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Antibacterial, brine shrimp lethality assay and GC-MS analysis of *Piper longum* Linn

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Abstract
Fruit part of *Piper longum* Linn. was subjected to extraction of essential oil by hydro distillation in Clevenger apparatus. Methanol was used to extract the dried and powdered plant. 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 germacrene (25.04%), heptadecene (11.71%), heptadecane (9.23%), β-bisabolene (8.36%) and caryophyllene (7.71%). Antibacterial activity of the essential oil of *P. longum* was studied. Oil exhibited moderate antibacterial activity against *Klebsiella pneumoniae*. The LD_{50} of the sample was found to be 251.1µg/mL.

Keywords: *Piper longum* Linn, essential oil, extract, GC-MS, activity

Introduction
Piper longum Linn. is popularly known as "long pepper", belongs to family Piperaceae [1]. It is called Pipla in Nepali. It is a native plant of Himalaya (Nepal to Bhutan), India, Sri Lanka, Malaysia and Indonesia [2]. *P. longum* is a slender, climbing, under shrub, creeping and rooting below. The young shoots are downy; the leaves are wide, ovate, cordate with broad rounded lobes at the base. The plant bears unisexual flowers and berries types fruits [3]. Flowers are yellow, in elongate spikes and the fruits are small, ovoid berries, shiny blackish green, embedded in fleshy spikes [4].
Phytoconstituents of *P. longum* fruits include volatile oil, starch, resin and other minor alkaloids such as piplartin, piperlogumine, piperidine, and pungent alkaloid piperine [5]. The volatile oil constituent of plant includes phyllandrene, caryophyllene, 3-carene, D-limonene, β-pinene and α-phellandrene [6, 7].
Roots are bitter, thermogenic, tonic, diuretic, purgative, expectorant, anthelmintic. Plant is useful for respiratory diseases, epilepsy, gonorrhea, cough, indigestion, disorders and chronic fever [2]. Fruits extract of *P. longum* shows antidepressant, antinociceptive, antiinflammatory, anticancer, antidiabetic, antibacterial, antifungal, antitumor, antiallergic, antiasthmatic, antifertility, antiulcer, antihypertensive, antiplatelet, antithyroid, antiamoebic, hepatoprotective, vasodilating and insecticidal activities [4, 5]. *P. longum* being used in traditional medicine system. Piperine enhance the bioavailability of several drugs, such as sulfadiazine, tetracycline, streptomycin, rifampicin, pyrazinamide, isoniazid, ethambutol and phenytoin [6].

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

Preparation of Plant Extract
The clean and dried fruits were grinded to powder and further proceeded via cold percolation process for 7 days for three times with 3 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 *P. longum* were crushed for hydro distillation and subjected to a Clevenger apparatus for three hours. By this process about 2 ml of pale yellow coloured essential oils were collected and stored in a sealed glass vials at low temperature (0-4°C) prior to analysis.

GC-MS Analysis

The essential oils sample of *P. longum* 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 280ºC 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.

Antibacterial activity assays

Antimicrobial assay of extracts 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 extracts (30 µl) were 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 methanol extracts were 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.

Brine Shrimp Lethality Assay

Ten nauplii were exposed to each of different concentrations of the plant extract and number of survivors were calculated the percentage of death after 24 hours.

Result and Discussion

GC-MS Analysis

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

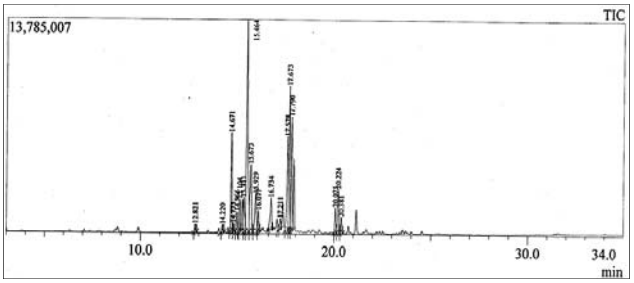

Fig 1: Chromatogram of essential oils of fruits of *P. longum*

The major contituents present in the essential oils sample were germacrene (25.04%), heptadecene (11.71%), heptadecane (9.23%), *β*-bisabolene (8.36%) and caryophyllene (7.71%). Constituents of essential oils of *P. longum* are tabulated as follows.

Table 1: List of compounds in essential oil of *P. longum*

S.No	Name of the compounds	Molecular Formula	Molecular Weight	Retention Time	Area %	Height %
1.	Tetradecane	$C_{14}H_{30}$	198	12.821	0.66	0.80
2.	*β*-Elemene	$C_{15}H_{24}$	204	14.220	0.61	0.73
3.	Caryophyllene	$C_{15}H_{24}$	204	14.671	7.71	9.55
4.	*α*-Bergamotene	$C_{15}H_{24}$	204	14.773	0.56	0.78
5.	*β*-Farnesene	$C_{15}H_{24}$	204	14.966	1.77	2.01
6.	*α* -Caryophyllene	$C_{15}H_{24}$	204	15.104	2.62	3.18
7.	3-Hexdecene	$C_{16}H_{32}$	224	15.311	3.54	3.37
8.	Germacrene	$C_{15}H_{24}$	204	15.464	25.04	20.59
9.	*β*-Bisabolene	$C_{15}H_{24}$	204	15.673	8.36	6.54
10.	*γ*-Cadinene	$C_{15}H_{24}$	204	15.929	4.98	3.71
11.	*α*-Caryophyllene	$C_{15}H_{24}$	204	16.077	1.69	2.11
12.	Caryophyllene oxide	$C_{15}H_{24}O$	220	16.734	3.68	3.13
13.	Spathulenol	$C_{15}H_{24}O$	220	17.211	1.20	1.07
14.	Heptadecene	$C_{17}H_{34}$	238	17.578	9.38	9.10
15.	Heptadecene	$C_{17}H_{34}$	238	17.673	11.71	14.02
16.	Heptadecane	$C_{17}H_{36}$	240	17.790	9.23	11.07
17.	Nonadecene	$C_{19}H_{38}$	266	20.075	2.08	2.42
18.	Nonadecene	$C_{19}H_{38}$	266	20.224	3.81	4.24

19.	Heneicosane	$C_{21}H_{44}$	296	20.381	1.36	1.58
					100.00	100.00

Antibacterial activity

Table 2: Antibacterial activity of the plant extract against *Klebsiella pneumoniae* (MDR) strains

Sample	MIC Values	MBC Values
Neomycin* (µg/mL)	0.0195 (µg/ml)	0.156 (µg/ml)
Extract (mg/mL)	0.09 (mg/ml)	12.5 (mg/ml)

*Control antibiotics

Brine Shrimp Lethality Assay

The LD_{50} of the sample was found to be 251.1µg/mL in Brine shrimp lethality assay

Conclusion

GC-MS analysis of essentials oil shows the presence of 17 major possible compounds. Among them germacrene (25.04%), heptadecene (11.71%), heptadecane (9.23%), *β*-bisabolene (8.36%) and caryophyllene (7.71%) are the major constituents. Extract of plant showed antibacterial activity against *Klebsiella pneumoniae* (MDR). The LD_{50} of the sample was found to be 251.1µg/mL.

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