C. excavata* leaf essential oil while working with plants of Taiwan. Similarly, a total of twenty-four compounds have been identified from the plants grown in China, corresponding to 73.2% of the area percentage by Guo *et al.*[18]. In this study, we found that myristicin, terpinolene, and β-phellanrene as three major compounds present in *C. excavata* leaf essential oil (**Table 1**). Out of thirty-seven identified compounds, myristicin (33.92%) was found to be a major compound, followed by terpinolene (21.29%) as the second major compound and sabinene (13.65%) as the third major compound in *C. excavata* leaf essential oil. Whereas the other minor components present in the essential oil of *C. excavata* leaf include germacrene D (3.24%), (-)-terpinen-4-ol (2.72%), 3-carene (2.0%), β-myrcene (1.96%), γ-elemene (1.92%), γ-terpinene (1.5%), (-)-spathulenol (1.27%), and β-ocimene (1.16%) (Table 1). In contrast, Cheng *et al*.[20] reported that safrole (75.85%), terpinolene (17.86%), and 3-carene (2.68%) as three major components of *C. excavata* leaf essential oil of Taiwan. But, the essential oil from the leaf of *C. excavata* grown in Vietnam has β-caryophyllene (25.3%), germacrene B (11.8%), and β-phellandrene (9.2%) as predominant components [38]. Recently, Guo *et al.*[18] reported that β-caryophyllene (30.7%) and elixene (9.7%) as two major compounds in the essential oil from the leaf of *C. excavata* grown in China. Thus, the major component of essential oil from the plant of Vietnam and China is β-caryophyllene as reported by Leclercq *et al*.[38] and Guo *et al*.[18] respectively in contrast to plants growing in Odisha, India where myristicin was found as the major component, as recorded in this study. However, safrole was recorded to be the major constituent of essential oil from the reported plant in Taiwan by Cheng *et al*.[20]. Furthermore, based on chemical classes, all the identified compounds were classified into different groups, such as monoterpenoid, sesquiterpenoid, phenylpropenoid, methoxyphenol, and others. Consistent with the abundance of compounds, monoterpenoid (49.18%) was the largest group among all the identified compounds followed by phenylpropanoid (34.22%) and sesquiterpenoid (6.73%). In contrast to our results, Cheng *et al*.[20] reported that phenolics compounds were at the highest concentrations (75.85%) followed by monoterpenoids (22.24%) present in the essential oil of *C. excavata* leaf. After considering the above studies, it was concluded that the compositional difference among essential oil of *C. excavata* grown in different countries is due to several factors, such as their genetic makeup, geographical location, age of the plants, growth conditions as well as climatic conditions [27].

Phytochemical analysis

Phytochemical constituents such as tannins, saponins, phenolics, flavonoids, alkaloids, etc. are playing key roles in the pharmacological activities of medicinal plants [31]. According to an ethnomedicinal survey, *C. excavata* has possessed several medicinal and pharmacological activities [3,4]. Thus, attempts have been made to check the phytochemical profiling in terms of tannins, saponins, flavonoids, phenolics, and alkaloids

Table 1. Chemical composition analysis of *C. excavata* leaf essential oil

No.	RT	$RI_{calculated}$	$RI_{literature}$	Compounds	Area (%)
1	13.569	921	921	α-Thujene	0.16±0.005
2	13.873	926	934	(-)-α-Pinene	0.42±0.016
3	15.857	958	961	Sabinene	13.65±0.223
4	16.696	971	981	β-Myrcene	1.96±0.078
5	17.335	981	996	α-Phellandrene	0.52±0.016
6	17.625	986	1005	3-Carene	2.00±0.036
7	18.353	997	1010	p-Cymene	2.22±0.017
8	19.036	1013	1032	*trans*-β-Ocimene	0.65±0.016
9	19.556	1027	1037	β-Ocimene	1.16±0.036
10	20.084	1041	1042	γ-Terpinene	1.50±0.047
11	21.674	1083	1097	Terpinolene	21.29±0.363
12	22.038	1093	1099	*trans*-Sabinene hydrate	0.20±0.005
13	22.728	1108	1111	p-Mentha-1,3,8-triene	0.17±0.004
14	23.575	1125	1131	*neo-allo*-Ocimene	0.59±0.008
15	25.982	1173	1175	(-)-Terpinen-4-ol	2.72±0.032
16	26.317	1180	1193	p-Cymen-8-ol	0.42±0.009
17	26.606	1186	1200	α-Terpineol	0.09±0.005
18	27.416	1202	1204	*cis*-Piperitol	0.05±0.004
19	32.497	1306	1315	p-Vinylguaiacol	0.18±0.014
20	36.553	1380	1382	(-)-β-Bourbonene	0.06±0.004
21	36.902	1386	1389	β-Elemene, (-)-	0.33±0.007
22	37.460	1396	1399	Methyleugenol	0.16±0.005
23	38.329	1414	1406	β-Ylangene	0.68±0.008
24	38.841	1424	1428	β-Copaene	0.34±0.012
25	39.012	1428	1431	γ-Elemene	1.92±0.013
26	40.416	1456	1440	Aromandendrene	0.08±0.000
27	40.528	1459	1469	(+)-*epi*-Bicyclosesquiphellandrene	0.07±0.000
28	41.174	1472	1474	γ-Muurolene	0.09±0.004
29	41.419	1477	1476	Germacrene D	3.24±0.063
30	43.499	1521	1509	Myristicin	33.92±0.518
31	44.487	1544	1537	Elemol	0.09±0.005
32	44.718	1549	1558	Elemicin	0.14±0.006
33	45.037	1556	1565	Nerolidol	0.05±0.004
34	45.772	1573	1582	(-)-Spathulenol	1.27±0.029
35	48.402	1636	1637	T-MuurolоI	0.19±0.005
36	48.922	1649	1650	α-Cadinol	0.33±0.014
37	50.185	1681	1681	Aromadendrene oxide-(1)	0.06±0.005
Monoterpenoid (Sl No. 1-13, 15-18)					49.18%
Sesquiterpenoid (Sl No. 20, 21, 23, 24, 26-29, 31, 33-36)					6.73%
Phenylpropanoid (Sl No. 22, 30, 32)					34.22%
Methoxyphenol (Sl No. 19)					0.18%
Others (Sl No. 14, 25, 37)					2.57%
Total area %					92.88%

RT: Retention Time; RI: Retention Index; All data represented in means with standard deviation (SD)

content of *C. excavata* leaf. In this study, it was observed that the phenolics content (165.23 mg GAE/g DW) was higher followed by alkaloids (89.30 mg/g DW) and saponin (72.11 mg/g DW) (**Table 2**). These compounds are responsible for several pharmacological activities including, but not limited to, free radical scavenging, anti-inflammatory, anticancer, antimicrobial, and antidiabetic activities [31,39]. This result is in agreement with an earlier study of Elumalai and Kasinathan [22] while working with *C. excavata* leaf extract from Tamil Nadu, India which was recorded as rich in phenolic compounds.

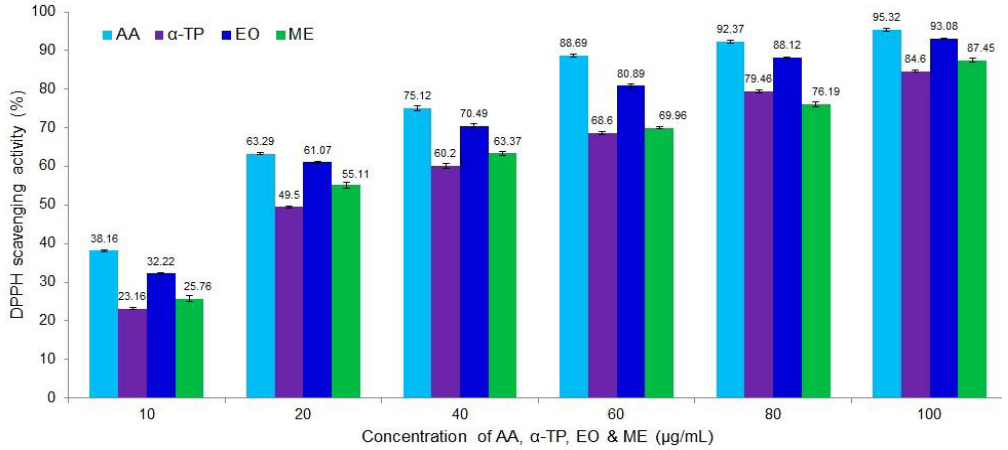
Antioxidant activity

Oxidative free radicals are dangerous byproducts of cellular oxidative phosphorylation and energy production. These radicals damage different intracellular macromolecules, such as DNA, protein, and lipids [24]. Antioxidants have the potential to prevent oxidative damage and inhibit inflammatory conditions by nullifying the activities of oxidative free radicals [27]. Medicinal plants are rich in antioxidant properties due to the presence of several phytochemical constituents. As mentioned earlier, *C. excavata* is a medicinal plant used in different traditional systems of medicine for the treatment of several illnesses. Therefore, the antioxidant activity of *C. excavata* leaf essential oil and methanol extract was evaluated using DPPH, ABTS, and FRAP assay. With increasing sample concentration, the scavenging activity steadily increased in all three assays. Leaf essential oil showed higher DPPH scavenging activity compared to leaf methanol extract and less activity compared to standard (ascorbic acid) but higher activity compared to the other standard (α-tocopherol) (**Fig. 1A**). ABTS and FRAP reduced the activity of leaf essential oil was higher in comparison to both the methanol extract (**Fig. 1B & C**). The IC_{50} values were 7.26, 33.59, 15.92, and 29.65 µg/mL for ascorbic acid (standard), α-tocopherol (standard), essential oil, and methanol extract, respectively for DPPH scavenging activity. For ABTS, the IC_{50} values were 54.3, 61.48, 41.85, and 50.13 µg/mL for ascorbic acid (standard), α-tocopherol (standard), essential oil, and methanol extract respectively. Similarly, for FRAP, the IC_{50} values were recorded as 45.75, 56.38, 39.9, and 50.56 µg/mL for ascorbic acid (standard), α-tocopherol (standard), essential oil, and methanol extract, respectively (**Table 3**). The antioxidant activity of essential oil was higher in comparison to extract may be due to the presence of a mixture of compounds in its essential oil which is rich in myristicin, terpinolene, and sabinene. Recently, Athipornchai *et al* [40] also reported the antioxidant activity of *C. excavata* essential oil against DPPH at IC_{50} value of 0.631 mg/mL. Whereas, Elumalai and Kasinathan [22] reported that *C. excavata* leaf methanol extract possesses stronger antioxidant activity than hexane and ethyl acetate extract.

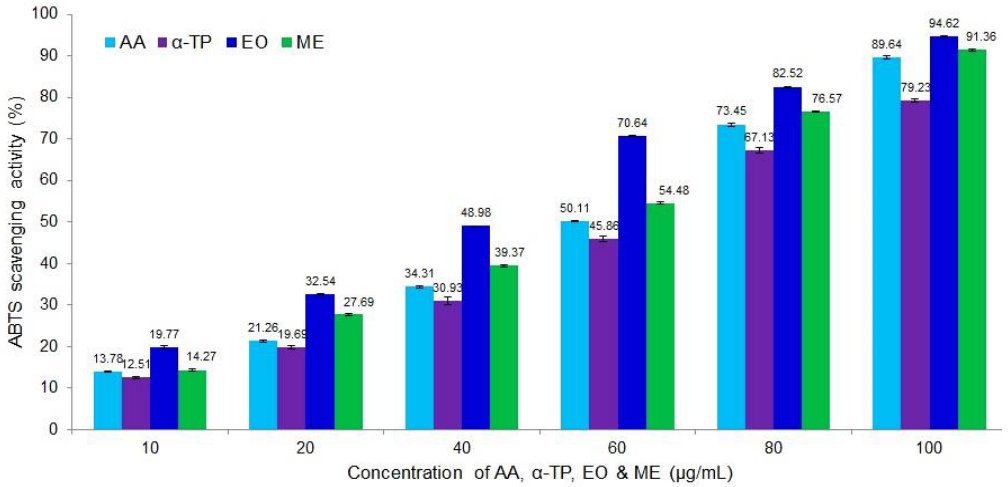
Table 2. Quantitative analysis of phytochemical constituents of *C. excavata* leaf methanol extract

Phytochemicals	Leaf sample
Alkaloids (mg/g DW)	89.30±5.10^{b}
Tannins (mg TAE/g DW)	64.45±3.31^{d}
Saponins (mg/g DW)	72.11±4.50^{c}
Flavonoids (mg/g DW)	43.4±3.56^{e}
Phenolics (mg GAE/g DW)	165.23±5.80^{a}

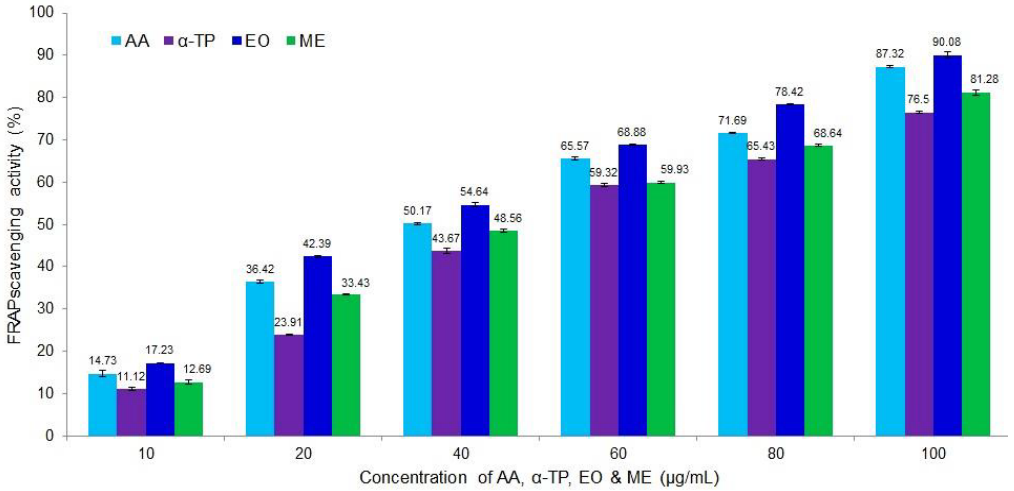
All data are represented in means with standard deviation (SD).
In a column, different letters in superscripts specify statistically significant differences between the means ($P \leq 0.05$; Duncan's new multiple range test)
DW: dry weight, GAE: gallic acid equivalent, TAE: tannic acid equivalent



(A) DPPH scavenging activity of *C. excavata* leaf essential oil and methanol extract



(B) ABTS scavenging activity of *C. excavata* leaf essential oil and methanol extract



(C) FRAP scavenging activity of *C. excavata* leaf essential oil and methanol extract

Figure 1 Antioxidant activity of *C. excavata* leaf essential oil and methanol extract. All data represented in means ± standard deviation
AA: Ascorbic acid, α-TP: α-tocopherol, EO: Essential oil, ME: Methanol extract

Table 3. Antioxidant activity of *C. excavata* leaf essential oil and methanol extract

Sample/standard	DPPH scavenging activity (IC_{50} in μg/mL)	ABTS scavenging activity (IC_{50} in μg/mL)	FRAP scavenging activity (IC_{50} in μg/mL)
Essential oil	15.92±0.34^{c}	41.85±0.45^{d}	39.9±0.60^{d}
Methanol extract	19.65±0.22^{b}	50.13±0.50^{c}	50.56±0.44^{b}
Ascorbic acid	7.27±0.41^{d}	54.30±0.21^{b}	47.75±0.53^{c}
α-tocopherol	33.59±0.71^{a}	61.48±0.57^{a}	56.38±0.37^{a}

All data represented in means with standard deviation (SD).
In each column, different letters in superscripts specify statistically significant difference between the means (P d ≤ 0.05; Duncan's new multiple range test)
IC_{50}: 50% of Inhibition concentration; DPPH : 2, 2-diphenyl-1-picrylhydrazyl; ABTS **:** 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP : Ferric reducing antioxidant power

Antidiabetic activity

Diabetes mellitus is commonly known as type 2 diabetes, which is a major and growing health issue worldwide due to peripheral insulin resistance and impaired insulin secretion [41]. Several therapies are available for treating diabetes, such as insulin and a number of oral antidiabetic drugs such as sulfonylureas, metformin, α-glucosidase inhibitors, etc. These oral antidiabetic drugs have various side effects [42]. Thus, there is an urgent need for a better therapeutic agent with a lower side effect. Medicinal plants are good sources of drugs; recently, a number of researchers have documented the antidiabetic activity of medicinal plants, including *Achyranthes aspera* [43], *Clausena cambodiana* [44], *Clausena indica*, *Znthoxylum rhetsa*, *Michelia tonkinensis* [45], and *Pogostemon cablin* [47]. A few studies have been documented about the antidiabetic activity of *C. excavata* in Myanmar [47] and also in Thailand [40]. In this study, we, first time reported antidiabetic activity, in terms of α-amylase inhibitory capacity, of leaf essential oil and methanol extract of *C. excavata* of India. Essential oil and methanol extract were used to evaluate their antidiabetic activity, which revealed that essential oil has strong α-amylase inhibitory effects in comparison to leaf extract and less than standard acarbose (**Fig. 2**). The IC_{50} values were 71.9, 97.50, and 133.79 μg/mL for acarbose (standard), essential oil, and methanol extract, respectively. Recently Quan *et al.*[45] reported that the myristicin of essential oil has possessed a major role in inhibiting α-amylase activity. In humans, α-amylase is a key enzyme, which hydrolyses polysaccharides into smaller glucose molecules. These glucose molecules are directly flowing into the bloodstream and cause postprandial hyperglycemia. In this study, we suggested that *C. excavata* essential oil is promising for preventing type 2 diabetes.

Anticancer activity

Natural plant products are playing a key role in cancer prevention and treatment without any side effects. To date, several herbal drugs or their derivatives, such as taxol, analogs docetaxel, cabazitaxel, vincristine, vinblastine, eldisine, navelbine, etc have been used for the treatment of cancer [48-52]. Furthermore, out of the 175 molecules approved internationally from 1940 to 2014, about fifty percent of anticancer molecules were planted secondary metabolites and their derivatives [49]. Therefore, natural plant products are considered a major source of anticancer agents whose therapeutic action and clinical application have proven successful in cancer treatment [50]. Several studies have documented on anticancer activity of essential oil against different cell lines, such as hepatocellular carcinoma, triple-negative breast cancer, acute myeloid leukemia, human colon cancer, ovarian adenocarcinoma cell, and lung carcinoma COR-L23, etc. [48,49,53].

To date, there are no reports on the anticancer activity of *C. excavata* essential oil. Thus, in

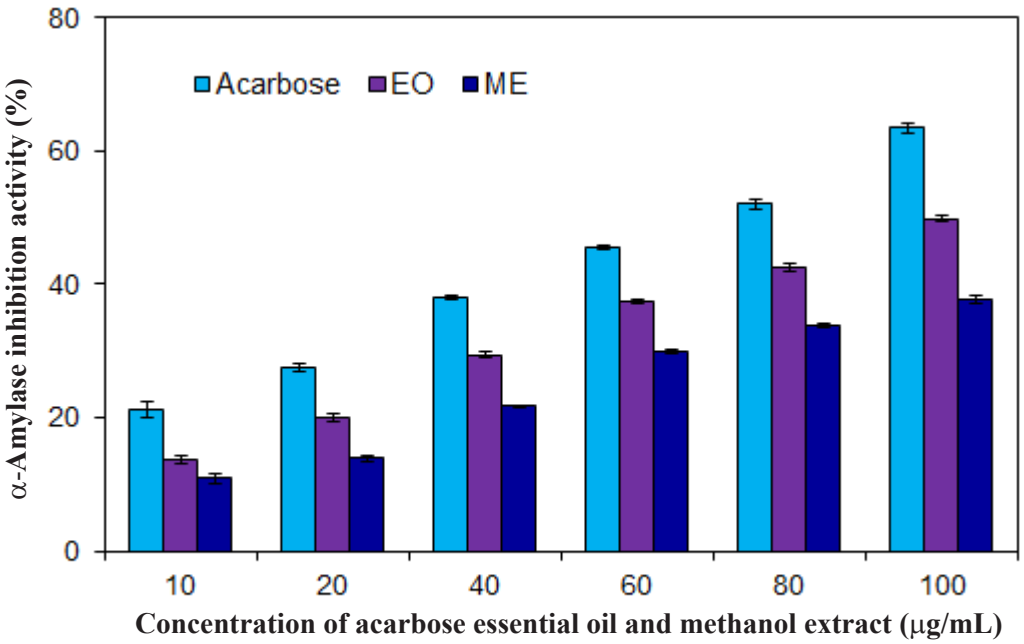


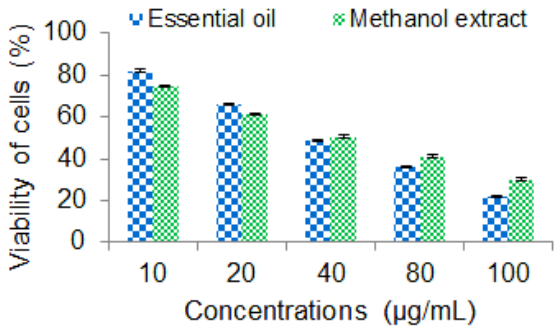
Figure 2. α-amylase inhibitory activity of *C. excavata* leaf essential oil and methanol extract
All data represented in means ± standard deviation. EO: Essential oil, ME: Methanol extract

the present study, we evaluated the anticancer activity in terms of cell viability of *C. excavata* leaf essential oil vis-à-vis methanol extract against four different cancer cell lines, such as PC-3, OVCAR-3, h-1299, and MCF-7 cells. A significant decrease in the rate of cell viability was noticed with treatment at increasing concentrations of leaf essential oil and methanol extract. The rate of cell viability (%) using leaf essential oil and methanol extract varied among the cell lines of different tissue origins (**Fig. 3A-D**). The IC_{50} values for the test samples (essential oil and methanol extract) for all the cell lines were collated in **Table 5**. The IC_{50} value amounted to 39.1, 55.7, 56.7, and 58.6 µg/mL for leaf essential oil against PC-3, OVCAR-3, MCF-7, and h-1299 cell line, respectively at 72 h of incubation. Parenthetically, a similar modest IC_{50} value of 43.7, 47.5, 48.1, and 45.9 µg/mL was found for methanol leaf extract against PC-3, OVCAR-3, MCF-7, and h-1299 cell line, respectively at 72 h of incubation (**Table 4**). Subsequently, the induction of apoptotic cell death to metastatic prostate cancer cell line, PC-3 with the treatment of IC_{50} concentration of leaf essential oils and methanol extracts of *C. excavata* was investigated by using merge dye (Acridine orange-Ethidium bromide; AO / EtBr). The apoptotic cell death of PC-3 cancer cells was identified based on morphological changes,

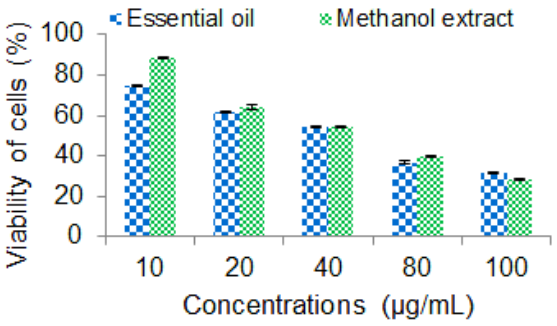
Table 4. Evaluation of anticancer activity of *C. excavata* leaf essential oil and methanol extract

Sample	IC_{50} values of cell line			
	PC-3	OVCAR-3	MCF-7	h-1299
Essential oil	39.1±1.2^{b}	55.7±1.2^{a}	56.7±1.2^{a}	58.6±1.2^{a}
Methanol extract	43.7±1.2^{a}	47.5±1.2$_{b}$	48.1±1.2^{b}	45.9±1.2^{b}

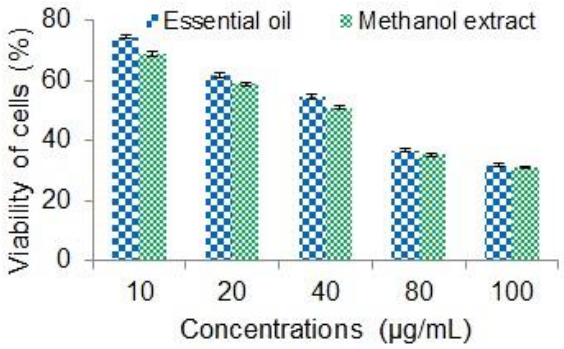
All data represented in means with standard deviation (SD).
In each column, different letters in superscripts specify statistically significant difference between the means (P d ≤ 0.05; Duncan's new multiple range test)
IC_{50}: 50% of Inhibition concentration



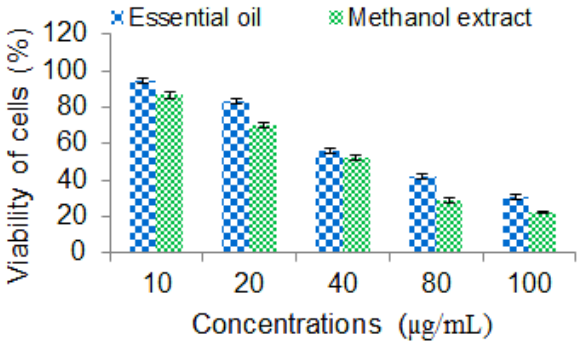
(A) PC3 treated with essential oil and methanol extract of *C. excavate* leaf



(B) MCF7 treated with essential oil and methanol extract of *C. excavate* leaf



(C) OVCAR3 treated with essential oil and methanol extract of *C. excavate* leaf



(D) h1299 treated with essential oil and methanol extract of *C. excavate* leaf

Figure 3. Anticancer activity of *C. excavata* leaf essential oil and methanol extract against four different cell lines, such as PC 3, MCF 7, OVCAR 3, and h1299 cells
All data represented in means ± standard deviation

Table 5. Assessment of antimicrobial activity of leaf essential oil and methanol extract of *C. excavata*

Sample / antibiotic	Inhibition zone in diameter (mm)						
	Gram-positive bacteria				Gram-negative bacteria		
	B. subtilis **(MTCC 1133)**	***S. pyogenes*** **(MTCC 1926)**	***S. mutans*** **(MTCC 497)**	***S. typhii*** **(MTCC 1252)**	***S. flexneri*** **(MTCC 1457)**	***V. cholerae*** **(MTCC 0139)**	***E. coli*** **(MTCC 40)**
Methanol extract (5 mg/well)	19±0.56^{c}	16±1.5^{c}	18±0.7^{c}	20±0.5^{b}	20±1.8^{bc}	17±1.0^{bc}	20±0.7^{c}
Essential oil (10 µL/well; 5 mg/mL)	22±0.8^{b}	19±1.3^{b}	20±1.1^{b}	22±0.7^{a}	21±0.6^{b}	18±0.9^{b}	23±0.68^{b}
Kanamycin (10 µg/well)	25±1.2^{a}	24±0.6^{a}	22±1.1^{a}	19±0.5^{bc}	25±1.3^{a}	21±1.0^{a}	27±1.2^{a}

All data represented in means with standard deviation (SD)

In each column, different letters in superscripts specify statistically significant difference between the means (P d ≤ 0.05; Duncan's new multiple range test)

including condensed chromatic formation, membrane blebs, and numerous fragmented nuclei compared to untreated cells (**Fig. 4**).

Essential oil showed promising activity against the cancer cell lines probably due to the presence of a mixture of compounds i.e., myristicin, terpinolene, sabinene, germacrene D, (-)-terpinen-4-ol, 3-carene, β-myrcene, γ-elemene, γ-terpinene, (-)-spathulenol, p-cymene, β-ocimene, etc. The antiproliferative activity of myristicin has been studied recently and reported that myristicin is responsible for anticancer activity by inducing cell apoptosis through changes in mitochondrial membrane potential, cytochrome C release, caspase-3 activation, PARP cleavage, as well as DNA fragmentation [54]. While, terpinolene has also been attributed to anticancer properties by targeting the proto-oncogene tyrosine-protein kinase ROS, which plays a key role in signal transduction and cellular communication in cancer cells [55]. Fascinatingly, terpinolene increases the intracellular production of ROS in cancer cells, consequently increasing expression of apoptotic markers, such as BCL2-associated X protein (BAX), Poly ADP (Adenosine Diphosphate)-Ribose Polymerase (cleaved-PARP), and pro-caspase-8 without promoting genotoxic effects [56]. Thus, the essential oil derived from *C. excavata* leaf could be used in the development of commercial drugs for the treatment of cancer. The potential of plant extract of *C. excavata* exhibiting anticancer activity was reviewed and studied by various workers. They reported that the anticancer activity of the plant may due to the presence of certain pharmaceutically important secondary metabolites, such as clausine-B, carbazole alkaloids, 3-carbomethoxy-2-hydroxy-7-methoxycarbazole, clausine-TY [57,58]. Further, a number of reports on the anticancer activity of this plant extract and its secondary metabolites against several cancerous cell lines, including MDA-MB-231, HeLa, CAOV3, HepG2, A549, KB, and KB-VIN cells are also available [6,57,59-62]. In addition, Arbab *et al*.[60] tested different extracts, such as hexane, ethyl acetate, chloroform, and methanol extract of *C. excavata* stem bark and root against five

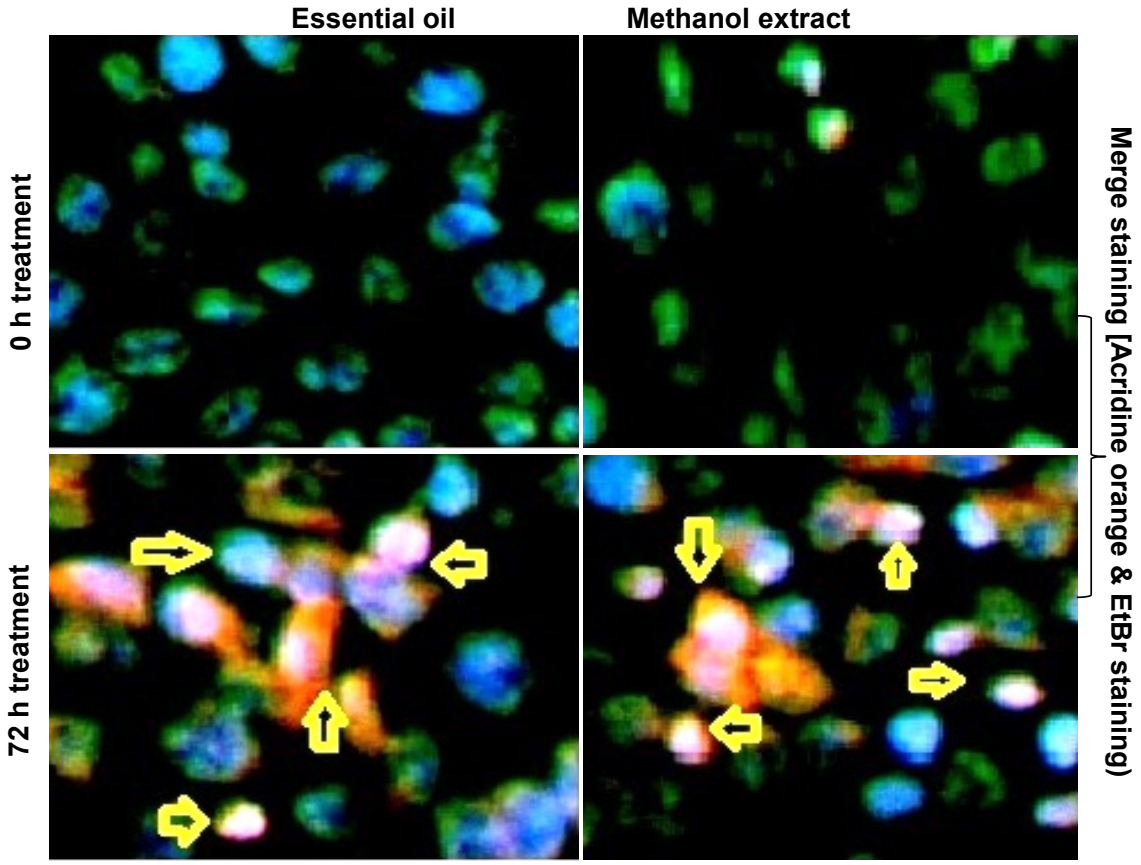


Figure. 4 Induction of apoptosis to PC 3 cancer cell line by *C. excavata* leaf essential oil and methanol extract, treated at IC_{50} concentration. The apoptotic cells are identified by changes in morphological characters i.e. chromatin condensation, blebbing of plasma membrane as well as apoptotic bodies (indicated by arrows). The changes in morphological characters are shown under fluorescence microscope after labeling with AO/EtBr staining. The untreated control cells were also included for the comparison

different cell lines i.e., HL-60, HT-29, HepG2, MCF-7, and HeLa cells and found that root extract was highly potent against all cell lines in comparison to stem bark. Recently, Albaayit *et al*.[57] investigated the anticancer activity of *C. excavata* leaf extracts (methanol, ethyl acetate, and chloroform) against the NCl-H460 cell line and it was shown that ethyl acetate extract has effective antiproliferative activity. Clausine K derived from the stem of *C. excavata* exhibited strong antiproliferative activity, which was better than etoposide against HepG2 [58]. Clausine B isolated from the stems and leaves of *C. excavata*, showed potent activity against HeLa, MDA-MB-231, HepG2, and CAOV3 cancer cell lines [63].

Antimicrobial activity

Since ancient times, essential oil has been used as therapeutic agent for the treatment of various diseases [64]. The essential oil has also been utilized in food and beverage industries as a flavoring agent and natural food-grade preservative agent [65]. It has played a key role in food preservation because of its antimicrobial activities [66]. The essential oil possesses antimicrobial activities against several pathogenic bacteria due to the presence of a complex mixture of different kinds of compounds, such as terpene, terpenoids, phenols, and aldehydes [67]. *C. excavata* is rich in terpenoids in comparison to other groups of compounds (**Table 1**). The antimicrobial activity

of leaf essential oil was evaluated for the first time against several pathogenic bacterial strains i.e., three Gram-positive bacteria strains (*B. subtilis*, *S. pyogenes*, and *S. mutans*) and four Gram-negative strains i.e., (*S. typhii*, *S. flexneri*, *V. cholerae*, and *E. coli*) in comparison to its leaf methanol extract. The antimicrobial activity was represented as an inhibition zone and kanamycin was used as a positive control in comparison to the inhibitory action of essential oil and methanol extract of *C. excavata* leaf. This study revealed that essential oil has more antimicrobial activity than methanol extract but less antimicrobial activity than kanamycin (control) in all the tested bacterial strains except *S. typhii* (**Table 5**). In the case of *S. typhii*, the leaf essential oil and methanol extract of *C. excavata* showed a higher inhibition zone than kanamycin. The results showed a promising zone of inhibition ranging from 18 to 23 mm and 16 to 20 mm for essential oil and methanol extract respectively. The antimicrobial activities of essential oil of this plant may be due to the presence of the highest percentage of terpenoids, such as myristicin, terpinolene, sabinene, p-cymene, 3-carene, germacrene D, terpinen-4-ol, and others compounds. Terpenoids are well-known for their antibacterial activities by disrupting the lipid assembly of the cell wall, leading to the disintegration of the cell membrane, denaturation of cell proteins, and leakage of cytoplasmic material, which in due course causes cell lysis and cell death. Terpenoids also inhibit bacterial growth by inferring oxidative phosphorylation or oxygen uptake in microbial cells [65,67]. Antimicrobial activities of essential oil and different solvent extract of *C. excavata* has also been reported by other workers. Jagadeesan and Elumalai [68] reported that *C. excavata* leaf methanol, hexane, and ethyl acetate extract showed promising inhibition against Gram-positive and Gram-negative bacteria. Thien *et al*.[69] reported that *C. excavata* leaf ethanol extract showed the best results compared to the other three Rutaceae species, such as *Acronychia pedunculata, Glycosmis pentaphylla,* and *Luvunga scandens* against six different pathogenic bacteria. Till now only a few reports documented the antimicrobial activities of essential oil of plants belonging to the Rutaceae family. Rahaman *et al*.[70] studied the antimicrobial activities of *Clausena suffruticosa* leaf essential oil and reported antimicrobial activity against five Gram-positive bacteria namely *B. subtilis*, *S. aureus*, *B. cereus*, *B. polymyxa*, and *B. megaterium* and four Gram-negative bacteria *S. typhi*, *S. flexneri*, *P. mirabilis*, and *E. coli*.

Conclusion

Clausena excavata is an important medicinal plant having immense industrial applications in the pharmaceutical sector. In the present report, the chemical composition of essential oil and the comparative therapeutic activities of leaf essential oil and methanol extract of *C. excavata* from the Eastern Ghats of India was reported. The essential oil of the plant is rich in monoterpenoids and the major compounds reported are myristicin (33.92%), terpinolene (21.29%), and sabinene (13.65%). Studies showed that the leaf methanol extract of *C. excavata* was rich in phenolic content (162.23 mg GAE/g DW) followed by alkaloid contents (89.30 mg/g DW). In this study, several biological properties including the antioxidant, antidiabetic, anticancer, and antimicrobial activity of leaf essential oil vis-à-vis methanol extract of *C. excavata* were examined. Leaf essential oil showed stronger biological activities than methanol extract. Hence, the essential oil derived from this plant could be useful as a therapeutic agent for the treatment of cancer, diabetes, and various microbial diseases. For this further research is required using animal models to authenticate the therapeutic activities of this important medicinal plant. Thus, this study could be helpful to explore the pharmaceutical activity of this plant and the development of new drug.

Author contributions

SB designed the study, carried out all experimental work, and wrote the manuscript. RKM helped the anti-cancer activity studies. SM helped in GC/MS data analysis to SKB. SKB and MN helped in the phytochemical analysis and antioxidant activity study. PKD field survey and collection of plant samples. KM prepared the

manuscript with SB. SKN and PKN edited the final manuscript. Finally, all authors approved the manuscript for publication.

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Conflicts of Interest

The authors declare no conflicts of interest.

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