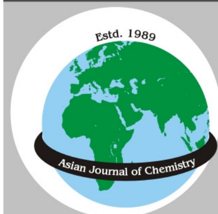


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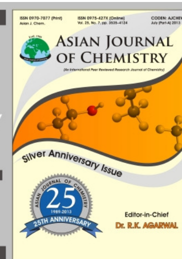



# Phytochemical Analysis of Saponifiable Matter of Petroleum Ether Extract of Leaves of *Butea monosperma*


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The present study designed for phytochemical analysis, antibacterial, antifungal activity of saponifiable matter of petroleum ether extract of leaves of *Butea monosperma* belongs to family Fabaceae. The extraction preformed by soxhlet extractor and identification was performed by phytochemical test. The petroleum ether extract of leaves of *Butea monosperma* was saponified using 1 M alcoholic KOH, to separate saponifiable (fatty material) and unsaponofibale matter. A comprehensive phytochemical investigation of the saponifiable matter of petroleum ether extract of leaves of *Butea monosperma* resulted in the isolation and identification of four known compounds Palmitic acid, tetradecanoic acid, oleic acid and α-linolenic acid. To our best of knowledge, these four compounds were isolated from leaves of plant for the first time. Their structures were identified by physical, chemical, elemental analysis and spectroscopic methods including UV, FTIR, MS, $^1$H NMR, $^{13}$C NMR. The study was further continued for screening of antibacterial activity against *E. coli, P. aeruginosa, B. subtilis, S. aureus* and antifungal activity screened against *A. niger* and *C. albicans* using agar diffusion assay.



**Key Words: *Butea monosperma*, Saponifiable matter, Antibacterial activity, Antifungal activity.**


## INTRODUCTION

In India a large number of plants are being used as drugs due to their medicinal properties. The plant kingdom still holds many species of plants containing substances of medicinal value, which are yet to be, discovered[1]. One of them is leaves of *Butea monosperma*. It is medium sized tree with 20-40 feet height belonging to the family Fabaceae, which is commonly known as 'Palas' in Hindi. It is found in mountain region of India, Burma and few Asian countries[2]. This plant is extensively used in India to treat various diseases. The flowers are used in the treatment of hepatic disorders, viral hepatitis, diarrhoea, anticonvulsive agent and tonic[3-5]. The roots are useful in treatment of night blindness, piles, ulcers, tumer[6]. The gum is powerful astringent. The stem bark possesses antifungal activity. Phytochemical investigation showed the presence of different classes of compounds *e.g.*, flavonoids[7,8] from flowers, sterols[9] from stem bark. β-Carotene and stigmasterol[10,11] were isolated from unsaponifiable matter of leaves of *Butea monosperma*. Chloroform extract also confirmed the presence of sterols by GC-MS analysis[12]. In continuation of our studies on medicinal plants the literature survey reveals that very less work is done on leaves of *Butea monosperma*. In present paper we are reporting the isolation, characterization and their antibacterial, antifungal activity of four known compounds isolated from saponifiable matter of petroleum ether extract of leaves of *Butea monosperma*.

## EXPERIMENTAL

Melting points were measured on melting point apparatus. UV spectra were recorded in $CHCl_3$ solution. FTIR was taken in KBr (MAGANA 550, range 4000 to 50 cm$^{-1}$ Make- Nicolet Instruments Corporation, USA), $^1$H NMR and $^{13}$C NMR spectra were taken on 300 MHz and 75 MHz respectively. (Mercury plus 300 MHz Make Varian USA). Mass spectra were taken on 410 Prostar Binary LC with 500 MS IT PDA Detectors. (Make Varian Inc, USA having specification of direct Infusion Mass with ESI & APCI Negative & Positive mode ionization, mass ranging from 50 to 2000 m/e). Elemental analysis was done on Flash EA 1112 series (Make-Thermo Finnegan, Italy).

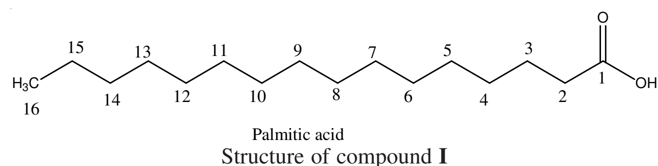
The *Butea monosperma* leaves were hand picked in the summer season of year 2007 from land which near to Village-Mhasawad, District-Nandurbar, Maharashtra, India. The land of this village is part of famous Satpuda ranges. The plant specimen was identified and authentificated from Botanical Survey of India Pune. A voucher specimen (No. BSI/WRC/

Tech/2010/1035) has been deposited at the department of botanical Survey of India, Pune.

**Extraction and isolation:** The leaves were carefully examined and old, insect damaged, fungus-infested leaves and twigs were removed. Healthy leaves were spread out and air dried at room temperature until they broke easily by hand. Leaf material was ground using mechanical grinder and the powder was sieved through sieve No. 14 (Mesh size-1410 μ) and stored in air tight containers. The sieved powder was used for extraction and evaluation purpose.

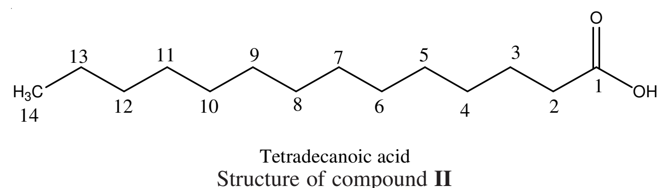
Accurately weighed 50 g of leaves powder was loaded in Soxhlet's extractor and defatted with petroleum ether[13] (60-80 ºC) in 20 batches (50-60 cycles in each batch). During extraction the temperature not exceeding the boiling point of solvent. The progress of the extraction was evaluated by applying spot of extract on thin layer chromatography plate. The thin layer chromatography was performed using silica gel plate and the plate was visualizing in UV-chamber followed by iodine chamber. Complication of extraction was confirmed by no appearance of spot on TLC plate by visualized in UV-chamber followed by iodine chamber. The extract was filtered and concentrated by rotary evaporator and finally dried at very low pressure then we obtained dark greenish black semisolid mass. The petroleum ether extract confirms the presence of sterols and triterpenes[10,14-16]. The petroleum ether extract of leaves of *Butea monosperma* was saponified using 1 M alcoholic KOH, to separate saponifiable (fatty material) and unsaponifiable matter[17]. Then unsaponifiable matter subsequently picked up in ether, solvent was evaporated, while aqueous layer contain saponifiable matter (fatty material). Aqueous layer was filtered through Whatman filter paper under vacuum, water was evaporated on rotary evaporator at 50-55 ºC, then extract kept in desiccators under vacuum to remove moisture until constant weight obtained. A small portion of saponified matter of petroleum ether extract was dissolved in $CHCl_3$ and solution was spotted on TLC plates. Then TLC plates were run by specific solvent system and were viewed individually under UV light[18]. TLC profile of saponifiable matter was developed for the presence of fatty acids using several solvent systems but hexane + diethyl ether (8.5 + 1.5) showed good resolution. It showed four different spots on TLC. Therefore, the saponifiable matter was separated by column chromatography using solvent system hexane + diethyl ether (8.5 + 1.5). Column chromatography of saponifiable matter was conducted using silica gel (mesh 60-120) the silica gel activated at 110 ºC for 1 h then it was mixed with mobile phase and slurry was prepared. The column was filled with slurry of silica gel without formation of any air bubble. The column was then allowed to stabilize for overnight. The 4 g of saponifiable matter vigorously mixed with silica gel and solvent (chloroform). The solvent was then evaporated to free flowing material. This material was charged in column. The column was run using hexane + diethyl ether (8.5 + 1.5) by gradient elution technicque. One hundred thirty fractions each of 5 mL was collected. TLC was used to monitor each fraction. Fraction shows similar separation (*i.e.,* same $R_f$ value) on TLC plates were mixed. The fraction showing single spot (*i.e.* same $R_f$ value) on TLC were mixed considering as single compound, solvent evaporator on rotary evaporator at 40-45 ºC. Then compounds kept in desiccators under vacuum until constant weight obtained. However fractions showing inseparable mixture of compound were rejected. Further purification is carried out by recolumn chromatography and finally by preparative thin layer chromatography, bands were identified, scraped and extracted with chloroform. Four pure compounds were isolated by column, recolumn and by preparative thin layer chromatography. They were numbered as **I**, **II**, **III** and **IV**. These isolated compounds have been characterized and their structures were identified by physical, chemical, elemental analysis and spectroscopic techniques like UV, IR, $^1$H NMR, $^{13}$C NMR, Mass and GC-MS.

Compound **I** was isolated as white crystals, m.p. 63-64 ºC, yield 80 mg (2.88 %), 256 [$M^+$ + 1]; 255 [$M^+$], Calcd. (found) (%): C 74.94 (75.11), H 12.58 (12.36), IR (KBr, $\nu_{max}$, $cm^{-1}$): 3400-3021, 2916, 1702, 1050, 2848. $^1$H NMR (300 MHz, $CDCl_3$), δ 0.86-0.89 (3H, t, H-16), 1.25-1.30 (24H, m, H-3-H-14), 1.59-1.67(2H, q, H-2)2.32 -2.36 (2H, t, H-1). $^{13}$C NMR (75 MHz, $CDCl_3$), d 14.3 (C-16), 22.9 (C-15), 24.9 (C-14), 29.28- 29.90 (C-3-C-13), 32.1 (C-2), 34.34 (C-1), 180.17 (C-1).



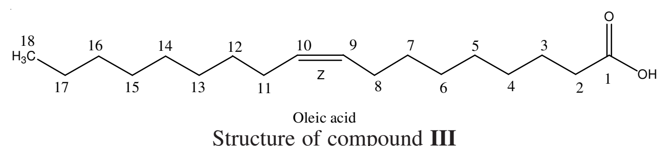
Structure of compound **I**

Compound **II** was isolated as white crystals, m.p. 63-64 ºC, yield 70 mg (2 %), 229 [$M^+$ + 1], Calcd. (found) (%): C 73.63 (73.69), H 12.36 (12.29), IR (KBr,$\nu_{max}$, $cm^{-1}$): 3316, 2916, 2849,1701,1471, 1092, 721. $^1$H NMR (300 MHz, $CDCl_3$), δ 0.86-0.89 (3H, t, H-16), 1.25-1.30 (24H, m, H-3-H14), 1.59-1.67 (2H, q, H-2), 2.32-2.36 (2H, t, H-1). $^{13}$C NMR (75 MHz, $CDCl_3$), δ 14.3 (C-16), 22.9 (C-15), 24.9 (C-14), 29.28-29.90 (C-3-C-13), 32.1 (C-2), 34.34 (C-1), 180.17 (C-1).



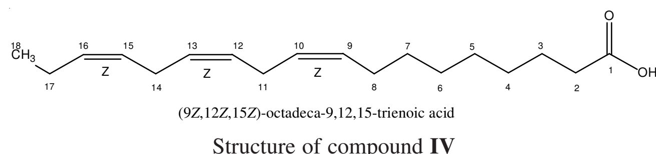
Structure of compound **II**

Compound **III** was isolated as colourless liquid, b.p. 286-288 ºC, yield 90 mg (2.57%), 283 [$M^+$ + 1], 282 [$M^+$], Calcd. (found) (%): C 76.54 (73.55), H 12.13 (11.76), IR (KBr, $\nu_{max}$, $cm^{-1}$): 1707, 1618, 1558. $^1$H NMR (300 MHz, $CDCl_3$), δ 0.86-0.89 (3H, t, H-1), 1.26 -1.31 (20H, m, H-3-H-7, H-12-H-16), 1.61-1.65 (2H, m, H-2), 1.61-1.65 (2H, q, H-11), 1.98-2.00 (2H, q, H-8), 2.32-2.36 (2H, t, H-17), 5.32-5.35 (1H, m, H-9-H-10). $^{13}$C NMR (75 MHz, $CDCl_3$), δ 14.3 (C-1), 22.90 (C-2), 24.87 (C-3,C-16), 27.37 (C-11), 34.34 (C-17), 32.14 (C-8), 129.90-130.20 (C-9-C10), 180.80 (C-18), 29.26-29.99 (C-4,5,6,7,12,13,14,15).



Structure of compound **III**

Compound **IV** was isolated as colourless liquid, b.p. is 230-232 ºC, yield 65 mg (1.85 %), 280 [M$^+$ + 1], 279 [M$^+$], Calcd. (found) (%): C 77.65 (71.61), H 10.8 (10.84),IR (KBr, $\nu_{max}$, cm$^{-1}$): 3400-3010, 2954, 2924, 1709, 1641, 1549, 1459. $^1$H NMR (300 MHz, $CDCl_3$), δ 0.87-0.90 (3H, t, H-18), 1.27-1.39 (10H, m, H-3, H-4, H-5, H-6, H-7), 1.61-1.65 (3H, t, H-8), 2.02-2.07 (4H, q, H-11, H-14), 2.32-2.36 (2H, t, H-2), 5.31-5.38 (6H, m, H-9, H-10, H-12, H-13, H-15, H-16). $^{13}$C NMR (75 MHz, $CDCl_3$), δ 14.27 (C-18), 22.9-34.2 (C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-11, C-14, C-15, C-16, C-17), 130.21 (C-12, C-13), 130.41 (C-9, C-10), 180 (C-1).




Structure of compound **IV**

**Antibacterial and antifungal activity:** The isolated compounds were preserved in labeled sterile screw capped bottles at -20 ºC. The test solution of isolated compounds and standard solution were prepared. Antibacterial and antifungal activity assays were performed by the modified disc diffusion method[19-22]. The bacterial and fungal strains used were obtained from National Collection of Industrial Microorganism (NCIM), Pune, India. Petri dishes (5 cm diameter) were filled up to a depth of 3-4 mm with sterile nutrient agar (Hi-Media) for bacteria and meat extract, glucose yeast extract, peptone medium *i.e.* yeast for fungi. A sterile Whatman filter paper disc of 6 mm diameter preloaded with 100 mcg of target compound in DMSO was placed in the centre of the nutrient agar plates of bacteria and MAYP plates of fungi. Four plugs of bacterial inoculums and fungal inoculums were placed upside down at the quarter circle points 20 mm radius around the drug loaded disc in the Petri dishes. Blank control disc were treated with DMSO, Chloramphenicol for bacteria and amphotericin-B for fungi was used as standard. The stringent aseptic conditions were maintained during microorganism inoculation and the plates were labeled. The Petri plates were incubated at 37 ± 1 ºC for 24 h for antibacterial screening and at 25 ºC for 2-7 days for antifungal screening. The diameter of zone of inhibition of each disk was recorded.

## RESULTS AND DISCUSSION

The saponifiable matter of petroleum ether extract afforded a compound **I**, m.f. $C_{16}H_{32}O_2$, m.p. 63-64 ºC. MS spectroscopy showed the molecular ion peak[23] at 256 [M$^+$ + 1], 255 [M$^+$], which corresponds to molecular formula $C_{16}H_{32}O_2$.

In the IR spectrum of compound **I** showed an intensively broad band at 3400-3021 cm$^{-1}$ which can be assigned to -COOH and band at 1702 cm$^{-1}$ represented >C=O vibration[24]. The intense band at 2916 cm$^{-1}$ and medium intensity band at 1458 cm$^{-1}$ noticed the stretching and bending vibration of methyl part. The vibration of the methylenic part was shown by band at 2848 cm$^{-1}$. The moderate intense band at 720 cm$^{-1}$ was attributed to rocking movement of methylene part, the corresponding carbon-carbon single bond vibrations showed weak intense band[16] at 1050 cm$^{-1}$.

The $^1$H NMR spectrum of compound **I** showed the multiplate of 24 H at 1.25-1.30 d which indicated the presence of twelve -$CH_2$- group in long chain[25]. It showed triplet of 3H at 0.86-0.89 δ which indicated terminal -$CH_3$ group attached to long chain -$CH_2$ group (*i.e.* $CH_3$-$CH_2$-). It also showed triplet of 2H at 2.32-2.36 δ which indicated -CO-$CH_2$-$CH_2$- group.

The $^{13}$C NMR spectrum of compound **I** showed doublet of 1C at 180.78 indicating the -COOH group[25]. It showed triplet at 34.34 indicates -CO-$CH_2$- group (α-carbon), triplet at 32.156 indicated the signal of -$CH_2$- group (β-carbon). It showed quartet of terminal -$CH_3$ group attached to long chain -$CH_2$- group (*i.e.* $CH_3$-$CH_2$-) at 14.313. It also showed triplet of ten -$CH_2$- in between 22.91-29.914.

The saponifiable matter of petroleum ether extract afforded a compound **II**, m.f. $C_{14}H_{28}O_2$, m.p. 60-61 ºC. It gives all the characteristic reactions of acids. MS spectroscopy of compound II showed the molecular ion peak[23] at 229 [M$^+$ + 1], which corresponds to molecular formula $C_{14}H_{28}O_2$.

In the IR spectrum[24] of compound **II** an intensively broad band at 3316 cm$^{-1}$ which can be assigned to -COOH and band at 1701 cm$^{-1}$ represented >C=O vibration. The intense band at 2916 cm$^{-1}$ and medium intensity band at 1471 cm$^{-1}$ noticed the stretching and bending vibration of methyl part. The vibration of the methylenic part was shown by band at 2849 cm$^{-1}$. The moderate intense band at 721 cm$^{-1}$ was attributed to rocking movement of methylene part, the corresponding carbon-carbon single bond vibrations showed weak intense band at 1092 cm$^{-1}$.

The $^1$H NMR spectrum of compound **II** showed the multiplate of 20 H at 1.21-1.24 δ which indicated the presence of ten -$CH_2$- groups in long chain[25]. It showed triplet of 3H at 0.91-0.93 δ that is strong evidence of terminal -$CH_3$ group attached to long chain -$CH_2$- group (*i.e.* $CH_3$-$CH_2$-). It also showed triplet of 2H at 2.32-2.36 δ, which indicated the alpha carbon -$CH_2$- group attached to -CO- group (-CO-$CH_2$-$CH_2$- group).

The $^{13}$C NMR-spectrum of compound **II** showed doublet of 1C at 180.78 indicates the -COOH group[25]. It showed triplet at 34.34 indicated -CO-$CH_2$- group (α-carbon), triplet at 32.156 indicates the signal of -$CH_2$- group (β-carbon). It showed quartet of terminal -$CH_3$ group attached to long chain -$CH_2$- group (*i.e.* $CH_3$-$CH_2$-) at 14.313. It also showed triplet of ten -$CH_2$- in between 22.91-29.914.

The phytochemical and spectroscopic assignments are in good agreement for the structure of tetradecanoic acid[26].

The saponifiable matter of petroleum ether extract afforded a compound **III**, m.f. $C_{18}H_{34}O_2$, m.p. 286-288 ºC. MS spectroscopy of compound **III** showed the molecular ion peak[23] at 283 [M$^+$ + 1], 282 [M$^+$], which corresponds to molecular formula $C_{18}H_{34}O_2$.

In the IR spectrum of compound **III** an intensively broad band at 2924 cm$^{-1}$ which can be assigned to -COOH[24] and band at 1707 cm$^{-1}$ represented >C=O vibration. The bands at 1618 and 1558 cm$^{-1}$ represented (>C=C<) vibration which indicates the presence carbon-carbon double bond.

The $^1$H NMR spectrum of compound **III** showed triplet of 3H at 0.86-0.87 and multiplate of 2H at 1.61-1.65 indicated the presence $CH_3$-$CH_2$-$CH_2$- group[25]. It showed δ value 5.32-5.35, multiplate of 1H this value indicated olefinic

proton $-CH_2-HC=CH-CH_2-$ group. In the IR spectrum of the compound there is strong evidence of -COOH group, therefore it showed triplet of 2H at 2.32-2.36 indicating $-CH_2-CH_2-CH_2-COOH$ group. It showed quartet of 2H at 1.98-2.00 which indicated the presence of $-CH_2-CH_2-HC=CH-$ group. It also showed quartet of 2H at 1.61-1.65 indicating the presence of another $-CH_2-$ group at another side of double bond. $-HC=CH-CH_2-CH_2-$ group. It showed multiplate of 20H (ten equivalent groups $-CH_2-$) at 1.26-1.31.

The $^{13}C$ NMR-spectrum of compound **III** showed 18 carbon signals[25]. It showed one signal for >C=O at 180.80 δ. indicates the -COOH group. It showed one signal at 34.34 δ indicates $-CO-CH_2-$ group (α-carbon), one single at 24.87 δ for two equivalent carbons indicates the presence of two equivalents $-CH_2-$ group. It showed one signals for >C=C< groups at 129.90-130.20 δ. It showed signal of terminal $-CH_3$ group attached to long chain $-CH_2-$ group ($CH_3-CH_2-$) at 14.3 δ. It also showed signal for thirteen $-CH_2-$ in between 22.90-34.34 δ.

The phytochemical and spectroscopic assignments are in good agreement for the structure of oliec acid (9-octadecanoic acid.)

The saponifiable matter of petroleum ether extract afforded a compound **IV**, m.f. $C_{18}H_{32}O_2$, m.p. 286-288 ºC. MS spectroscopy of compound IV showed the molecular ion peak[23] at 280 [$M^+$ + 1], 279 [$M^+$], which corresponds to molecular formula $C_{18}H_{30}O_2$.

In the IR spectrum[24] of compound **IV** an intensively broad band at 3010-3400 and 2954 $cm^{-1}$ which can be assigned to -COOH and band at 1709 $cm^{-1}$ represented >C=O vibration. The intense band at 2924 $cm^{-1}$ and weak intense band at 1459 $cm^{-1}$ noticed the stretching and bending vibration of methyl part. The band at 1641 and 1549 $cm^{-1}$ represent >C=C< stretching. This indicated the presence of unsaturated acid.

The $^1H$ NMR spectrum of compound **IV** showed the multiplate of 6H at 5.31-5.38 δ this signal is strong evidence of olefinic proton[25]. As it showed multiplate of 6H means it may have three equivalent olefinic protons (three carbon-carbon double bond.) It showed of 2H at 2.32-2.36 and IR spectrum showed the band at band at 3010-3400 and 2954 $cm^{-1}$ which can be assigned to -COOH therefore the group may be $-CH_2-COOH$. It showed triplet of 3H at 0.87-0.9 indicating the $CH_3-CH_2-$ group. It showed multiplate of 10H at 1.27-1.39 which indicated the presence of five equivalents $-CH_2-CH_2-$ group. It showed multiplate of 2H at 2.75-2.78 which indicated that it has $CH_3-CH_2-CH=CH-$ group. It showed quartet of 2H at 1.65-1.67 it is evidence of $-CH=CH-CH_2-$ on another side of double bond.

The $^{13}C$ NMR-spectrum of compound **IV** showed the presence 18 carbon in the compound[25]. It showed one signal for >C=O at 180.00 indicates the -COOH group. It showed one signal at 34.32 indicates $-CO-CH_2-$ group (α-carbon). It showed signal for ten $CH_2$ groups at 22.9-29.80. It showed three signals for >C=C< groups at 128.12-129.29, 130.21, 130.41. It showed signal of terminal $-CH_3$ group at 14.27. The phytochemical and spectroscopic assignments are in good agreement for the structure of (Z,Z,Z)-9,12,15-octadecatrienoic acid (α-linolenic acid).

**Antibacterial and antifungal activities:** The results of antibacterial activity were tabulated in Table-1. The results of antifungal activity were tabulated in Table-2.

TABLE-1
ANTIBACTERIAL SCREENING

| Compound | Zone of inhibition (mm) | | | |
|---|---|---|---|---|
| | *E. coli* | *P. aeruginosa* | *B. substalis* | *S. aureus* |
| Control (DMSO) | - | - | - | - |
| **I** | 9.45 | 8.02 | 9.62 | - |
| **II** | - | - | - | 8.05 |
| **III** | - | 41.17 | - | 16.19 |
| **IV** | - | - | 8.83 | 9.09 |
| Chloramphenicol[1] | 24.79 | 19.54 | 18.15 | 33.66 |

[a]Zone of inhibition in mm, diameter in mm calculated by venire caliper; (–) Means no zone of inhibition.
[1]Concentration of chloramphenicol was set to10 μg/disc.

TABLE-2
ANTIFUNGAL SCREENING

| Compound | Zone of inhibition (mm) | |
|---|---|---|
| | *A. niger* | *C. albicans* |
| **I** | - | - |
| **II** | - | - |
| **III** | - | 12.22 |
| **IV** | - | - |
| Amphotericin-B[1] | 16.04 | 14.23 |

[a]Zone of inhibition in mm, diameter in mm calculated by venire caliper; (–) Means no zone of inhibition.
[1] Concentration of amphotericin-B was set to10 μg/disc.

The compound **III** showed good inhibition zone for *P. aeruginosa*. Other compounds do not showed significant antibacterial activity as compared to standard. Only compound **III** (oleic acid) showed antifungal activity, while other compounds did not show antifungal activity.

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