Volatile Constituents in the Essential oil of *Piper longum* Seeds by GC-MS and the Separation of Barbituric and Tannic acids from its Aqueous Methanolic Extract

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Abstract

Indian Long Pepper or Pippali *(Piper longum)*, indigenous to Indian subcontinent, is a powerful stimulant to digestive and the respiratory systems including rejuvenating effects on lungs. It is an essential constituent of many Ayurvedic formulations including *Trikatu*. Hydro distillation of Pippali yielded greenish oil which was subjected to Gas Chromatography-Mass spectrometric (GC-MS) analysis. Fragmentation pattern studies were used to identify ten compounds: 2, 2-dimethyl propanoic acid, decane, 1-decyne, 3,4, 8-trimethyl 1-nonene, undecane, bis- (1-methylpropyl) disulfide, 2,4-nonynoic acid, 2,4-decadienal, nonanoic acid and tetradecanoic acid. Further, it was Soxhlet extracted with 1:1 aqueous methanol followed by column chromatography and preparative Thin Layer Chromatography (TLC), whereby two colourless crystalline compounds- barbituric acid (R_f = 0.74) and tannic acid (R_f = 0.59) were separated. These were confirmed by elemental analysis, m. pt and characteristic infrared spectral studies. Barbituric acid was further confirmed by GC-MS fragmentation analysis. Both the separated acids are of medicinal importance as these are used in many formulations. Some compounds identified by GC-MS are strong antioxidants attributed to medicinal value of *P longum*.

Keywords: *Piper longum*, Essential oil, Barbituric acid, Tannic acid, Preparative TLC, GC-MS

1. Introduction

Piper longum, commonly known as long pepper, pippalimool and pippali in Indian language belongs to the genus *Piper* and family *Piperaceae.* It is of South Asian origin and found in evergreen forests almost all over India, Sri Lanka and Middle Eastern countries. It is a

slender, aromatic, trailing dioecious plant with creeping jointed stems and perennial woody roots cultivated on a large scale in lime stone soil and in heavy rainfall in the hotter parts of India[1, 2]. The fruits and berries are extensively used as spice for culinary purposes and a traditional medicine in Asia and Pacific islands including China. Pippali plays an important role in aiding thermogenic response by releasing metabolic heat energy. When fresh and wet, Pippali is sweet in taste with cold potency but on drying it becomes black and pungent with semi-hot potency. Literature refers to four types of pippali; *pippali, vanapippali, saimhali*, and *gajapippali.* Former three are known as *P. longum, P. sylvaticum* (Himalayan species), and *P. retrofractum* (indeterminable taxon) respectively and the fourth one has controversial identity. It must be used with utmost care because of its pungent taste. The main application of long pepper is its usage in spicy vegetable pickles and Ayurvedic, Unani, Siddha and Chinese systems of medicine as a stimulant and carminative. It is good for constipation, gonorrhea, and paralysis of the tongue, diarrhea, cholera, scarlatina, chronic malaria and viral hepatitis. It is most commonly used to treat respiratory infections such as bronchitis, cough, asthma and spleen diseases. It soothes and relieves muscular pain and inflammation when applied topically. In Ayurvedic medicine, it is described as good rejuvenator because it stimulates our digestive and respiratory systems. Ancient Indian sage Charaka described it as an appetizer, beneficial for throat and for cleansing nasal therapy[3]. *Piper Longum* helps stimulate the appetite and dispels gas from the intestines[1-3]. Pippali is beneficial as brain tonic and its decoction is effectively used in sciatica and hemiplegia[3]. Kumar et al[4] emphasized its dietary importance as a herb and spice. Zaveri et al[5] extensively reviewed its chemical composition, medicinal and pharmacological activities including antimicrobial, antidiabetic, analgesic and anticancer activity. Importance of *P. longum* in Ayurveda is essentially due to its bioavailability enhancement and safety profile. Powdered pippali taken with honey relieves cough, cold, asthma, hoarseness and hiccup. It is considered more powerful than black pepper. It is one of the constituents of *Trikatu*- the commonly used Ayurvedic herbal formulation along with black pepper and ginger, widely used for anti-cold, anti-asthmatic properties and as remedy of *kapha dosha*[1, 3].

Chemical composition of *Piper longum* has been comparatively less investigated. Only scanty reports are available on its chemical composition and phytoconstituents. Most studies relate to its pharmacological activity. Koul et al[6] reported isolation and characterization of three new pyrrolidides; brachyamide A, brachyamide B and brachystine. Kumar et al[7] reported the isolation and characterization of two active constituents from the combined

hexane and chloroform extracts; ethyl 3', 4', 5'-trimethoxycinnamate and piperine,. Lee et al[8] carried out a bioassay-guided isolation of piperine from the ethanol extract and found it to be a monoamine oxidase (MAO) inhibitor. A bioactivity-guided fractionation from the chloroform extract by Lee et al[9] (2004) for ACAT inhibitor led to the isolation of guineensine. Trinnaman et al[10] identified several terpene and sesquiterpene compounds along with a waxy alkaloid (N-isobutyl deca trans-2-trans-4-dienamide) and a terpenoid from the volatile oil (1%) of the fruit. In another study, Shankaracharya et al[11] found about 1% volatile oil, 1.25% piperine and 40% starch. Park et al[12] attributed its antifungal activity due to a piperidine alkaloid- piperoctadecalidine. Matsuda et al[13] investigated the melanogenesis stimulation activity of its aqueous ethanolic extract. Sunila and Kuttan[14] studied the antiangiogenic activity using *in vivo* and *in vitro* models. Lakshmi et al[15] have identified pure compounds from the hexane extract of powdered fruits useful for the antifertility effect in female rats. Liu et al[16] isolated 11 compounds by extraction of *P. longum* with 95% ethanol and identified these by mass spectral and NMR studies: coumaperine, N-5-(4-hydroxy-3-methoxyphenyl)-2E-pentenoyl piperidine, piperolactam A, 1-[1-oxo-5 (3,4-methylenedioxyphenyl) -2E,4E-pentadienyl] –pirrolidine, 1-[1-oxo-5 (3,4-methylenedioxyphenyl) -2E-pentenyl] -pirrolidine, 1-[1-oxo-9 (3,4-methylene dioxyphenyl)-2E, 8E-nonadienyl] -pyrrolidine, (R)-(-) -turmerone, octahydro-4-hydroy-3alpha-methyl-7-methylene-alpha-(1-methylethyl)-1H-indene-1-methanol,(+)-aphanamol I, bisdemethoxycurcumin, demethoxycurcumin. Singh et al[17] have reported its pharmacognostical and phytochemical analysis suggesting the constituents such as alkaloids, hydrocarbons, p-methoxy acetophenone, sesquiterpenes, piperine, piplartin, triacontane, glycosides etc.

In recent years GC-MS has become an important tool widely used for the identification of organic compounds in natural products and complex mixtures. Earlier we have used GC-MS for the identification of three new phytoconstituents in methanolic extract of *Terminalia arjuna*-a heart tonic[18]. In a recent study from Algeria, Tebbal et al[19] have used GC-MS for the identification of 16 compounds in acetone and ethanol extracts of *Quercus canariensis L*. leaves.

As a part of our trace element analysis studies of *Trikatu* and its constituents, we have analyzed long pepper for 31 elements including essential, minor, trace and toxic elements using INAA and AAS methods[20]. In continuation, we have identified 10 organic compounds; four long chain hydrocarbons, four fatty acids, an aldehyde and a disulfide

from the essential oil of *P. longum* by GC-MS studies. Also, barbituric acid and tannic acid were isolated from its aqueous methanolic (1:1) extract followed by column chromatographic separation. These were further confirmed by elemental analysis and infrared (IR) spectroscopy and GC-MS.

2. Materials and methods

2.1 Sample preparation;

Dried fruits of Pippali, as shown in photograph 1, were purchased from the local market of Roorkee.

Photograph 1. Dried fruits of long pepper, pippali or *P. longum*

These were washed with distilled water, wiped with tissue paper to remove any dirt, dried under Infrafil lamp and then powdered in Agate mortar. It was passed through a 100 mesh sieve, oven dried at 80 ^{o}C for 2h and stored in a precleaned polythene capped bottle for further analysis.

2.2 Gas chromatography-Mass spectrometric (GC-MS) analysis;

50 g air-dried finely powdered pippali sample was distilled in water using a Clevenger apparatus for 4h when pale yellow greenish, odourless oil (yield 2.31% v/w) was obtained. It was dried over anhydrous sodium sulphate, filtered and analyzed by GC-MS using a Perkin-Elmer Clarus-500 gas chromatograph (66 cm x40 cm x72 cm) coupled with a mass spectrometer[18]. It consisted of a fused silica capillary column (HP-5MS) (5% phenyl methyl siloxane), 30 mm X 0.32 mm, 0.25 μm film thickness, in a temperature program from 50 (2 min hold) to 250 °C (10 min hold) at a rate of 8 °C/min. The injector temperature was 250 °C, and the flow rate of carrier gas (He) was 1mL/min. The interface of the capillary column end into the ion source block was kept at 280 °C. Diluted samples (1/100 in acetone v/v) of 1.0 μL were injected manually and, in the split,-less mode. Individual organic compounds

were identified on the basis of comparison of their relative retention time and mass spectra with those of built-in NIST 2.0 mass spectral database[21].

2.3 Soxhlet extraction and chromatographic separation;

An aqueous methanolic (1:1) extract of pippali was used for the separation of phytoconstituents. 100 g dried powder was Soxhlet extracted with 1:1 aqueous methanol successively for 6h at approximately 65 ^{o}C. The extract was filtered using Whatman filter paper and concentrated. Silica gel-G column chromatographic separation was carried out using a set of solvents with increasing polarity in the order; petroleum-ether < chloroform < ethyl acetate < ethanol < methanol < water. Two distinct spots at R_f =0.74 and 0.59 were obtained with methanol-water fraction. It was later developed on a preparative TLC plate (20x20 cm^2) in a methanol/water (50:1) mixture. Separated fractions of two compounds were scrapped out, dissolved, filtered and distilled. These were finally recrystallized in acetone. Whole separation scheme of the two compounds is illustrated in flow sheet in Fig. 1.

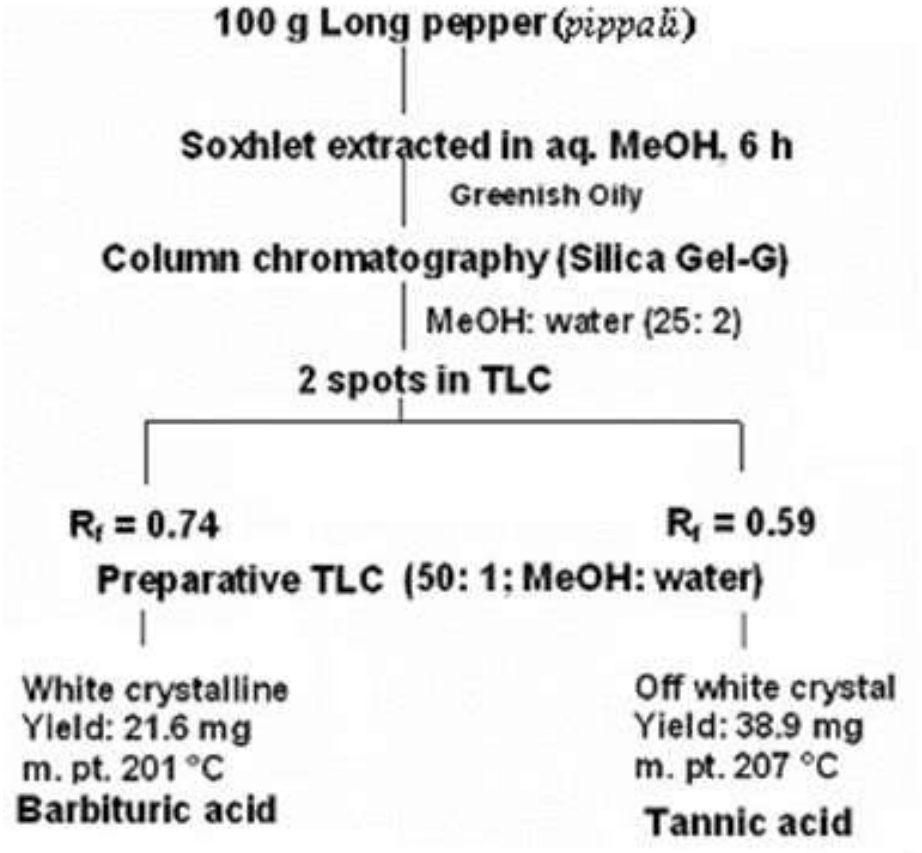


Fig. 1. Flow sheet for the separation scheme of barbituric and tannic acids.

C, H and N contents of the separated compounds were determined by using Elementar Vario EL III (Germany) with TCD detector. Infrared spectra in KBr thin film were recorded using Thermo Nicolet (Nexus, USA) FT-IR spectrophotometer in the range 400-4000 cm^{-1}.

3. Results and discussion

3.1 GC-MS analysis;

Several workers have studied *P. longum* for its phytoconstituent contents using a variety of techniques[22-25]. Chandra et al[22] developed an Ultra Performance Liquid Chromatography (UPLC) coupled with triple quadrupole electrospray tandem mass spectrometry (EST-MS) with continuous polarity switching for the analysis of piperamides, phenolics and terpernoids. During last few years, several review articles[23-25] have reported phytoconstituents of alkaloids, flavonoids, esters and steroids exhibiting neuroprotective, anti-inflammatory, analgesic, antibacterial, antidiabetic and antiulcer properties including its use in treating nervine disorders such as Parkinson's, Alzheimer's/ dementia, epilepsy, insomnia and others diseases. Li et al[26] have reported eight bioactive compounds from 95% EtOH-H_2O extract of fruits of *Piper longum* and explored their potential vascular relaxation activity on rat mesenteric arteries.

A Mass chromatogram of the essential oil obtained by hydro distillation of pippali was reported earlier from our laboratory[20]. It is very complex in nature suggesting the presence of a large number of compounds. Ten major phytoconstituents corresponding to retention times (R_t) of 2.92, 5.16, 5.45, 5.64, 7.41, 9.40, 10.23, 10.46, 10.54 and 12.81 min. were identified on the basis of comparison with NIST 2.0 mass spectra library[21]. In order to further identify the phytoconstituents, their GC-MS fragmentation patterns were studied. Data so obtained for various fragments in 10 compounds in terms of m/z (relative intensity) are listed here;

(I) 2,2-dimethyl propanoic acid, $C_5H_{10}O_2$: 102 (M^+) (54), 87 (100), 57 (51), and 42 (17).

(II) Decane, $C_{10}H_{22}$: 142 (M^+) (21), 127 (14), 113 (7), 99 (9), 85 (19), 71 (28), 57 (83), and 43 (100).

(III) 1-decyne, $C_{10}H_{18}$: 138 (M^+) (12), 123 (11), 109 (9), 95 (22), 81 (92), 67 (74), 57(41), and 43(62).

(**IV**) 3, 4, 8-trimethyl 1-nonene, $C_{12}H_{24}$: 168 (M^+) (18), 125 (2), 111 (3), 85 (48), 83 (27), 71(64), 57 (69) and 43 (100).

(**V**) Undecane, $C_{11}H_{24}$: 156 (M^+) (32), 141 (2), 127 (12), 113 (11), 99 (8), 85 (14),

71(33), 43 (93) and 57(100).

(**VI**) Bis(1-methylpropyl)disulfide, $C_4H_{10}S_2$: 122 (M^+) (21), 107 (42), 93 (15), 57 (100).

(**VII**) 2, 4-nonynoic acid, $C_9H_{14}O_2$: 154 (M^+) (35), 139 (11), 125 (19), 111 (17), 83 (100) and 69 (52).

(**VIII**) 2, 4-decadienal, $C_{10}H_{16}O$: 152 (M^+) (21), 137 (22), 123 (14), 109 (3), 95 (8), 55 (32) and 81(100).

(**IX**) Nonanoic acid, $C_9H_{18}O_2$: 158 (M^+) (7), 129 (13), 115 (9), 101 (5), 87 (14), 45 (22),73 (72), 59(100).

(**X**) Tetradecanoic acid, $C_{14}H_{28}O_2$: 228 (M^+) (4), 185 (9), 171 (6), 157 (3), 143 (7), 129 (24), 115(11), 101 (4), 87 (19), 73 (73), and 59(43).

Based on the mass spectra of these compounds, fragmentation patterns are suggested as shown in Figs. 2-11 where m/z values match very well with those of the suggested fragments.

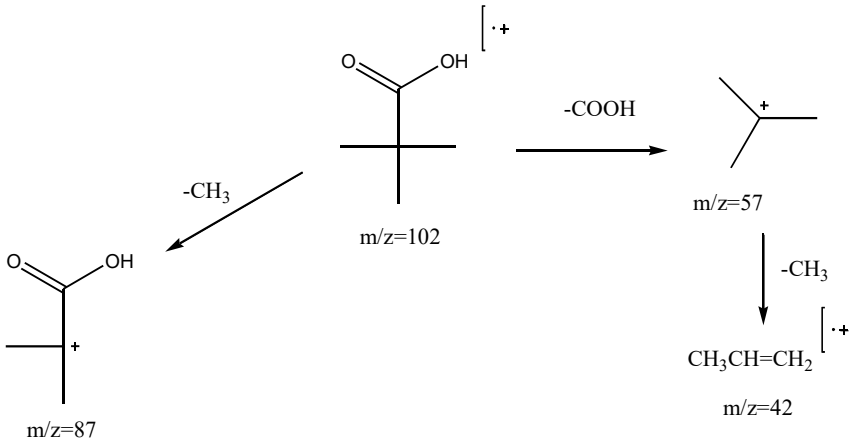


Fig. 2 Mass fragmentation pattern of 2, 2-dimethyl propanoic acid (**I**), R_t = 2.90 min

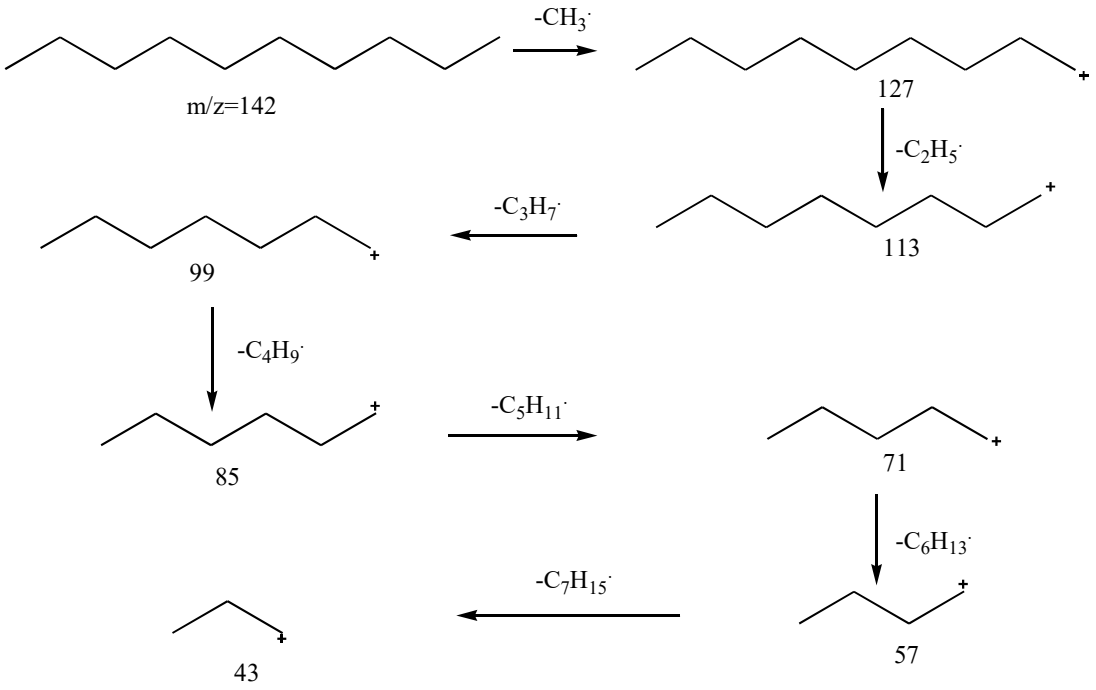


Fig. 3 Mass fragmentation pattern of decane (**II**), R_t = 5.14 min

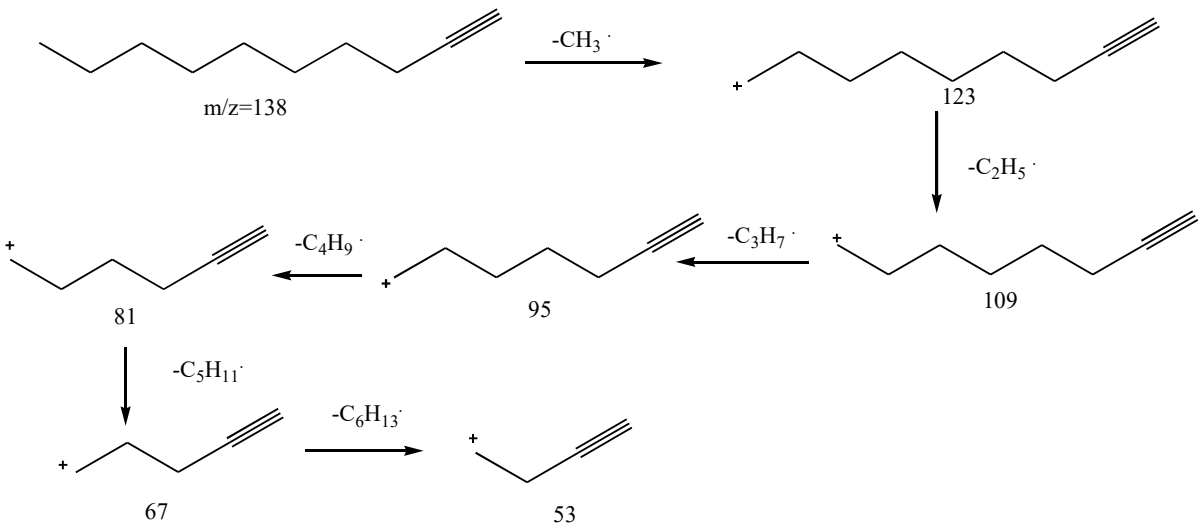


Fig. 4 Mass fragmentation pattern of 1-decyne (**III**), R_t = 5.46 min

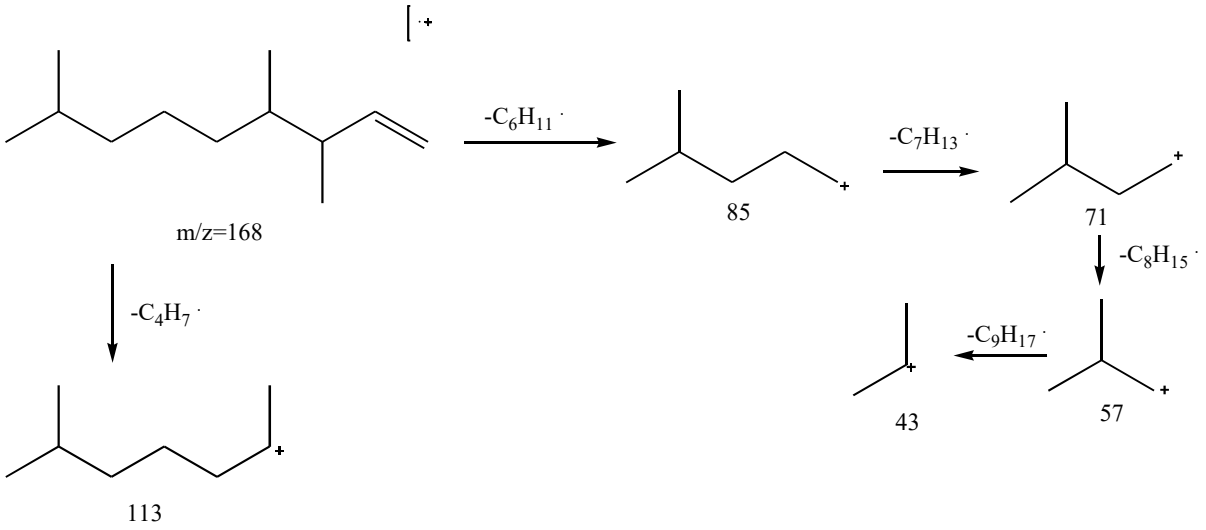


Fig. 5 Mass fragmentation pattern of 3,4, 8-trimethyl 1-nonene (**IV**), R_t = 5.64 min

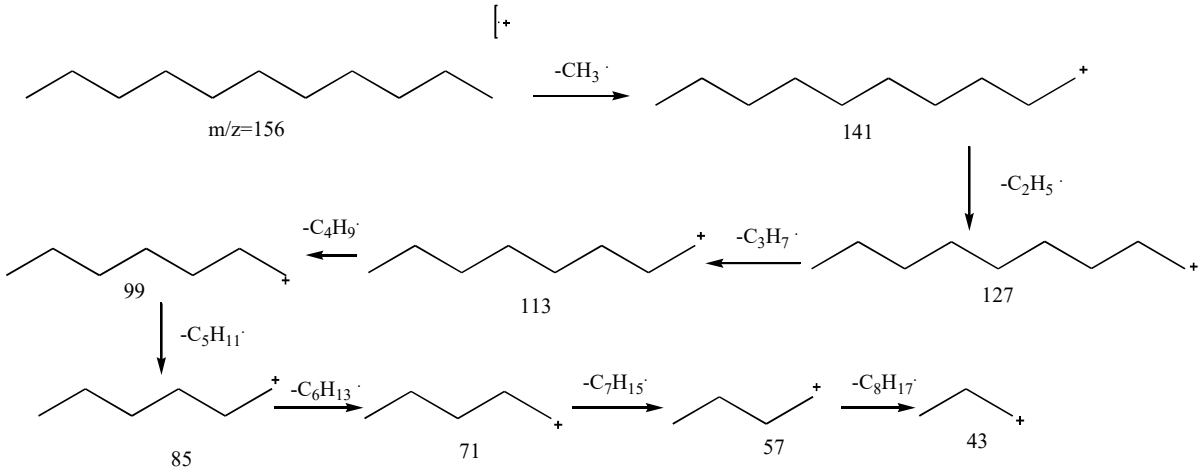


Fig. 6 Mass fragmentation pattern of undecane (**V**), R_t = 7.43 min

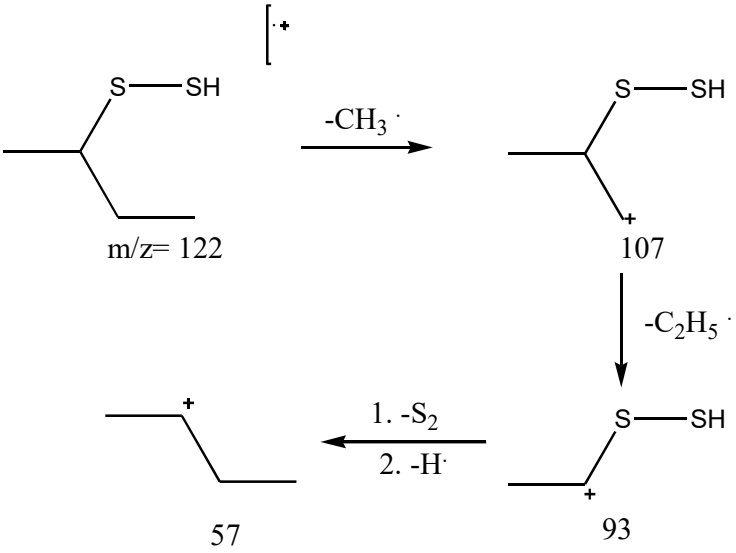


Fig. 7 Mass fragmentation pattern of bis- (1-methylpropyl) disulfide (**VI**), R_t = 9.41 min

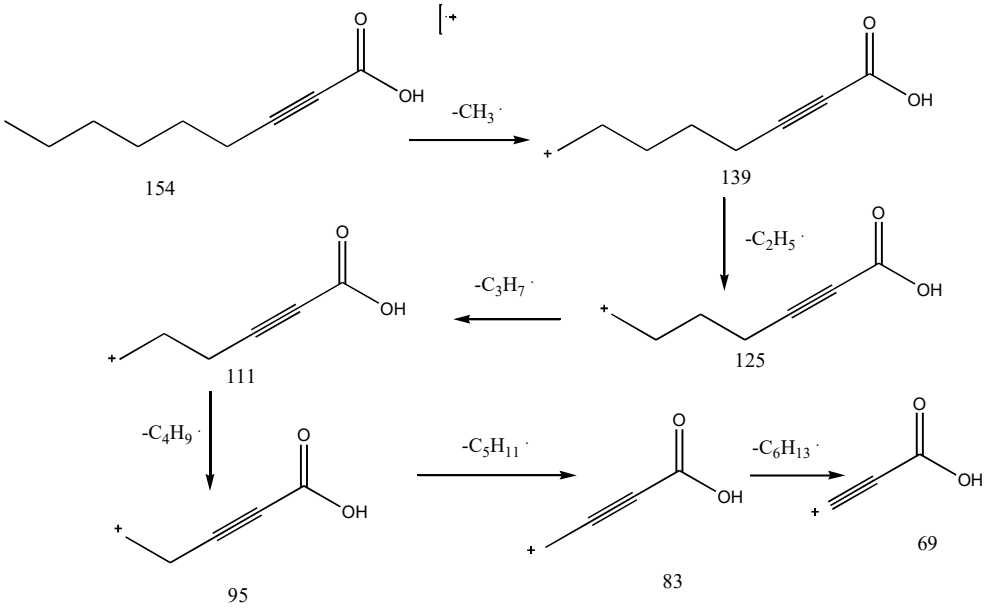


Fig. 8 Mass fragmentation pattern of 2, 4-nonynoic acid (**VII**), R_t = 10.27 min

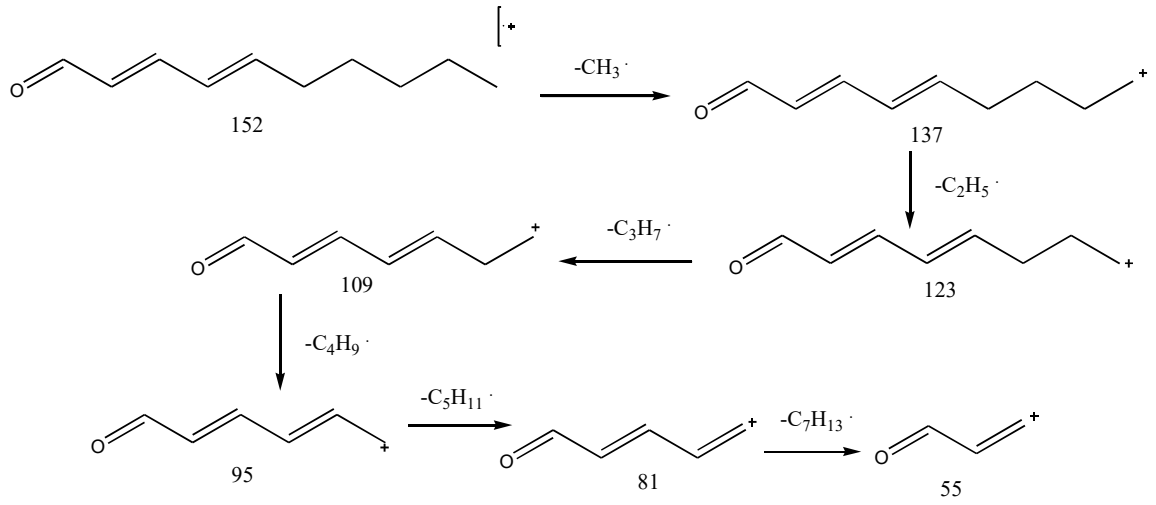


Fig. 9 Mass fragmentation pattern of 2, 4-decadienal (**VIII**), R_t = 10.45 min

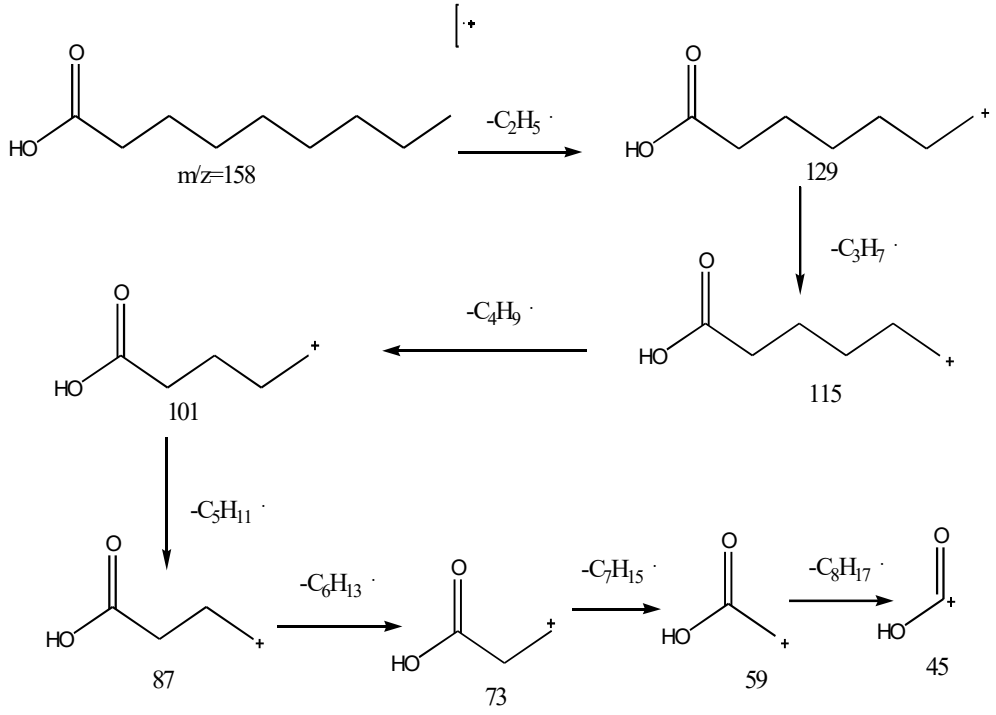


Fig. 10 Mass fragmentation pattern of nonanoic acid (**IX**), R_t = 10.55 min

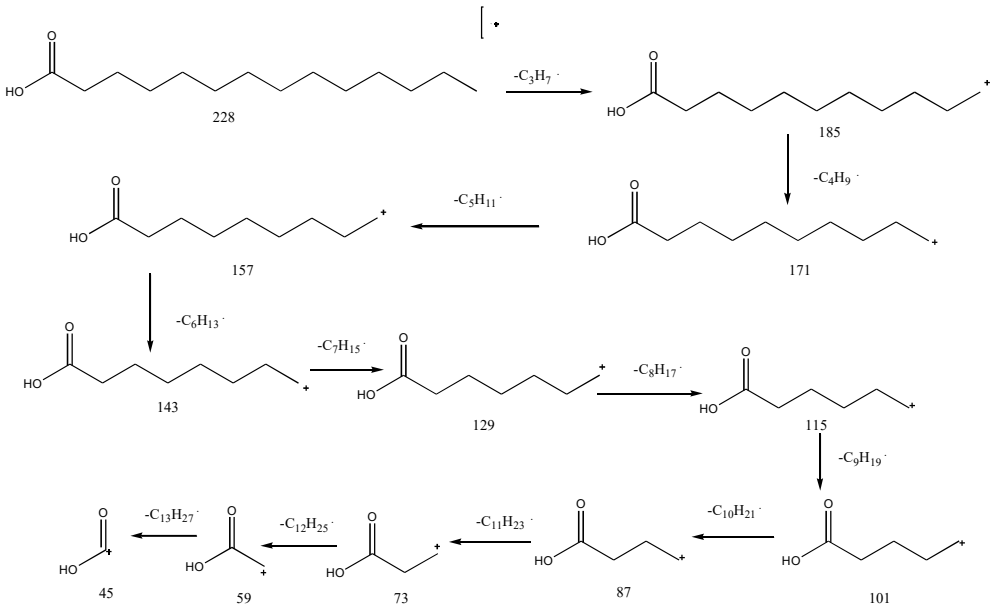


Fig. 11 Mass fragmentation pattern of tetradecanoic acid (**X**), R_t = 12.81 min

Compound **I,** 2, 2-dimethyl propanoic acid, also known as pivalic acid was earlier detected by Toth[27] in the essential oil of fennel seeds. It is produced in large quantities by the pharmaceutical industry and is thought to have minimal toxicity. Lewin et al[28] have found this compound to reduce the fertility of male hamsters. Thus, Munshi et al[29] have suggested it to be responsible for the antifertility properties of pippali.

Literature survey shows that three each of hydrocarbons [decane (**II**), 1-decyne (**III**), and undecane (**V**)] and carboxylic acids [2, 4-nonynoic acid (**VII**), nonanoic acid (**IX**) and tetradecanoic acid (**X**)] were all reported earlier in the essential oil of pippali[8, 9]. **IX** is also called pelargonic acid and used as an active ingredient of environment friendly herbicide with quick effects[30]. Chadeganipour and Haims[31] studied its antifungal activity on *Microsporum gypseum*. Tetradecanoic acid (**X**), commonly known as myristic acid, a 14-carbon straight chain saturated fatty acid [$CH_3(CH_2)_{12}COOH$] occurs in most animal and vegetable fats, particularly butterfat, coconut, palm, and nutmeg oils. It is used to synthesize flavor and as an ingredient in soaps and cosmetics[32].

Sefidkon et al[33] observed bis(1-methylpropyl) disulfide (**VI**) as the main constituent in *Ferula assafoetida* gum collected from Iran. It is an aroma chemical, which could be attributed for the use of *Piper longum* as a spice. 2, 4-decadienal (**VIII**) has been reported in the volatile compounds of red pepper[34] and is used commercially to impart a deep fat

flavour in beef, lamb, chicken, potato chips and French fries. Out of ten compounds identified here, 3, 4, 8-trimethyl 1-nonene (**IV**) an unsaturated aliphatic hydrocarbon, is the only one being **reported for the first time.** It has not been reported earlier in any natural product.

3.2 Aqueous Methanolic extract;

Two crystalline compounds were separated after 1:1 aqueous methanolic extraction followed by column chromatographic separation. On the basis of their elemental analysis, m. pt and characteristic ir functional group frequencies, we suggest these to be barbituric acid and tannic acid.

Barbituric acid, **$C_4H_4N_2O_2$** (Mol wt, 128): White crystalline, Yield: 21.6 mg, m. pt. 201 ^oC (Lit.198 ^oC);

CHN analysis; C (calc.%): 38.16 (37.51), H (%): 3.07 (3.15), N (%): 22.04 (21.87).

IR spectrum KBr (cm^{-1}): 3437 (ν_{N-H}), 2966(ν_{C-H}), 1653 ($\nu_{C=O}$), 1277 (ν_{C-N}).

GC-MS of barbituric acid showed a retention time (R_t) of 15.3 min in methanol. Its mass spectrum and fragmentation studies showed four fragments at; M^+ 128 (25), 100 (45) 72 (100), 44 (22) which corresponded to fragmentation scheme shown in Fig. 12.

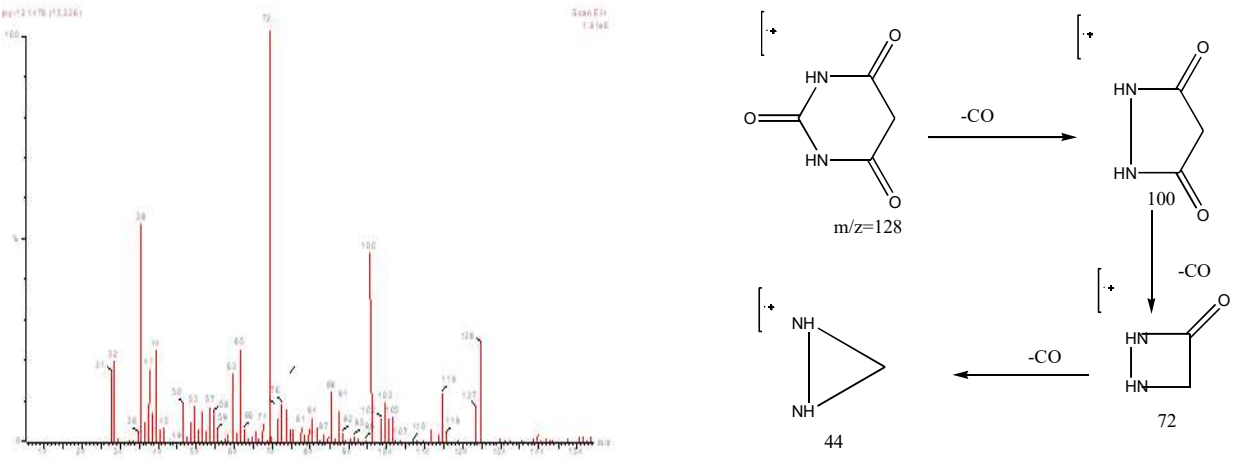


Fig. 12 Mass spectrum and fragmentation pattern of barbituric acid, R_t = 15.3 min

All the m/z values match well with those of the suggested fragments. Barbiturate drugs are known to be sedative on central nervous system (CNS). Although it is not itself pharmacologically active but it has a role as an allergen and a xenobiotic. Barbituric acid is the parent compound of barbiturate drugs widely used for various ailments. It produces wide spectrum of effects such as mild sedation to anesthesia[35]. Some barbiturates are also used as

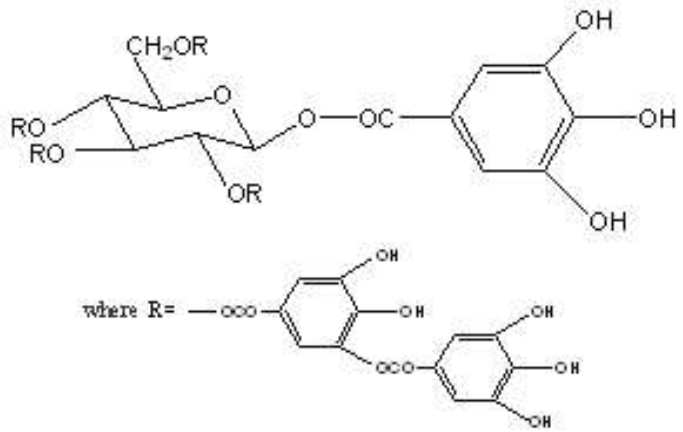
anticonvulsants. An exhaustive review by Kaur et al[36] deals with derivatives of barbituric acid and its wide ranging bio-applications. It is used in the production of a wide range of bioactive drugs such as antioxidant, anticonvulsant, anticancer. Singh et al[37] have shown that pippali extract had a central stimulant action in frogs, mice, rats, and dogs. It may be noted that barbituric acid is also not reported earlier in pippali. Its sedative action may possibly be attributed to barbituric acid.

Tannic acid: Off white crystalline, Yield: 38.9 mg, m.pt. 207 ^{o}C (Lit. 210 ^{o}C);

CHN analysis (calcd. %); C: 26.18 (25.76), H: 1.61 (1.47)

IR spectrum in KBr (cm^{-1}): 3425 (ν_{O-H}), 2852(ν_{C-H}), 1712 ($\nu_{C=O}$), 1205 (ν_{C-O})

Tannic acid is a type of tannin, known for its astringent taste and ability to bind and to precipitate proteins. It is found in all foods derived from plants and widely used in medicine and leather tanning. Tannic acid is found in plants like oak tree, gall nuts, and tara pods. It is also present in commonly used foods like tea, coffee, wine, and chocolate. It is a polyphenolic compound (as shown in structure below) that acts like a weak acid often found in the nutgalls formed by insects on twigs of oak tree. It has been widely investigated as a chemopreventive agent because of many health-promoting properties such as antioxidant behaviour[38] and CNS activity[39]. Chen et al[40] have reviewed its health benefits and special chemical properties leading to versatile functional polymeric



Tannic acid

networks. Calixto et al[41] investigated its pharmacological action and its effect on blood pressure. Sehrawat et al[42] observed that its oral treatment of rats with tannic acid resulted in significant recovery of hepatic glutathione content, antioxidant and phase-II metabolizing

enzymes thereby suppressing the tumour promotion stage. Sunila and Kuttan[43] suggested tannic acid as chemo preventive agent which may be attributed to its immunomodulatory and antitumour activity in alcoholic extract. Li et al[26] have already suggested *P longum* as vascular relaxant candidate which is worthy of studying its molecular mechanism.

3.3 Correlation with elemental analysis;

In our earlier studies on minor, trace and toxic element analysis of pippali[20], it was found to contain significant amounts of Ca (6.30±0.04 mg/g), Mg (0.90±0.07 mg/g), K (16.0±2.1 mg/g), P (2.93±0.69 mg/g), Fe (172±16 µg/g), Mn (50.3±8.1 µg/g), Cu (28.1±2.6 µg/g), and Zn (14.5±1.1µg/g) besides others. Minor elements such as Ca, K and Mg are electrolytic and essential for cellular transport. However, trace elements Fe, Mn, Cu, Zn are essential for bio enzymatic processes. The phytoconstituents, barbituric and tannic acids including other carboxylic acids may possibly act as ligands to form complexes with various elements[44]. Mahmudov et al[45] have reviewed the ability of barbituric acid as a tool forming coordination and supramolecular compounds. These organic molecules may help in the bioavailability of essential elements thus making these easily assimilable in our body[20].

4. Conclusions;

Piper longum also known as pippali or long pepper is an important culinary spice and medicinal herb being extensively used in Ayurvedic, Unani and Siddha medicine systems since long. It has several phytoconstituents that help in drug development for the treatment of a variety of ailments. GC-MS analysis and fragmentation pattern studies have been used to identify of 4 each of long chain hydrocarbons and fatty acids and one each of an aldehyde and a disulfide. Further, Soxhlet extraction in aqueous methanolic (1:1) followed by column chromatography and preparative TLC yielded two carboxylic acids- barbituric and tannic acids. Several phytoconstituents identified in *P. longum* are of immense medicinal importance. Barbituric acid is responsible for sedative action and tannic acid as chemo preventive agent. Nonanoic acid is an antifungal agent and teteradecanoic acid (myristic acid) is used as an ingredient in soaps and cosmetics.

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Author's contribution: RPC: Experimental work, Methodology, generation of data, writing rough draft of manuscript.

ANG: Conceptualization, supervision of experimental work and data validation, checking and finalization of manuscript.

Conflict of Interest: Authors declare no conflict of interest.

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