Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating 2017: 3.53
JMPS 2017; 5(6): 126-129

Received: 25-09-2017
Accepted: 27-10-2017

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GC-MS analysis, antibacterial and antioxidant study of *Amomum subulatum* Roxb

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Abstract
Fruit part of *Amomum subulatum* Roxb. was subjected to extraction of essential oil by hydro distillation in Clevenger apparatus. The dried powdered fruit was subjected to extraction with methanol as well. The composition of essential oil so collected were determined by GC-MS system and showed the presence of 17 different compounds. The most abundant were *α*-Terpineol (27.85%), Terpine-4-ol (11.19%), Pinocarvone (8.02%), Nerolidol (6.90%) and Pinocarveol (6.32%). Antibacterial and antioxidant activity of the essential oil of *Amomum subulatum* Roxb. were studied. Essential oil exhibited moderate antibacterial activity. Plant extract also showed average DPPH antioxidant activity with IC_{50} 431.2 ± 21.3µg/mL.

Keywords: *Amomum subulatum*, Essential oil, GC-MS, Activity

Introduction
Amomum subulatum Roxb. belongs to the family Zingiberaceae with approximately 150 species [1]. *A. subulatum* is native to the Eastern Himalayas, commonly known as large cardamom and locally known as Alaichi in Nepal and India.[2,3] It is cultivated commercially in Nepal, India, China and Bhutan. Nepal, India and Bhutan are the largest producer of Alaichi.[4] It is cultivated in marginal, sloppy and degraded land about 700 to 2100m high at (4- 20)ºC temperature with (2000- 2500)mm rainfall. It requires sufficient water supply throughout the year however, water logging may damage the crop. Aster (*Alnus nepalensis*) is the most widely used and preferred shade trees for the large cardamom cultivation in Nepal [5].
The large cardamom is perennial herb with large, green, glaborous leaves and subterranean rhizomes. Leaves are oblong and lanceolate 30-60 cm in length. Rhizomes are creeping and branched. Inflorescence is spike. Flowers are yellow bisexual, zygomorphic & pollinated by bumble bees. Calyx and corolla tube segments are sub-obtuse. Seeds are nearly round, dark grey and 3mm in diameter. Capsules are 2-5cm long, irregular, dark red brown in colour containing several aromatic seeds [3, 6].
A. subulatum revealed the presence of carbohydrates, flavonoids, amino acids, steroids, triterpenoids, glycosides and tannins as chemical constituents [2]. Phyto constituent varies with region, variety and age. The major constituent of large cardamom is oleoresins and essential oil. Essential oil contain 1,8-cineole (65-80%), monoterpene (5-17%), while the content of terpenyl acetate is low. Seed contain cardamonin, alpinetin and glycosides [6, 7].
In Ayurveda and Unani, it is used for gastric ulcers and gastrointestinal disorders, as a liver tonic, hypnotic and appetiser, diuretic and as a treatment for migraine [1]. It is as an aromatic, cardiac, stomachic and carminative stimulant. It is also used as a flavouring agent in tea and food preparations. Cardamom oil is a precious ingredient in food preparations, perfumery and beverages [2-4, 7].
Plant extract shows analgesic, anti-inflammatory, antimicrobial, antioxidant, antiulcer, cardio-adaptogen, hypolipidaemic and inhibition against *Aspergillus flavus* activities. Dry seeds of this plant is used to cure dyspnea, cough, vomiting, nausea, itching and indigestion [6, 7].

Experimental
Collection of Plant Materials
The plant material (fruits) was collected from Kavre, Nepal. The plants were identified by Department of Botany, Amrit Campus, Lainchour, Kathmandu, Nepal.

Preparation of Plant Extracts

The clean and dried fruits were grinded to powder and further proceeded via cold percolation process for 7 days for three times with 2.5 liters methanol. The methanol extract was concentrated by evaporation on rotavapour. Plants extracts were stored at 4 °C.

Extraction of Essential Oil

The mature fruits of *A. subulatum* were crushed for hydro distillation and subjected to a Clevenger apparatus for three hours. By this process about 2ml of pale yellow coloured essential oils were collected and stored in a sealed glass vials at low temperature(0-4 ^{o}C) prior to analysis.

GC-MS Analysis

The essential oils sample of *A. subulatum* was subjected to GC-MS analysis. GC-MS analysis was performed on a gas chromatography mass spectrometer GCMS-QP2010 under the following condition: injection volume 1µL with split ratio 1:50; Helium as a carrier gas with a Rtx-5MS column of dimension 30m×0.25mm×0.25µm, temperature programmed at 40, 200 and 280 ^{o}C with a hold time of 2.0, 3.0 and 4.0 min identification was accompanied by comparison of MS with those reported in NIST 05 and FFNSCI.3 libraries. It was performed in Department of Food Technology and Quality Control, Nepal Government, Babarmahal, Kathmandu, Nepal.

Antioxidant Assay (DPPH method)

First of all, 1mg of sample to be tested was dissolved in 1ml methanol to get stock solution of concentration 1mg/ml. 100µl of these solutions were added to 100µl of 0.1mM DPPH (prepared in methanol) and was left for 30 minutes in dark room. After 30 minutes, their absorbance was taken at 517 nm against DPPH and DMSO as a blank. Quercetin was prepared as standard. The extracts or essential oil, which do not show antioxidant property was discarded and for the sample with the yellow colour (more than 50% inhibition then control) was taken for further testing as they were expected to be the potential antioxidants.

Different concentration of the extracts were prepared by two-fold dilution method to find the IC_{50} value.

Antibacterial activity assays

Antimicrobial assay of essential oil of plants was performed by agar well diffusion method in Muller Hilton Agar (MHA) and the minimum bactericidal concentration of those extract was determined by micro dilution method. All the strains of bacteria was cultured in Nutrient broth (NB) and incubated at 37 ^{0}C for 18 hours. After incubation each stain were diluted with sterile distilled water. The turbidity of dilution was compared with 0.5 McFarland standards (approximately 10^{8} CFU/ml). The suspensions were then diluted (1:100) in Muller Hilton Broth (MHB) to obtain 10^{6} CFU/ml. Prepared inoculums were incubated for 30 minutes at 37 ^{0}C prior to use.

Plant oil (30 µl) was loaded into the respective wells with the help of micropipette. The solvent (50% DMSO) was tested for its activity as a control at the same time in the separate well. The Neomycin 20 µg/ml was used as a positive control. The plates were then left for half an hour with the lid closed so that extracts diffused to the media. The plates were incubated overnight at 37 °C. After proper incubation (18-24 hours) the plates were observed for the zone of inhibition around well which is suggested by clean zone without growth. The ZOI were measured with the help of the ruler and mean was recorded for the estimation of potency of antibacterial substance.

Determination of Minimum Bactericidal Concentration

The Minimum Bactericidal Concentration (MBC) was determined by micro dilution method. The essential oil was diluted by two fold to get series of concentrations from 0.048 to 25 mg/ml in freshly prepared sterile nutrient broth. 20µl of the microorganism suspension (correspond to 10^{6} CFU/ml) was added to each of the sample dilutions. These were incubated for 18 hours at 37 °C and each tube content was subculture in fresh nutrient agar separately and minimum bactericidal concentration was determined that showed no growth at all.

Determination of the Minimum Inhibitory Concentration

The smallest amount of compounds required to kill or inhibit the growth of micro-organism *in vitro* can be determined by the dilution method. This amount is referred as minimum inhibitory concentration (MIC). It is a measure of potency which is expressed in terms of either µg or mg/ml. A stock solution of 25 mg/ml was prepared. This was serially diluted to obtain various ranges of concentrations between 25 mg/ml to 0.048 mg/ml.

Result and Discussion

GC-MS Analysis

GC-MS analysis of essential oils of fruits of *A. subulatum* shows the presence of 17 different compounds. The chemical compound identified in essential oils of the the fruits of the *A. subulatum* plant are presented below:

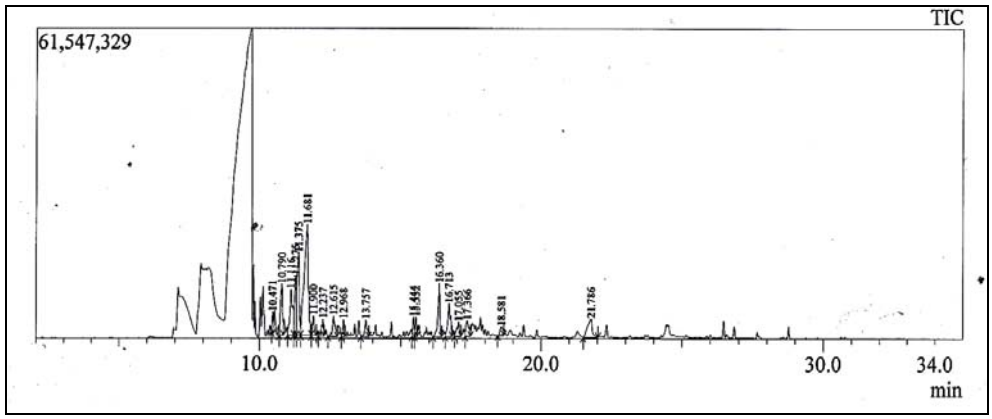

Fig 1: Chromatogram of essential oils of fruits of *A. subulatum*

The major constituents present in the essential oils sample were α-Terpineol (27.85%), Terpine-4-ol (11.19%), Pinocarvone (8.02%), Nerolidol (6.90%) and Pinocarveol (6.32%). Constituents of essential oils of *A.subulatum* are tabulated as follows.

Table 1: List of compounds in essential oils of *A. subulatum*.

S.N.	Name of the compounds	Molecular Formula	Molecular Weight	Retention Time	Area%	Height%
1.	4-Isopropyl-1-methyl-2-cyclohexen-1-ol	$C_{10}H_{18}O$	154	10.471	1.51	3.22
2.	Pinocarveol	$C_{10}H_{16}O$	152	10.790	6.32	7.89
3.	Pinocarvone	$C_{10}H_{14}O$	150	11.116	8.02	7.12
4.	α-Terpineol	$C_{10}H_{18}O$	154	11.275	6.27	9.22
5.	Terpine-4-ol	$C_{10}H_{18}O$	154	11.375	11.19	12.74
6.	α-Terpineol	$C_{10}H_{18}O$	154	11.681	27.85	17.12
7.	cis-Carveol	$C_{10}H_{16}O$	152	11.900	1.99	3.04
8.	Carvone	$C_{10}H_{14}O$	150	12.237	1.94	2.67
9.	Cinnamaldehyde	C_9H_8O	132	12.615	3.50	3.21
10.	Perilla alcohol	$C_{10}H_{16}O$	152	12.968	1.42	2.67
11.	Eugenol	$C_{10}H_{12}O_2$	164	13.757	2.39	2.69
12.	Germacrene	$C_{15}H_{24}$	204	15.444	1.61	3.03
13.	β-Selinene	$C_{15}H_{24}$	204	15.532	1.53	3.02
14.	Nerolidol	$C_{15}H_{26}O$	222	16.360	6.90	8.24
15.	Spathulenol	$C_{15}H_{24}O$	220	16.713	5.33	5.13
16.	Caryophyllene oxide	$C_{15}H_{24}O$	220	17.055	2.31	2.26
17.	Caryophyllene oxide	$C_{15}H_{24}O$	220	17.366	2.91	2.47
18.	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	18.581	1.52	1.37
19.	Palmitic acid	$C_{16}H_{32}O_2$	256	21.786	5.48	2.91
					100.00	100.00

Antibacterial activity

Table 2: Antibacterial activity of the *A. subulatum.*

Sample	MIC Values				MBC Values			
	EC	MM	MRSA	KP	EC	MM	MRSA	KP
Neomycin*(µg/ml)	1.25	5	0.156	0.0195	1.25	5	5	0.156
Oil(mg/ml)	6.25	12.5	3.125	6.25	12.5	50	12.5	25

*Control Antibiotics
EC = *Escheriachia coli* MDR
MM = *Morganella morganii* (MDR)
MRSA = Methicillin resistance *Staphalococcus aureus* (MRSA)
KP = *Klebsiella pneumoniae* (MDR)

Antioxidant Activity

The antioxidant potential is in an inverse relation with IC_{50} value, which can be calculated from linear regression of the% inhibition verses antioxidant activity. Lower the IC_{50} value indicates high antioxidant activity.

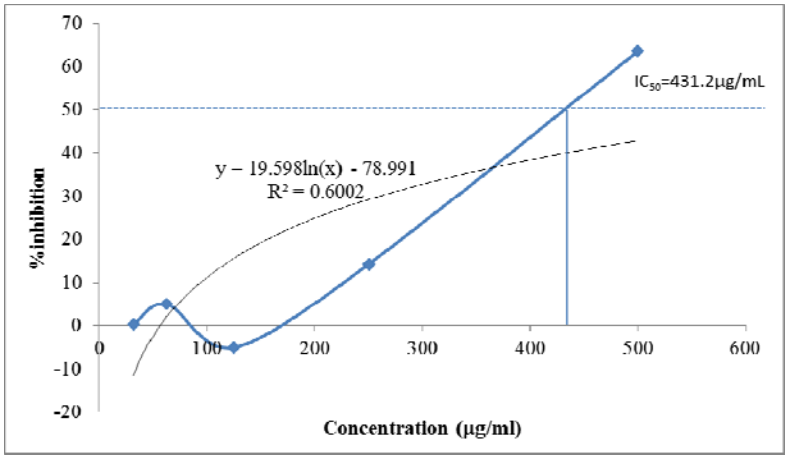

Fig 2: Graphical representation of DPPH assay of the methanolic extract of *A. subulatum.*

The IC_{50}±SEM of the oil was found to be 431.2 ± 21.3µg/mL and the standard, quercetin was 2.28±0.1µg/mL.

Conclusion

GC-MS analysis of essential oils of fruits of *Amomum subulatum* Roxb. showed the presence of 17 different compounds. The major contituents present in the essential oils sample were α-Terpineol (27.85%), Terpine-4-ol (11.19%), Pinocarvone (8.02%), Nerolidol (6.90%) and Pinocarveol (6.32%). Essential oils show antibacterial activity against

Escheriachia coli MDR, *Morganella morganii* (MDR), Methicillin resistance *Staphalococcus aureus* (MRSA) and *Klebsiella pneumoniae* (MDR). The IC_{50}±SEM of the essential oil was found to be 431.2 ± 21.3µg/mL.

Acknowledgement
I am thankful to Kathmandu Valley School and College, Chhauni, Kathmandu for providing the research lab, Dr. Bishnu Marasini and Ms. Shrimita Shrestha Department of Biotechnology, National College, Kathmandu for bioactivity test and Mr. Tayer Mohamad Miya, Department of Food Technology and Quality Control, Nepal Government for GC-MS analysis.

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