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Research Article

Antibacterial, antioxidant, and phytochemical analysis of *Piper longum* fruit extracts against multi-drug resistant non-typhoidal Salmonella strains *in vitro*

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Abstract
Most bacteria are becoming resistant to almost all of the currently recommended drugs, leading to difficulty in their treatment. The present study focused on evaluating the therapeutic potential of *Piper longum* fruit extracts in terms of bactericidal, antioxidant and phytochemical evaluation by conducting antibacterial sensitivity tests against four multidrug-resistant Salmonella strains (*Salmonella enterica* subsp. *arizonae*, *Salmonella* Newport, *Salmonella enterica* ser. Typhi, and *Salmonella enterica* ser. Paratyphi) obtained from the Institute of Microbial Technology (IMTECH), Chandigarh. The Agar Well Diffusion method and the Minimum Inhibitory concentration (MIC) methods were performed to implement the anti-sensitivity test of crude extracts of the plant. The present study showed that the MIC of the *P. longum* was between 0.25-0.0625mg/ml, which was lowest in the aqueous extract at 0.5mg/100µl, and the highest in the methanol extract (1mg/100µl). The Minimum Bactericidal Concentration (MBC) was lowest in aqueous (0.5mg/100µl) and highest in methanol plant extract (1mg/100µl). The methanol extract had the maximum antibacterial potency, whereas the aqueous extract had the lowest. The antioxidant capacity of the plant extracts was determined using a DPPH assay. Methanol plant extract revealed the highest antioxidant power (81.92%) and the lowest was found in the aqueous extract (62.84%). The GC-MS approach identified active bioingredients, important botanicals including caryophyllene, eicosane, and piperazine (potent antibacterial agent) as naphthyridine (having antimicrobial, anticancer, and anti-inflammatory activities), among others. The unique aspect of the study was the effectiveness of *P. longum* against *Salmonella* strains that are resistant to multiple antibiotics. This suggests that *P. longum* can be a great source of novel antibacterial compound for the development of herbal formulations.

Keywords: Antioxidant, DMSO, DPPH, Multidrug-resistant, *Piper longum*

INTRODUCTION

Salmonella is a common bacterium that may live in all environmental conditions for several weeks (World Health Organization, 2018); this pathogenic bacterium can persist and proliferate in faeces (Guerrero *et al.,* 2020). Furthermore, NTS with its antimicrobial quality has become a major threat worldwide (World Health Organization, 2018), along with its property of being impervious to more than three antimicrobial groups (World Health Organization, 2017; McDermott *et al.,* 2018; Tack *et al.,* 2020).

Piper longum Linn, basically recognized as (Pipalli in India) Long Pepper *and* a representative of a Piperaceae family. *It* is distributed in West Bengal, Maharashtra, Eastern Uttar Pradesh, Madhya Pradesh, Kerala, Tamil Nadu, Karnataka, Andaman & Nicobar Islands and is recognized to have antibacterial qualities (Reddy *et al.,* 2001; Yadav *et al.,* 2020). *P. longum* is a valuable medicinal plant used to treat respiratory and digestive disorders, cancer, snake and insect bites and other ailments (Khushbu *et al.,* 2011; Mgbeahuruike *et al.,* 2017). The fruit comprises enormous alkaloids and chemically effective compounds, the first most prevalent is piperine and recent research revealed the content present in *P. longum* has anti-cancer properties and also antioxidant properties (Kumar *et al.,* 2011).

Recent research (Choudhary and Singh, 2018) used a computational method to assess the impact of *P. longum* phytochemicals on human proteomics and discovered 434 target proteins in the nervous system engaged in sending signals and developmental pathways. Piperlongumine strengthens cognitive performance in a dose-dependent way in a murine model by deacetylating the sirtuin gene and lowering amyloid precursor protein (Go *et al*., 2018). The plant has already been listed on the IUCN Red List of Threatened medicinal plants of India in the category of drugs for both human and veterinary use (Gowthami *et al*., 2021).

It holds a variety of organic elements including tannins, alkaloids, carbohydrates, and mineral nutrients like potassium, sodium, manganese, zinc, magnesium, and cobalt, all of which are necessary for metabolic activities, healthy growth, and good lipid profile which are important for humans' health (Hwang *et al.,* 2016). Regularly, *P. longum* has pharmacologically helpful properties against amoebic diseases, different cancer varieties, treating depression, ulcer illness, heart illnesses, etc. (Zaveri *et al.,* 2010; Brundha, 2015; Brundha *et al.,* 2019). The *P. longum* leaves are of great importance to treat various viral diseases such as cancer, depression, obesity, hepatotoxicity, inflammation, etc. (Kumar *et al.,* 2011; Sharma *et al.,* 2020; Moradi and Yousofvand, 2016).

Antioxidant qualities are found in a variety of naturally occurring plant resources and their derivatives, which help to reduce and counteract the oxidative stress imposed by reactive oxygen species (Rajeshkumar, 2016). This study aimed to investigate alternative strategies using a very effective *Piper longum* to manage various infections such as gastroenteritis, focal infection and bacteremia caused by non-typhoidal *Salmonella* strains.

MATERIALS AND METHODS

Chemicals and reagents used:

Pure and analytical grade DMSO (Dimethylsulphoxide), Chloroform, Methanol, Hexane, Petroleum ether, DPPH, Tris HCL, and ethanol. Muller Hinton Agar and Broth brought from (HiMedia, Mumbai) were used throughout the study.

Test organisms

MDR *Salmonella* strains (*Salmonella enterica* subsp. *arizonae* (MTCC 660), *Salmonella* Newport (MTCC 3225), *Salmonella enterica* ser. Typhi (MTCC 733), *Salmonella enterica* ser. Paratyphi (MTCC 735) were procured from the Institute of Microbial Technology (IMTECH), Chandigarh.

Plant material collection and extract preparation:

The *P. longum* plant was brought from the local nursery of Dehradun (Uttarakhand), for identification while the matured dried fruits were bought from a herbal market. The dried fruits were washed with normal tap water, air-dried, transformed into powdery form and safely stocked in a tightly packed vessel or vial. Methanol, chloroform, hexane, petroleum ether, and aqueous were employed as solvents for the extraction of dry powdered fruits of the plant. Soxhlet extraction technique was used to prepare solvent extracts of plant fruits. In a thimble of an extractor, 25g of fruit powder was placed and immersed in a round bottom flask containing 250ml of solvents for 48hrs at optimum temperature. The filtered slurry was concentrated by the rotary evaporator apparatus. Different plant crude extracts were stocked at 4°C before use in an airtight bottle (Akhtar *et al.,* 2014).

Screening for antibacterial action

To assess the sensitivity of all bacterial strains, the agar well diffusion procedure was employed with antibiotics and various extracts of *P. longum* fruits (Wayne, 2002; NCCLS, 1998). All the strains to be tested were suspended in Muller Hinton broth at 37°C for 18h and the cell density was adjusted to 10^5 CFU/ml by the McFarland Turbidity standards (Sawhney *et al.,* 2011). A suspension of 20 µl of all bacterial isolates was spread on MHA plates to generate a uniform bacterial lawn, then fresh agar plates were punched with 9mm wells. 100µl of all extracts (0.5mg/100µl and 1mg/100µl concentrations) were placed directly on wells. The zone of inhibition (ZOI) was determined after 18 h incubation at 37°C. Commercially available antibiotic chloramphenicol was employed as a positive control and DMSO as a negative control. This test was conducted in triplicates and at the end, different zones of inhibition were measured.

Minimum inhibitory concentration testing on broth dilution

By using a macro broth dilution experiment, the minimal inhibitory concentration (MIC) values of all crude ex-

tracts were determined against four multidrug-resistant Salmonella strains. Based on the agar well diffusion method results, extracts were serially diluted in a 2-fold range in well plates using dilution with Mueller-Hinton Broth. The positive and negative controls were the same as taken in MIC. 20µl of freshly generated bacterial suspension (5 x 10^5CFU/ml) was inoculated and plates were kept at 37°C for 24h. The lowest concentration at which the plant extract showed no growth was taken as MIC. (Wayne, 2018; NCCLS, 2000).

Determination of minimum bactericidal concentration

20µl was taken from all broth and spread on MHA plates, and kept for incubation at 37°C for 24h. The dilution, which showed no bacterial growth was taken as minimum bactericidal concentration (MBC) and the particular concentration is said to be bactericidal. The test was conducted in three replicates, and the mean values of MIC and MBC were estimated. The MIC index (difference between MIC and MBC value) was developed to know whether the extracts are naturally bactericidal (<4) or bacteriostatic (>4) (Radhakrishnan *et al.,* 2011).

DPPH antioxidant activity

The DPPH test is a low-cost, simple, and quick method for determining the anti-oxidative activity of a given chemical or extract (Koleva *et al.,* 2002). The anti-oxidative capacity of all extracts was concluded via DPPH radical scavenging property (Jindal and Rani, 2022). The concentrations of fruit crude extracts were prepared such as 25, 50, 75, 100, 125, 150, 175, 200, 225, 250 µg/ml. DPPH is a free radical chemical compound that is stable, purple, and absorbed at 517nm. 1% stock solution of the test sample was made. Measurements at 11 concentration points, including control, were required in the analytical technique. The DPPH scavenging property of the analytical sample solution was measured in triplicates at each concentration.

DPPH suspension was made by mixing DPPH (7.89mg) with 95 % ethanol (100ml) and then kept for 2hr in a dark place. In the test solution, 1ml freshly prepared DPPH (in 95% distilled ethanol) was mixed with a 200µl sample and about 800 µl of 0.1M(1M) Tris HCL. Mixed it for 10 sec and kept it in the dark for approximately half an hour then optical density was recorded at 517nm against a suitable blank. Higher scavenging of reactive oxygen species (free radicals) is proportionate to the reaction mixture's absorbance. The difference between the control absorbance (DPPH mixed with ethanol) and the test sample was evaluated and expressed as an inhibition percentage. In this analysis free radical scavenger, gallic acid was used as standard.

$$\text{Inhibition percentage} = (\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}}) / \text{Absorbance}_{\text{Control}}) \times 100 \quad \ldots \text{Eq.1}$$

If free hydroxyl radicals are present and scavenged then the colour of DPPH changes from purple to yellow.

The IC50 value was calculated from the graph of inhibition % plotted against the concentration of all extracts tested. It was characterized as the concentration of a sample necessary to scavenge 50% DPPH.

Gas chromatography and Mass spectroscopy analysis

HP-5MS column with measurement of 30m × 250 µm; 0.25 µm was applied to perform Gas chromatography and Mass spectroscopy analysis (GC-MS) on chloroform, methanol, aqueous, hexane, and petroleum ether extracts. At a rate of 1 ml/min, a 1 µl sample infused with helium gas was taken as a carrier. The split ratio was 1:10, and the temperature was 280 °C for the transfer line and 250 °C for the ion source. The scan range of the samples was 40 to 500amu (Atomic mass unit). The components were identified by computer searches in a commercial library (NIST) National Institute of Standards and Technology.

RESULTS

The present study observed that yield of *P. longum* extracts varied in various solvents, which can be due to the differences in the polarity of the solvents employed in the Soxhlet extraction procedure. The maximum yield was obtained from an aqueous extract followed by chloroform and the minimum was obtained from petroleum ether (Table 1).

Various antibiotics and fruit extracts of *P. longum* were screened to assess antibacterial activity against the four multidrug-resistant Salmonella strains. Four antibiotics (chloramphenicol, amoxicillin, cefixime, and ofloxacin) were tested against the bacterial strains and the preeminent antibiotic was selected as a positive control. Chloramphenicol demonstrated the highest ZOI (34mm) against MTCC 660, MTCC 733, and MTCC 735 followed by MTCC 3225 (32mm) (Table 2), so was

Table 1. The polarity index and yield% of *Piper longum*

S. No	Solvent Used	Polarity Index	Yield %
1.	Hexane	0.1	24.48
2.	Petroleum ether	0.1	10.36
3.	Chloroform	4.1	29.04
4.	Methanol	5.1	20.84
5.	Aqueous	9	59.76

Table 2: Sensitivity analysis for selecting the suitable antibiotic as a reference against multidrug-resistant NTS bacterial strains

Bacterial culture	Chloramphenicol		Amoxicillin		Cefixime		Ofloxacin	
	0.5mg/ 100µl	1mg/ 100µl	0.5mg/ 100µl	1mg/ 100 µl	0.5mg/ 100 µl	1mg/ 100µl	0.5mg/ 100µl	1mg/ 100µl
Salmonella Newport (MTCC 3225)	32	35	9	12	14	18	26	32
Salmonella enterica subsp. Arizonae (MTCC 660)	34	36	8	15	10	15	22	26
Salmonella enterica ser. Typhi (MTCC 733)	34	37	7	11	9	12	24	29
Salmonella enterica ser. Paratyphi (MTCC 735)	34	37	13	18	12	17	29	32

selected as the reference antibiotic for the study. Methanol extract of the fruits of *P. longum* showed maximum ZOI against all *Salmonella* strains and minimum was observed in the case of the aqueous extract. MTCC 660 showed the highest ZOI (29mm) with the 0.5mg/100µl concentration of methanol extract followed by MTCC 733 (22mm), MTCC 3225 (22mm), and minimum ZOI (21 mm) was recorded against MTCC 735. At a concentration of 1mg/100µl, the methanolic extract showed maximum ZOI (31mm) against MTCC 660 followed by MTCC 735 (25mm), and minimum ZOI was seen against MTCC 733 (24mm), MTCC 3225 (24mm). The aqueous extract (0.5mg/100µl) was found to be greatly effective against MTCC 660 (17mm), moderately effective against MTCC 3225 and MTCC 733 (14mm & 12mm), and the least effective was noticed in the case of MTCC 735 (10mm). MTCC 660 was best suppressed by aqueous plant extract at 1mg/100µl with ZOI values of 20mm followed by MTCC 3225 (16mm) and minimum ZOI (14mm) was seen against MTCC 733 and MTCC 735. All the crude extracts employed in the study exhibited significant potency in inhibiting the *Salmonella* strains (Fig.1).

Minimum inhibition concentration and minimum bacterial concentration

The MIC assay was performed on all extracts using a two-fold serial dilution technique. The minimal dosage of a test sample/antibiotic required to inhibit or suppress the growth of microorganisms is referred to as the MIC. The control extracts MIC and MBC were 0.0156mg/ml and 0.0312mg/ml, respectively. MIC Indices of control and extracts were 2.0 for all the bacterial strains. Methanol plant extract had the highest concentration of ZOI followed by chloroform, petroleum ether, hexane and aqueous. According to the MBC data, MBC levels var-

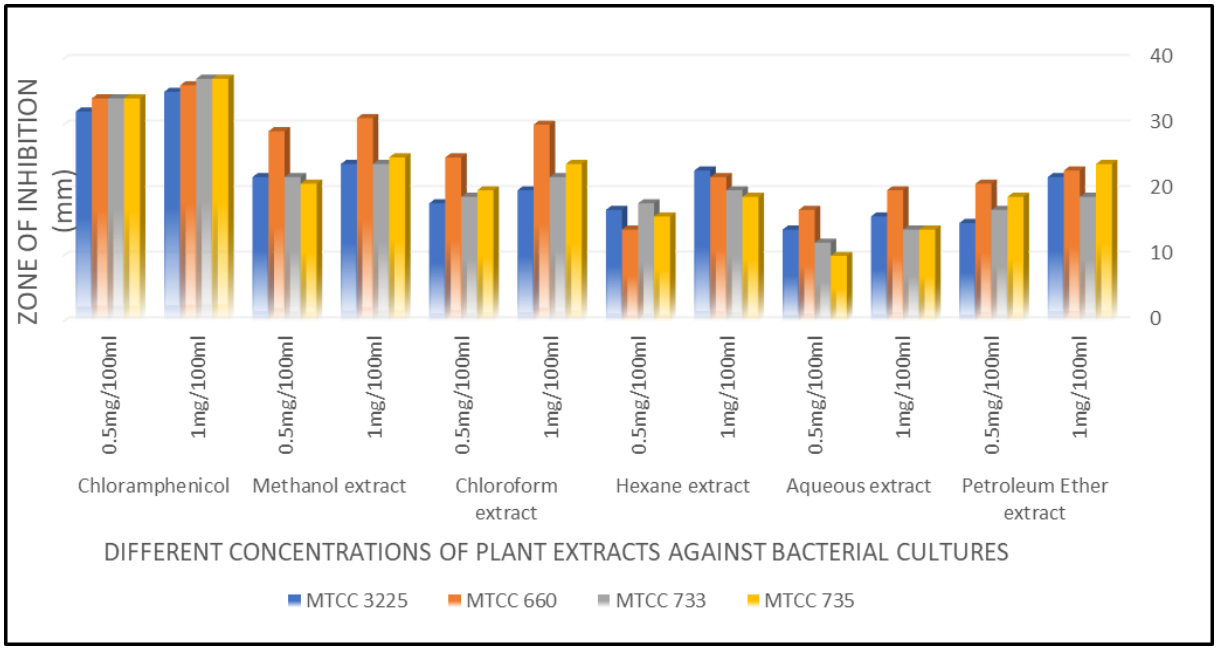

Fig. 1. *Zone of inhibition in mm of methanol, chloroform, hexane, aqueous, petroleum ether extracts of P.longum against Salmonella strains*

Table 3. *Piper longum* extracts showing MIC, MBC, and MIC Index values against MTCC 660 (*Salmonella enterica* subsp. *arizonae*) bacterial strains

***P.longum* extracts**	**MIC**		**MBC**		**MIC/MBC Index**	
	Control (mg/mL)	**Extract (mg/mL)**	**Control (mg/mL)**	**Extract (mg/mL)**	**Control**	**Extract**
Methanol	0.0156	0.0625	0.0312	0.125	2	2
Hexane	0.0156	0.125	0.0312	0.25	2	2
Aqueous	0.0156	0.25	0.0312	0.5	2	2
Chloroform	0.0156	0.125	0.0312	0.25	2	2
Petroleum ether	0.0156	0.125	0.0312	0.25	2	2

Table 4. *Piper longum* extracts MIC, MBC, and MIC Index values against MTCC 3225 (*Salmonella* Newport) bacterial strains

***P.longum* extracts**	**MIC**		**MBC**		**MIC/MBC Index**	
	Control (mg/mL)	**Extract (mg/mL)**	**Control (mg/mL)**	**Extract (mg/mL)**	**Control**	**Extract**
Methanol	0.0156	0.0156	0.0312	0.0312	2	2
Hexane	0.0156	0.0625	0.0312	0.125	2	2
Aqueous	0.0156	0.25	0.0312	0.5	2	2
Chloroform	0.0156	0.125	0.0312	0.25	2	2
Petroleum ether	0.0156	0.125	0.0312	0.25	2	2

Table 5: *Piper longum* extracts MIC, MBC, and MIC Index values against MTCC 733 (*Salmonella enterica* ser. Typhi) bacterial strains

***P.longum* extracts**	**MIC**		**MBC**		**MIC/MBC Index**	
	Control (mg/mL)	**Extract (mg/mL)**	**Control (mg/mL)**	**Extract (mg/mL)**	**Control**	**Extract**
Methanol	0.0156	0.0625	0.0312	0.125	2	2
Hexane	0.0156	0.125	0.0312	0.25	2	2
Aqueous	0.0156	0.25	0.0312	0.5	2	2
Chloroform	0.0156	0.0625	0.0312	0.125	2	2
Petroleum ether	0.0156	0.125	0.0312	0.25	2	2

Table 6: All *Piper longum* extracts MIC, MBC, and MIC Index values against MTCC 735 (*Salmonella enterica* ser. Paratyphi) bacterial strains

***P.longum* extracts**	**MIC**		**MBC**		**MIC/MBC Index**	
	Control (mg/mL)	**Extract (mg/mL)**	**Control (mg/mL)**	**Extract (mg/mL)**	**Control**	**Extract**
Methanol	0.0156	0.0625	0.0312	0.125	2	2
Hexane	0.0156	0.0625	0.0312	0.125	2	2
Aqueous	0.0156	0.25	0.0312	0.5	2	2
Chloroform	0.0156	0.125	0.0312	0.25	2	2
Petroleum ether	0.0156	0.25	0.0312	0.5	2	2

ied from 0.5 to 0.0312mg/ml (Table 3, 4, 5, 6). The MIC Index was used to assess whether the extract is either bactericidal (<4) or bacteriostatic (>4).

DPPH free radical scavenging activity

The selected plant for the study presented notable anti-oxidant activity at different concentrations that were used to calculate the % inhibition concentrations and IC50 values. Table 7 and Fig. 2 showed the graphical representation of anti-oxidative properties as inhibitions %. Results showed that methanol extract exhibited the highest antioxidant power (81.92±0.002), followed by hexane extract (80.09±0.013), chloroform extract (73.40±0.001), petroleum ether extract (65.06±0.002) and Aqueous extract (62.84±0.002). The values of % inhibition were found to be concentration-dependent.

IC_{50} value, referred to as the substrate concentration at which the DPPH activity reduced to 50%, was calculat-ed by linear regression in which inhibition % versus the concentration of the tested chemicals was plotted. The

Table 7: *Piper longum* (fruit) DPPH scavenging activity in various solvent extracts and gallic acid standard

Concentration (µg/ml)	Inhibition%					
	Gallic Acid (Standard)	Methanol	Chloroform	Hexane	Aqueous	Petroleum ether
25	90.99±0.003	41.73±0.003	16.85±0.002	34.44±0.002	10.26±0.002	19.81±0.002
50	91.47±0.002	45.50±0.002	17.16±0.003	36.38±0.002	16.69±0.002	20.68±0.002
75	92.55±0.003	47.73±0.004	17.50±0.002	48.42±0.003	29.05±0.001	27.69±0.002
100	93.03±0.002	57.27±0.003	27.47±0.002	56.67±0.001	45.29±0.003	28.49±0.002
125	93.51±0.002	64.35±0.003	39.44±0.002	59.44±0.002	46.70±0.002	39.60±0.002
150	94.83±0.002	68.75±0.004	51.39±0.002	64.72±0.002	49.43±0.002	40.09±0.002
175	95.19±0.001	72.36±0.003	56.39±0.003	65.96±0.002	49.76±0.002	51.08±0.002
200	95.55±0.003	76.52±0.002	62.28±0.002	76.82±0.002	54.90±0.062	61.30±0.002
225	96.03±0.002	80.73±0.002	72.93±0.002	77.25±0.002	57.10±0.003	61.79±0.002
250	96.51±0.002	81.92±0.002	73.40±0.001	80.09±0.013	62.84±0.002	65.06±0.002

Table 8. IC50 Value of *Piper longum* (fruit) in different solvents

Solvents	Crude extract (g)	IC50 Value (µg/ml)
Methanol	2.59	2.68
Chloroform	5.21	6.38
Hexane	6.12	3.605
Aqueous	7.26	6.927
Petroleum ether	14.94	6.996

IC50 values of all extracts are reported in Table 8, in which petroleum ether extract had 6.996 µg/ml, aqueous 6.927 µg/ml, chloroform 6.380 µg/ml, hexane 3.605 µg/ml, and in methanol 2.68 µg/ml.

GC-MS-Analysis

The phytochemical estimation was done by the GC-MS technique of all five extracts, which exhibited the presence of remarkable bioactive compounds with significant biological activity (Table 9,10,11,12,13) and chromatogram (Fig 3,4,5,6,7). Different compounds showed different properties according to their chemical structures and nature. On the basis of GC-MS analysis, the maximum compounds (48) were observed in chloroform extract and minimum compounds (26) were observed in hexane extract. A Total of 186 compounds were characterized in all the solvent extracts. The important differences in the extraction of compounds were that the compounds which were only observed in methanol extract were verbenone, octacosane, beta-bisabolene, pyridinethiol, benzohydrazide, oxirane, benzoquinoline and alloaromadendrene while the compounds observed only in chloroform extract were cyclooctatraene, decadiyne, spathulenol, cadinene, eudesma, angelicin, phenanthrene. The compounds observed only in petroleum ether were cahydronaphthalene, zolpidem, linalyl isobutyrate and the compounds observed only in aqueous were glycin, propanenitrile and tetrazene. Glycine plays a great role in suppressing the formation of free radicals and inflammatory cytokines. (Zhong *et al.,* 2003). Benzohydrazide has significant anticancer, antibacterial and antifungal properties (Jubie *et al.,* 2016). Angelicin has a very effective role in curing cancer (Wang *et al.,* 2019). Octacosane has antiplatelet, antioxidant, antiangiogenic, and neurological property due to its long alcohol chain (Kabir, 2020). Spathulenol has many properties like antioxidant, anti-inflammatory, antiproliferative and antimycobacterial (do Nascimento *et al.,* 2018).

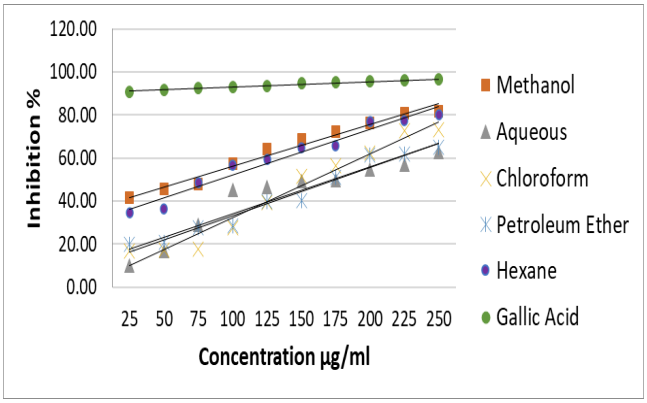

Fig. 2. *Linear regression of methanol, chloroform, hexane, aqueous, petroleum ether extracts, and gallic acid by plotting inhibition % against a concentration of the tested compound*

DISCUSSION

The findings showed that *P. longum* has robust and efficient antibacterial and antioxidant properties in a variety of solvent extracts, including methanol, aqueous, hexane, chloroform, and petroleum ether. As the ZOI values ranged from 31 mm to 14 mm at a dose of 1mg/100µl, all of the extracts demonstrated high effec-

Table 9. Different compounds detected in GC-MS of *Piper longum* methanol extract

GC-MS of Methanol			
Peak#	**R. Time**	**Area%**	**Name**
1	5.025	0.23	Piperidine, 3-methyl-Piperidine, Pinacolborane, 2-ethyl-4-(3-oxiranylpropyl)-
2	7.699	0.79	Benzenepropanoic acid
3	9.228	1.25	Isocaryophyllene
4	9.887	0.33	1,4,7, -Cycloundecatriene, 1,5,9,9-tetramethyl-
5	10.011	0.17	Oxirane, 2-decyl-3-(5-methylhexyl)-, 1,1-dicyclohexyl-, 2-Hexenal,
6	10.125	0.21	Cyclopentadecane, 2-Nonenal, 2-pentyl- n-Nonadecanol-1
7	10.271	0.17	Dimethylamphetamine, Benzeneethanamine, alpha.,2,6-trimethyl-, Methyl 10,12-heptadecadiynoate
8	10.373	0.43	Pentadecane, Hentriacontane
9	10.444	1.72	Germacrene D isomer- Bicyclosesquiphellandrene 1H-, beta-Cuvebene,
10	10.557	0.53	alpha-Longipinene, 1,3-Cyclohexadiene, Zingiberene, alpha-cedrene
11	10.692	0.17	alpha-Himachalene
12	10.773	0.21	Caryophyllene, Alloaromadendrene
13	10.86	0.52	β-bisabolene
14	11.286	0.29	Aromadendr-1-ene, alpha Selinene
15	12.642	0.16	Verbenone
16	13.982	0.54	8-Heptadecene, E-14-Hexadecenal
17	17.677	0.8	Pyridinethiol
18	17.925	0.32	Cyclohexanebutanoic acid, Tetradecanoic acid
19	19.751	2.61	1-Indanone
20	20.443	0.3	Cyclohexanone
21	24.699	0.24	1-Ethanone, o-Anisic acid
22	26.45	0.19	Silicic acid, 2-Pyridinemethanol, 2,4-Cyclohexadien-1-one
23	27.422	4.01	Benzohydrazide, Methylenedioxybenzylamphetamine.
24	27.584	1.02	Naphthyridines, Piperine
25	28.465	1.1	Benzoquinoline, 2,3-Dimethylindole, 2-Methyl-2-phenylindolizine
26	28.637	2.56	1,2-Bis(trimethylsily)benzene 4-Hydroxyphenyl pyrroldinyl thion
27	31.225	2.79	Benzoic acid, 4-Thiazolidinone, 4-methoxy-, pentadecyl ester,
28	35.79	3.17	2-Ethylacridine, Indole

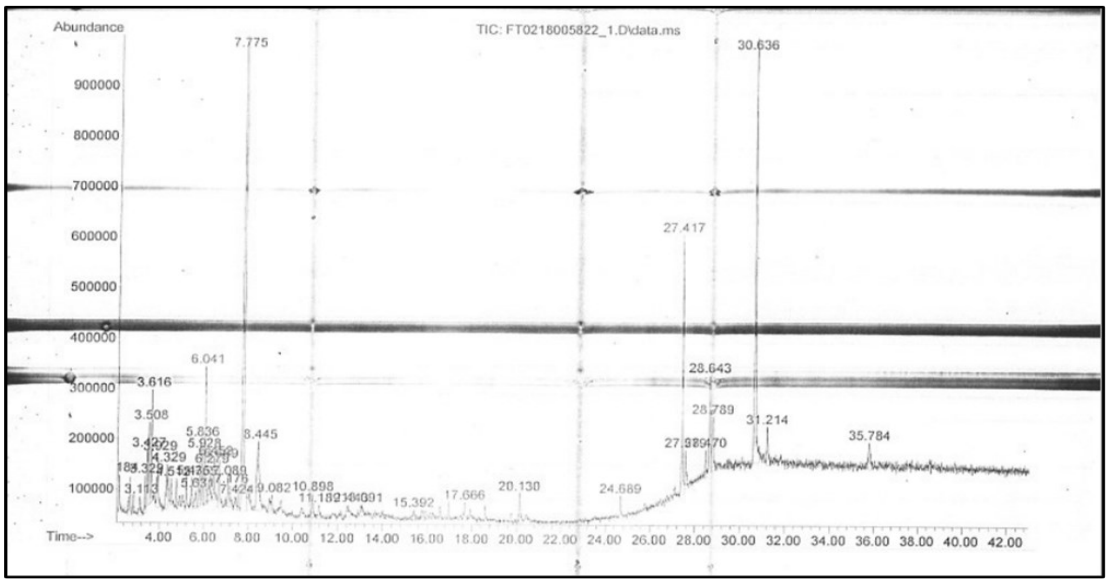

Fig. 3. *GC-MS chromatogram of P. longum aqueous extract*

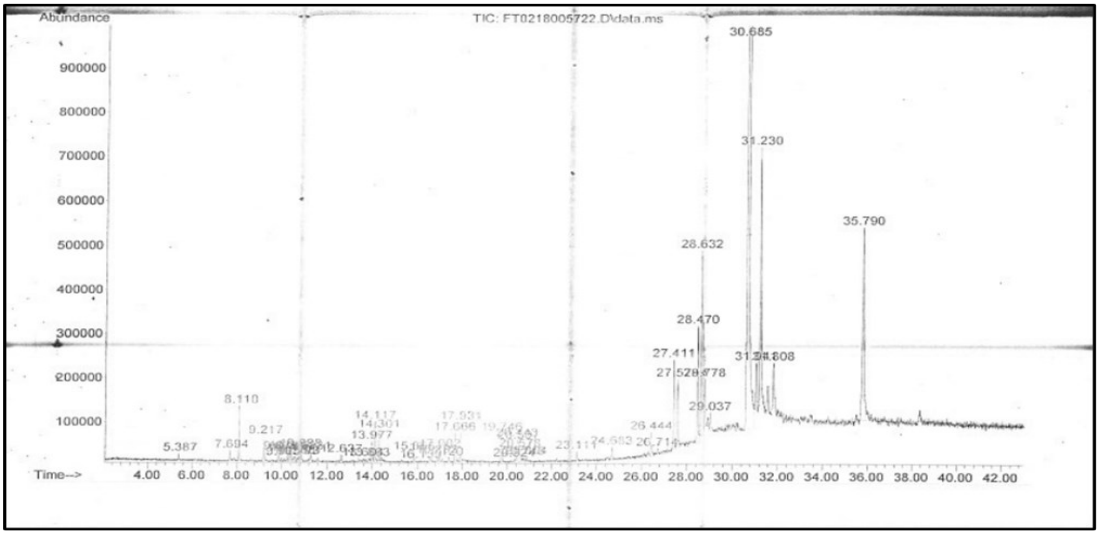

Fig. 4. *GC-MS chromatogram of P. longum chloroform extract*

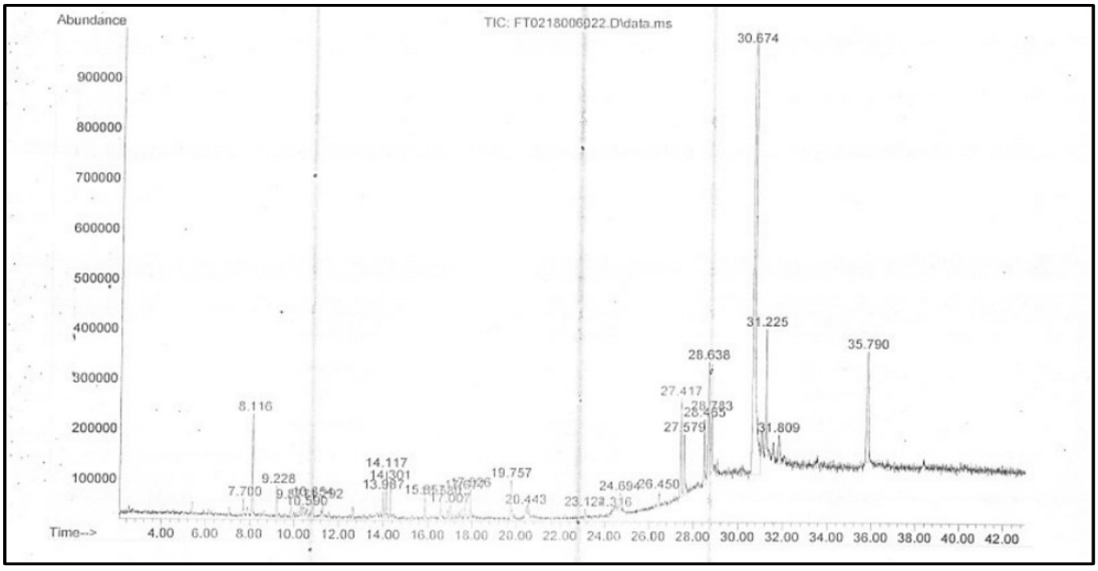

Fig. 5. *GC-MS chromatogram of P. longum hexane extract*

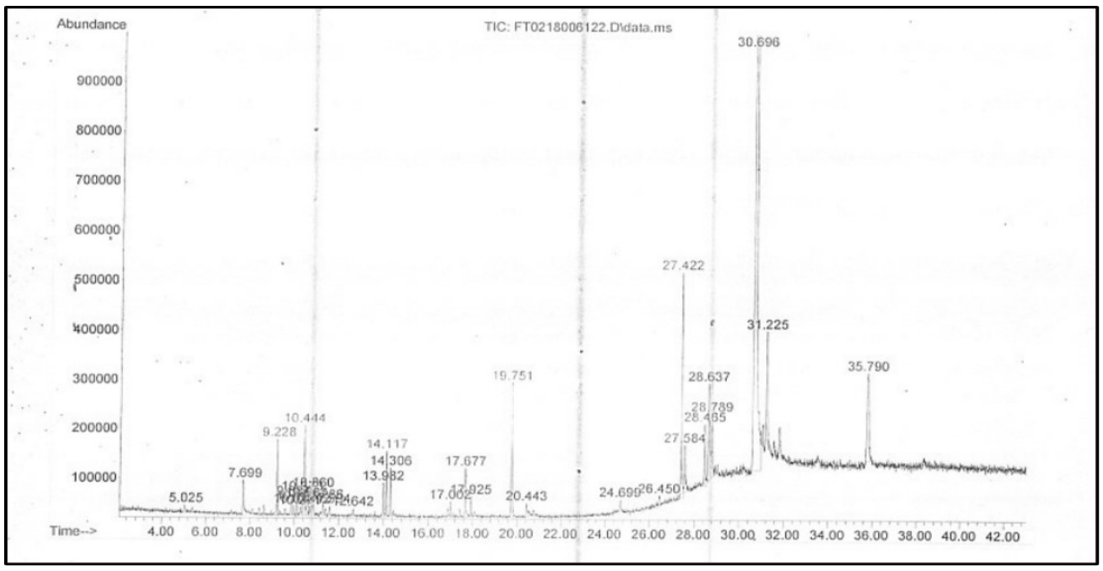

Fig. 6. *GC-MS chromatogram of P. longum methanol extract*

Table 10. Different compounds detected in GC-MS of *Piper longum* chloroform extract

GC-MS of Chloroform			
Peak#	**R. Time**	**Area%**	**Name**
1	5.387	0.09	Cyclooctatraene
2	7.694	0.21	Benzenepropanoic acid
3	8.11	1.02	Phenol, Eugenol
4	9.217	0.52	Caryophyllene
5	9.995	0.09	Acetamide, 2- cyano-N- [{ethylamino}carbony]-2- {methoxyimino}- Urea, 1- ethyl-3 - {propylsulfonyl} -2-thio- 1- Heptadecanamine
6	10.271	0.18	Acetonitrile, Quinolinediol
7	10.363	0.23	Pentadecane, Octacosane, Octane, 5- ethyl-2- methyl-
8	10.438	0.2	Naphthalene
9	10.568	0.2	Alpha- Himachalene
10	10.703	0.1	28- Decadiyne, Thujopsene
11	10.773	0.1	Spathulenol, Cadinene
12	10.838	0.34	Beta- Bisabolene, beta-Farnesene
13	11.281	0.25	Eudesma-4(14), 7(11)-diene, alpha. -Cubene
14	12.637	0.24	alpha. -Bisabolol, Ethanol, 2-(3.3-dimethylcyclohexylidene)-, (Z)-
15	13.604	0.09	2-Naphthalenol, 2- Hydroxy-5-methylisophthalaldehyd
16	13.863	0.13	Semicarbazone, N, N- Dimethyl-dimethyl phosphoric amide, Dewar benzene
17	13.977	0.41	E-14-Hexadecenal, 3- Heptadecene, (Z)-, Cyclopentene, decyl-
18	14.117	0.7	3- Heptadecene, (Z)- 8- Heptadecene
19	14.301	0.52	Heptadecane, Pentadecane, Octadecane
20	15.856	0.25	Angelicin, 2H-Naphtho[1,8-bc] thiophen-2-one
21	16.159	0.1	Picolinyl acetate, 1,2,5-Oxadiazole
22	16.883	0.11	1- Dodecanesulfonyl chloride, 9- Nonadecene, Cyclohexane, 1,3-dimethyl-, cis-
23	17.002	0.21	4-Methyl-E-9-octadecene, n-Tetracosanol-1, 1-Heneicosanol
24	17.12	0.12	Nonadecane, Dodecane, 2,6,10-trimethyl-Tetracosane
25	17.666	0.69	2 (3H)- Benzothiazolone, 3-Ethoxy-4-methoxybenzaldehyde, 2(3H)- Benzothia- zolone
26	17.931	0.75	n- Decanoic acid, Piperidine, Cinnamoylcocaine- Adipic acid, hexyl iso hexyl ester
27	19.746	0.68	1- Phenyl-1-cyclopropanecarbonitril, 1- Napthalenol, 4- chloro-N- Vinylthio- naphthalene-1-amine
28	20.324	0.1	3-Methylthio-thiophene-2-carboxamide, Rabenzazole, 2- Pyrrolidinone, 5-hydroxy- 5-methyl-1-phenyl-
29	20.443	0.59	Calcium linoleate, Cyclodecene
30	20.507	0.51	Oliec Acid, 6- Octadecenoic acid
31	20.578	0.36	O-Ethyl methylethylphosphinate, 1, 3- cis, 5-cis-Octatriene, 1,3-trans, 5- cis- octa- triene
32	20.745	0.11	Phenanthrene, benzoic acid
33	20.864	0.14	D- Erythro- Pentose, 2-deoxy- N- (3-Methylbutyl) acetamide Octadecanoic acid
34	23.111	0.24	p- Toluic acid, decyl ester Isolongifolene
35	24.683	0.28	3,4- Methylenedioxy- 3- Methyl-4-piperonyl-5-isoxazolone
36	26.444	0.49	N- (2-phenylethyl)- 4- Hydroxy-3- methyl- beta-phenylcinn amonitrile
37	26.714	0.16	2- Ethylacridine Benzenamide, 2- (cyclopropylmethyl)- 4,5-dimethoxy- 2,3,4- Tri- methoxyphenylacetonitrile
38	27.411	1.87	Benamide, 2- methoxy-N-(2,4-dimethoxyphenethyl)- 1,2- Benzenediol, O-(4- methoxybenzoyl)-
39	27.579	1.63	1,4- Naphthalenedione, 2-acetyl-3-hydroxy- (1- Cyclohexyl- 1H- benzoimidazol-5- yl) carbamic acid methyl ester
40	28.47	2.52	Pyrido [2,3-d] pyrimidine, 4-phenyl-
41	28.632	6.18	Pyrimidine-5-on 4, 4'- Bis[phenylthiocarbomate] 3,4- Dimethoxytolune
42	28.778	1.2	Piperine
43	29.037	0.41	Silicic acid, diethyl bis (trimethylsilyl) ester 5-Methyl-2-trimethylsilyloxy- acetophenone Anthracene, 9,10- dihrdro-9.9,10-trimethyl-
44	30.685	54.46	Piperine
45	31.041	1.17	2- Ethylacridine, 1,2- Benzisothiazol-3-amine tbdms
46	31.23	7.83	Benzoic acid, 3-methoxy-, Serratinan-5-one, 8,13-dihydroxy- (8. alpha.,13S)-
47	31.808	1.75	N-methyl-1-adamantaneacetamide
48	35.79	8.99	Acetic acid, 2- (4-propylphenoxy)-N'- [(3-nitrophenyl) methylidene] hydrazide, oth- er isomer Pyrido[2,3-d] pyrimidine, 4-phenyl-

Table 11. Different compounds detected by GC-MS technique of *Piper longum* hexane extract

GC-MS of Hexane			
Peak#	**R.Time**	**Area%**	**Name**
1	7.7	0.52	Benzenepropanoic acid
2	8.116	2.21	Eugenol
3	9.228	0.73	Caryophyllene
4	9.877	0.43	. alpha. - Caryophyllene, cis-. Alpha. -Bisabolene
5	10.59	0.33	Methyl 6,9,12,15,18-heneicosapentaenoate
6	10.854	0.45	Isocaryophillene
7	11.292	0.44	1H-Cyclopropa[a] naphthalene
8	13.987	0.68	8-Heptadecene, Cyclopentene, 1-pentyl-2-propyl-Pentafluoropropionic acid, tetradecyl ester
9	14.117	0.91	EE-14-Hexadecenal, 3-Heptadecene, (Z)-
10	14.301	0.66	Heptadecene, Heneicosane, Heptadecane, 9-octyl-
11	17.007	0.37	2-Dodecen-1-yl (-) succinic anhydride, Cyclohexane, (1-hexyltetradecyl)-
12	17.677	0.66	Hexadecanamide, N-(6-hydroxyl-9H-purin-2-yl)-
13	17.926	0.6	n-Hexadecanoic acid
14	19.757	1.15	2-Quinolinecarboxaldehyde, 8-hydroxy-, 3-n-Butyrylcoumarin
15	20.443	0.39	9,12-Octadecadienoic acid (Z, Z)-
16	23.122	0.38	Benzoic acid, 3-amino-2,5-dichloro Pyridine
17	24.316	0.34	2-(Acetoxymethyl)-3-(methoxycarbonyl) biphenylene, 2-Propen-1-one, 1,3-diphenyl-
18	24.694	0.64	Ethyladamantane-1-carboxylate
19	26.45	0.33	1,1,1,3,5,5,5-Heptamethyltrisiloxane, Cyclotrisiloxane, hexamethyl, Silicic acid,
20	27.417	2.33	Benzamide
21	27.579	1.72	Thieno(2,3-b) quinoline-9-oxide
22	28.465	1.63	5-Methyl-2-phenylindolizine
23	28.638	4.17	Piperine
24	31.225	3.9	2-[4-Cyclohexybutanoylamino]-3-chloro-1,4-naphthoquinone
25	31.809	1.29	Cycloheptatrien
26	35.79	5.11	Phenol, 3-methoxy-2,4,5-trimethyl-, 5-Methyl-2-phenylindolizine

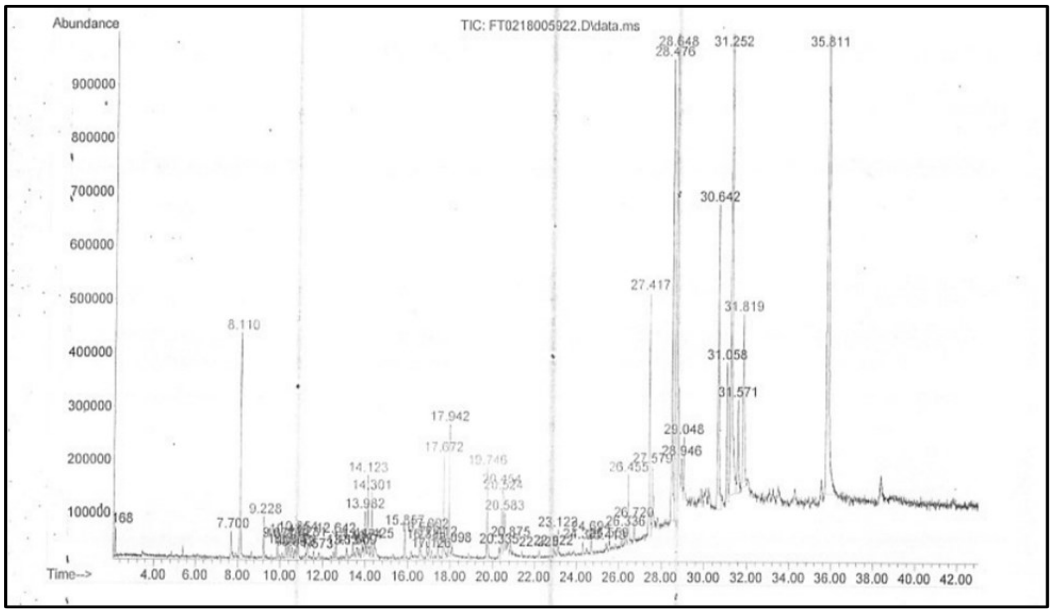

Fig. 7. *GC-MS chromatogram of P. longum petroleum ether extract*

Table 12. Different compounds detected in GC-MS of *Piper longum* petroleum ether extract

GC-MS of Petroleum ether			
Peak#	**R. Time**	**Area%**	**Name**
1	2.168	0.2	2,4-Hexadiyne, Benzene
2	7.7	0.44	Benzenepropanoic acid
3	8.11	2.64	Phenol, 2-methoxy-3- (20propenyl)- Eugenol
4	9.877	0.28	Linalyl isobutyrate
5	10.357	0.2	Pentadecane, Di-n-decylsulfone
6	10.438	0.29	Copane, 2,4-Quinolinediol, alpha. -Cubebene
7	10.573	0.24	Seychellene, 1,1, 4a-Trimethyl-5,6-dimethylenede, cahydronaphthalene
8	10.692	0.12	1H-Benzocycloheotene
9	11.573	0.13	Cyclohexanemethanol
10	13.134	0.23	3-Cyclohexene-1-carboxaldehyde, 3,4-dimethyl- , 3-Octyne, 2,2-dimethyl- , Cyclohexene, 4-butyl-
11	13.404	0.13	2,8-Decadiyne, s-Triazolo [4,3-a] pyrazine, 3,5,8-trimethyl
12	13.609	0.22	3,5-Dimethylanisole, Benzaldehyde, 2-methoxy- 3,5-Dimethylanisole
13	13.874	0.33	. delta. -Selinene, beta. -Guaiene
14	13.982	0.51	8-Heptadecene, 3-Heptadecene, (Z)-, E-14-Hexadecenal
15	14.123	0.84	2-Methyl-Z-7-hexadecene, 3-Heptadecene, (Z)-
16	14.301	0.76	Heptadecane, Eicosane,
17	14.425	0.43	5-Amino-i-phenylpyrazole, 2(1H)- Quinolinone, 3-methyl- 2,3,4-Trifluorobenzoic acid, dodec-9-ynyl ester
18	16.883	0.19	1-Nonadecene
19	17.002	0.4	Z-5-Nonadecene, Tetradecyl trifluoroacetate, 9-Nonadecene
20	17.126	0.12	Pentadecene, 2-methyl-Octadecene, 2-methyl-Octane, 2-methyl-
21	17.412	0.26	Vinyl trans-cinnamate, 1-Penten-3-one, 1-phenyl-
22	17.672	1.48	2', 4'-Dihydroxy-3'-methylpropiophenone, 2{3H}- Benzothiazolone, 4-Amino-2,1,3-benzothiadiazole
23	18.098	0.15	Isoquinoline
24	20.335	0.25	2,4-Dimethyl-6- (2furyl -(2-furyl) pyridine, 1,4,6,7-Tetramethyl 1,2,3,4-tetrahydronaphthalene
25	20.454	1.07	9,12-0ctadecadienoic acid
26	20.583	0.51	7,10,13-Hexadecatrienoic acid, methyl ester, Methyl 7,10,13-hexadecatrienoate, Cyclooctene, 3-ethenyl-
27	20.875	0.25	Octadecanoic acid
28	22.225	0.15	2H-Pyran-2-one, 3-Decanone,1-(4-hydroxy-3-methoxyphenyl)-
29	22.922	0.15	Hentriacontane, Sulfurous acid, butyl tetradecyl ester, Undecane, 3-ethyl-
30	23.122	0.44	4-Amino-2, 3-xylenol, Benzaldehyde, 3-hydroxy-, oxime
31	24.305	0.13	11, Methylnonacosane
32	24.694	0.32	3,4-Methylenedioxy-N, N-diethylbenzylamine Benzene, (3-iodo-1-methoxy-1-methylpropyl)-
33	25.413	0.23	4-cyano-1,5-dihydro-1-oxo-, methyl ester
34	26.455	0.91	Zolpidem, Pyrazole, 5-amino-1,3-diphenyl-
35	26.72	0.23	Benzenamine, 2-(cyclopropylmethyl)-4,5-dimethoxy- Dodecahydropyrido [1,2-b] isoquinolin-6-one
36	27.417	3.54	Benzamide, 2-methoxy-N-(2,4-dimethoxyphenethyl)- Benzamide, N-(2,5-dimethoxyphenyl)-4-methoxy-
37	27.579	1.11	Carbamic acid, 1-naphthalenyl-, methyl ester
38	28.476	6.02	1-Iododec-1-yne-
39	28.648	14.8	trans-Decalin
40	28.946	0.42	Quinoline, 4-chloro-6-methoxy-2-methyl- 2-Methyl-7-phenylindole, 2-Ethylacridine
41	29.048	0.95	5,7-Dihydroxy-4-methylcoumarin Benzeneacetonitrile, 3,4,5-trimethoxy-
42	30.642	5.84	Piperine
43	31.058	2.46	2-Methy-7-phenylindole Methanol, [4-(1,1-dimethylethyl) phenoxy]-, acetate
44	31.252	18.21	Benzoic acid, 3,4-dimethoxy-, perhydro-1-quinolizinylmethyl ester 2,3-Dimethoxytoluene (Octahydroquinolizin-1-yl) methyl 4-nitrobenzoate
45	31.571	2.04	2,3,4-Trimethoxyphenylacetonitrile 1,3-Dimethyl-3-hydroxy-5-methoxyoxindole 1H-Indole, 1-methyl-2-phenyl-
46	31.819	4.04	5,7-Dihydroxy-4-methylcoumarin Dibenzothiophene, 1,2,3,4,6,7,8,9-octahydro-
47	35.811	18.35	Benzene, (2-chloroethenyl)- 2-Chlorostyrene Benzene, (2-chloroethenyl)-, (Z)-

Table 13. Different compounds detected in GC-MS of *Piper longum* aqueous extract

GC-MS of Aqueous			
Peak#	**R. Time**	**Area%**	**Name**
1	2.184	0.8	2-Propanone, 1,2- Epoxy-3-propyl acetate
2	3.113	0.69	d1-Glyceraldehyde Methanamine, N-hydroxy-N-methyl-Urea, N-methyl-N-nitosotroso-
3	3.329	0.62	2-Furanmethanol, 3-Furanmethanol
4	3.427	1.29	2-Pentanol
5	3.508	2.03	N-Methoxymethyl-N-methyl acetamide Dimethyl-ethyl-cyano-phosphine, N-Methyl-N- (2-hydroxyethyl) carbamic acid, phenyl ester
6	3.616	2.03	d1-Glyceraldehyde dimer 2- Propanone, 1,3-dihydroxy-1,3-Dihydroxyacetone dimer
7	3.929	1.95	1H-Imidazole-2-methanol 2-Cyclopenten-1-one, 2-hrdroxy-. alpha. -Pyrrolidone, N-methyl-5-bromomethyl-
8	4.329	1.4	Glycerin
9	4.512	0.65	Pentanal, Formic acid, 2-propenyl ester Butanal, 3-methyl-
10	5.436	0.91	1-Propanol, 2-amino-2- Thiophenecarboxylic acid, 5-(1,1-dimethylethoxy)- 1-Pentanol, 4-amino-
11	5.631	0.66	Cyclohexanone, 3-hydroxy-1,3-Dimethyl-3-n-propyldiaziridine 1,2-Cyclopentanediol, trans-
12	5.755	0.74	Galacto-heptulose Carbamic acid, ethylnitroso-, ethyl ester
13	5.836	1.03	2-Tetrazene
14	5.928	1.29	Pentanal
16	6.279	1.34	Benzenediol
17	6.452	1.49	4-aminobenzoate (ester)
18	6.689	1.48	1,2,3-Propanetriol, monoacetate Acetic acid, trans-4-Nitroso-2,3-morpholindiol, 3-acetate
19	7.089	0.78	2-Propyl-tetrahydropyran-3-ol, D-Alanine, N-butoxycarbonyl-, tetradecyl ester
20	7.176	0.78	. alpha. - Piperidinomethyl-6-benzothiazole methanol, Glutamine, N-methyl-, N-[2-(Perfluorophenoxy) ethyl] piperidine
21	7.424	0.6	2-Propen-1-amine, N-ethyl-Cyclopropanecarboxylic acid, Piperazine, 2-methyl-
22	7.775	32.2	Benzenepropanoic acid
23	8.445	4.73	2-Imidazolidinethione, Glycine, N-ethoxycarbonyl-, nonyl ester
25	10.898	0.96	1,3-Benzodioxole-5-carboxylic acid
26	11.189	0.6	Indan, epoxide, 2-Methylbenzyl cyanide
27	12.443	0.73	Pterin-6-carboxylic acid, Phenethylamine, p-methoxy-. alpha. -methyl-, (. +/-.)-
28	13.091	0.72	Propanenitrile, 3- (methylamino)- Silane, (bromomethyl)-
29	15.392	0.72	5'-O- [N, N-Dimethylsulfamoyl] adenosine, Benzamide, 2-methoxy-N, N-dimethyl-Propargite
30	17.666	0.63	Cyclohexene, 1-heptyl-m-Methoxybenzamide, p-Toluthioamide
31	20.13	1.39	Crabonic acid, thio-, O-methyl O-1-naphthyl ester, 1-phenyl-1-cyclopropanecarbonitril
32	24.689	0.71	Benzene, (3- iodo-1-methoxy-1-methylpropyl)-, Adamantane, 2-(dicyanocyclohexylmethyl)-, Benzamide, 2-methoxy-N-allyl-
33	27.417	6.23	2-methoxy-N- (2,4-dimethyloxyphenethyl)- 4, O-Anisic acid, 4-cyanophenyl ester
34	28.47	0.6	5-Methyl-2-phenylindolizine, -carboniyrile nitrile
35	28.643	3.12	Piperine
36	31.214	1.32	Cyclotrisiloxane
37	35.784	1.32	2'- (trimethylsiloxy)-, Indolizine, 2-(lizine, 2- (4- methylphenyl)-

tiveness in suppressing the non-typhoidal *Salmonella* strains. Methanol extract revealed the maximum zone of inhibition against *S. enterica* subsp. *arizonae* (31mm), whereas Sultana et al. (2019) showed the maximum zone of inhibition of 10 mm only against *Salmonella* typhi.

The current investigation results showed that the MIC values ranging from 0.5 mg/ml to 0.0156 mg/ml were significant for preventing the spread of the four Salmonella strains. The Methanol extract was found to be the most efficient extract, followed by hexane, chloroform, petroleum ether, and aqueous. The Aqueous extract was least effective. Since the MIC index value was 2.0, all extracts were bactericidal. A study carried out by Sawhney *et al.* (2011) reported that the MIC value of all the three extracts of *P. longum* was 0.0625mg/ml and was bactericidal. However, the strains taken for the study were *Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus mutans, Streptococcus pyogenes, Streptococcus pneumoniae* which were different from the present investigation. Chauhan et al. (2017) observed that the *P. longum* ethyl acetate,

aqueous, and methanol extracts were most potent against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas*, and *Salmonella typhi*, with MIC value of 0.25 mg/ml and MBC value of 0.5mg/ml, whereas in the present study, the most potent MIC value was 0.0156mg/ml and MBC value 0.0312 mg/ml.

The methanolic fruit extract of *P. longum* displayed the maximum scavenging activity (81.92%), followed by hexane (80.09%), whereas, Kumar *et al*. (2021) claimed that the scavenging activity of methanol extract of *P. longum* had the highest antioxidant activity at 75%.

Twenty six compounds were found in the hexane extract of *P. longum*, followed by 47 compounds in the petroleum ether, 48 compounds in the chloroform, 28 compounds in the methanol extract, and 37 compounds in the aqueous extract, according to the GC-MS analysis of the various solvent-based extracts of *P. longum,* The following were the evaluated compounds with their Retention time employing all five extracts:

Eugenol (8.11), caryophyllene (9.228), isocaryophyllene (9.228), copane (10.438), naphthalene (10.438), alpha-Longipinene (10.557), 28-Decadiyne (10.703), verbenone (12.642), ecosane (14.301), piperidine (17.931), 1-Indanone (19.751), cyclohexanone (20.443), methylnonacosane (24.305), cyclotrisiloxane (26.45), benzenediol (27.411), naphthyridines (27.584), silicic acid (29.037), and piperine (30.642). Naphthyridines have various properties like antimicrobial, antipsychotic, anti-Alzheimer's, antimalarial, anti-platelets and CB2 receptor agonist (Ojha *et al.,* 2021). Thus, *P. longum* can be a novel possibility for the preparation of herbal medication to treat multi-drug-resistant non-typhoidal *Salmonella* infections due to the high levels of bactericidal, anti-tumour, anti-inflammatory, anti-fungal, and antiviral characteristics of the above-indicated compounds.

Conclusion

The present study concluded that methanol extract of *P. longum* had the highest ZOI values among the five solvent extracts (aqueous, hexane, chloroform, petroleum ether, methanol) and can be particularly effective in suppressing the growth of multidrug-resistant non-typhoidal Salmonella strains . It also showed strong free radical scavenging abilities, with an inhibition of 81.92 %. The study identified a number of notable botanicals based on the solvent polarity index, such as caryophyllene, piperazine, eicosane, and piperine. Thus, the present findings hold great promise for developing a wide range of herbal pharmacological formulations with a high safety profile containing important active bioingredient compounds in *P. longum.*

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Conflict of interest

The authors declare that they have no conflict of interest.

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