Volume 65, Issue 2, 2021
Journal of Scientific Research
Institute of Science,
Banaras Hindu University, Varanasi, India.

# Phytochemical Screening of *Calendula Officinalis (Linn.)* Using Gas-Chromatography-Mass Spectroscopy with Potential Antibacterial Activity

Darekar D. P.[*1], Hate M. S.[2]

[*1]Ramnarain Ruia Autonomous College, Matunga, Mumbai 400 019, ddipika1992@gmail.com
[2]Ramnarain Ruia Autonomous College, Matunga, Mumbai 400 019, manishhate@ruiacollege.edu

***Abstract:*** ***Calendula officinalis L.*** **( Pot Marigold ) which belongs Asteraceae family is a yellow to orange flowers and known medicinal plant used in folk medicine from the ancient times. It has been found to have lots of medicinal properties, particularly for its antibacterial and antioxidant activities. The primary goal of this study is to evaluate phytochemicals present in *Calendula Officinalis (Linn.)* using Gas-Chromatography-Mass Spectroscopy technique with potential antibacterial activity. A total 48 compounds were identified and analysed from chloroform extract of *C.officinalis*, using GC-MS. The major components of the *C. officinalis* extract were Caryophyllene (12.97%), Lupeol (9.45%), and Stigmasterol (9.38%), Gamma.-Sitosterol (5.07%). Antibacterial effects of different extracts of *C.officinalis* flowers against gram negative and gram positive bacteria were determined by disc diffusion method. Chloroform extracts showed excellent antibacterial effects against tested strains of gram positive (Staphylococcus aureus, Bacillus subtilis and Enterococcus faecalis) and one strains of gram negative (Klebsiella pneumonia) bacteria.**

***Index Terms:*** ***Calendula officinalis L.*****, Caryophyllene, Enterococcus faecalis, Gamma.-Sitosterol, Klebsiella pneumonia. Lupeol, Staphylococcus aureus, Stigmasterol *Bacillus subtilis***

## I. INTRODUCTION

Analytical science in recent years has progressed spectacularly with the discovery of new separation methods, (Sahingil D. et al., 2019; Sivananthan M. et al., 2013). It is an important and integral components of analytical science because few instrumental methods can be directly used for quantitative analysis on account of the presence of interfering substances (Agrawal et al., 2006; James et al., 1952), Plant phytoconstituents as a source of medicinal actives have been reported in literature, since ancient times (Tewari D. et al., 2012; Tosun G. et al., 2012). Many plants are used as folk medicines to infectious diseases (Jan AK et al., 2019; Moghtader M. et al., 2016). The most important of these bioactive constituents of plants are alkaloids, tannins, flavanoids and phenolic compounds (Khalid KA et al., 2012; Markham KR et al., 1975). *Calendula Officinalis (Linn.)* family Asteraceae is a known medicinal plant and is used in folk medicine from the ancient times (Ourabia I et al., 2019; Wilken JN et al., 2016). It has been found to have lots of medicinal properties, particularly for its antimicrobial and antioxidant activities (Ali EM et al., 2017; Hamza LF et al., 2018). Therefore, investigation of the chemical compounds within medicinal plants has become desirable (Pandey A et al., 2014). Gas-Chromatography-Mass Spectroscopy (GC-MS) has been used widely for analysis of herbal medicinal extracts (Mohammed GJ et al., 2018). Due to its simplicity of operation, speed, versatility and reproducibility, as a number of samples can be analysed simultaneously on a single run using only a small amount of sample (Al Mussawi et al., 2019; Chalchat JC et al., 1991). The main aim is to separate and identify phytochemicals from *C. Officinalis* flowers with respect to caryophyllene, lupeol, stigmasterol and gamma.-sitosterol and to study its antibacterial activities (Bogdanova NS et al., 1970; Jalill A et al., 2014; Prasad S et al., 2008). (Refer Fig.1, Fig.2, Fig 3 and Fig.4).

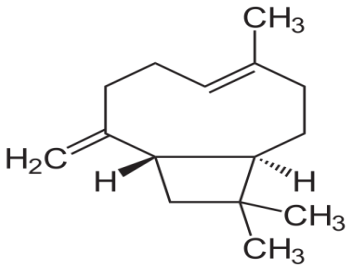


Fig. 1. Chemical structure of Caryophyllene

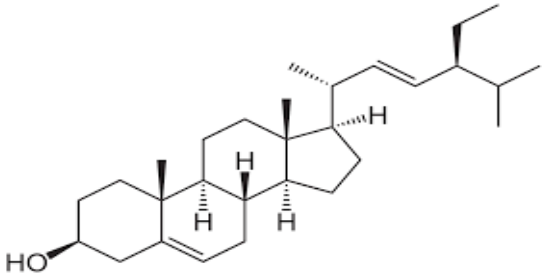


Fig. 2. Chemical structure of Stigmasterol

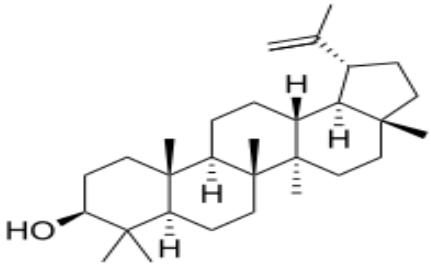


Fig. 3. Chemical structure of Lupeol

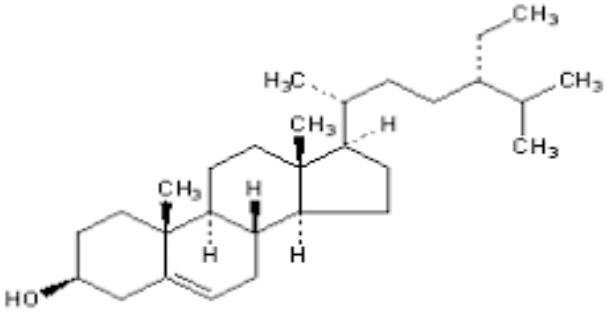


Fig. 4. Chemical structure of Gamma.-Sitosterol

## II. EXPERIMENTAL

### A. Materials and Methods

#### 1) Collection of Plants

The fresh flowers of *Calendula Officinalis (Linn.)* were collected from Lonavala, Pune, in the month of February 2016. The plant was botanically authenticated. A Voucher specimen of the plant has been deposited at the herbarium of the Botanical Survey of India, Pune. No. BSI/WRC/IDEN.CER./2017/600.

#### 2) Chemicals and Standards

HPLC grade chloroform were procured from E. Merck, Mumbai, India, Analytical grade ethyl acetate (purity 99.6%), Methanol (purity 99.9 %), DMSO, n-Hexane and HPLC grade water were obtained from E.Merck, Mumbai, India

#### 3) Equipments

Shimadzu System, AOC-20i auto injector, GC for mass spectrometer, GCMS-QP 2010 Ultra

#### 4) Preparation of the Extracts

The dried flowers of *Calendula officinalis (Linn.)* powder was extracted using soxhlet extraction. Five grams of plant powder was weighed and packed in a whatman paper thimble. It was then extracted with 100 ml chloroform for 12 hours using soxhlet extractor. Extracts were filtered through a syringe filter of pore size 0.45 µm before further analysis.

### B. Chromatographic Conditions

GC-MS analysis of the chloroform extract of *Calendula officinalis (Linn.)* was performed using Shimadzu GCMS-QP2010 system comprising a gas chromatograph interfaced to a mass spectrometer equipped with Rtx-5ms column (5% diphenyl/ 95% dimethyl polysiloxane) a capillary column having the length of 30 m, internal diameter of 0.25 mm and film thickness of 0.25 µm. For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1.25 ml/min, and an injection volume of 1 µl was employed (a split ratio of 5:1). The injector temperature was maintained at 250 °C, the ion-source temperature was 200 °C, the oven temperature was programmed from 200 °C (isothermal for 2 min), with an increase of 4 °C/min to 280°C with 10 min isothermal. Total program time was 36 minutes. Mass spectra were taken at 70 eV and recorded over the range of 35 to 600 m/z. Identification of components was based on comparison of their mass spectra with those of Wiley and NIST libraries and those described by Adams as well as on comparison of their retention indices with literature. (Refer Table I)

### C. Antibacterial Activity Assay

The antibacterial assay of *Calendula officinalis (Linn.)* flowers extracts were carried out against *Staphylococcus aureus* ,*Bacillus subtilis* and *Enterococcus faecalis* and *Klebsiella pneumonia* by the disc diffusion method. All the bacteria were collected from the culture collection of Microbiology department of Ramnarain Ruia Autonomous College, Matunga, Mumbai. (Refer Fig.5 Fig.6, Fig.7 and Fig.8) In this assay, the positive control without extracts (solvent) and with flower extracts used .The extracts inhibitions were corrected based on positive

Table I. Optimized Chromatographic Conditions

| **Parameters** | **Description** |
|---|---|
| Instrument | GC-MS QP 2010 Ultra Shimadzu |
| Stationary phase | Rtx-5ms (5% Diphenylpolysiloxane 95% dimethylpolysiloxane) |
| Carrier Gas | Helium |
| Injector Mode | Split |
| Split Ratio | 5:1 |
| Sample Volume | 1 µL |
| Flow Rate | 1.25ml/min |
| Flow control Mode | Linear Velocity |
| Purg Flow | 3ml/min |
| Interface temperature | 320ºC |
| Ion Source Temperature | 200 ºC |
| Run Time | 36 minutes |
| Start m/z | 35 |
| End m/z | 600 |

Fig. 5. Bacillus subtilis (Gram-positive)

Fig. 6. Enterococcus faecalis) (Gram-positive)

Fig. 7. Staphylococcus aureus (Gram-positive)

Fig. 8. Klebsiella pneumonia (Gram-negative)

control values and compared to those of reference control values given in references. The experiments were run in triplicate. (Refer Table II)

*D. Inoculums Preparation*

Each bacterial strain was subcultured overnight at 35 ºC in Mueller-Hilton agar slants. The bacterial growth was harvested using 5 ml of sterile saline water, its absorbance was adjusted at

Table II. Antimicrobial screening test of Calendula officinalis (Linn.) Flowers with different solvents extracts (10 mg/ml) against some bacterial Strains

| Plant used | Solvent extract | Bacterial Stain inhibition zone (mm) | | | |
|---|---|---|---|---|---|
| | | **Bacillus subtilis** | **Enterococcus faecalis** | **Klebsiella pneumonia** | **Staphylococcus aureus** |
| ***Calendula Officinalis (Linn.)*** | Chloroform | 18.5 ± 0.13 | 18.7 ± 0.61 | 15.2 ± 0.57 | 18.8 ± 0.14 |
| | Methanol | 16.8 ± 0.37 | 15.7 ± 0.17 | 14.9 ± 0.0 | 13.7 ± 0.12 |
| | Ethanol | 15.6 ± 0.53 | 11.9 ± 0.34 | 14.1 ± 0.36 | 15.6 ± 0.72 |
| | Water | 13.4 ± 0.11 | 13.1 ± 0.35 | 6.2 ± 0.10 | 15.2 ± 0.62 |
| | n-Hexane | 14.7 ± 0.25 | 14.2 ± 0.20 | 12.1 ± 0.13 | 13.8 ± 0.22 |
| | Ethyl Acetate | 9.5 ± 0.74 | 8.3 ± 0.46 | 0.0 ± 0.0 | 9.1 ± 0.35 |
| | Toluene | 12.3 ± 0.51 | 11.8 ± 0.25 | 13.2 ± 0.25 | 11.3 ± 0.17 |
| | DMSO | 11.4 ± 0.15 | 13.2 ± 0.15 | 14.6 ± 0.37 | 14.3 ± 0.26 |

580 µm and diluted to attain viable cell count of 107 CFU/ml using spectrophotometer.

## III. RESULT AND DISCUSSION

In the present research work, a Gas Chromatography-Mass spectroscopy (GC-MS) method for the simultaneous identification of caryophyllene ,lupeol ,stigmasterol and gamma.-sitosterol from flowers powder of *Calendula Officinalis (Linn.)* has been developed. Simultaneous identification of caryophyllene ,lupeol ,stigmasterol and gamma.-sitosterol is not reported so far in literature. Initial trial experiments were conducted to select a suitable temperature program for accurate analysis of the standards of the various stationary phases i. e. various columns (Rtx-1ms, Rtx-5ms, Famewax) Rtx-5ms gave best results in terms of resolution between analytes of interest. Isothermal analysis, temperature programmed analysis and different flow rates of carrier gas were also tried. Out of which temperature programmed run gave the best resolution between the caryophyllene ,lupeol ,stigmasterol and gamma.-sitosterol.(Refer Fig. 9 and Fig. 10).

The antibacterial activity of *Calendula officinalis (Linn.)* flowers extract were tested using the disc diffusion method at concentration of 10mg/ml . The results, shown in Table II indicate significant difference ($p < 0.05$) in inhibitory activity between Calendula officinalis (Linn.) flowers. These extracts exhibited moderate to appreciable antibacterial activities against three Gram-positive and one Gram-negative bacteria. In all, chloroform extracts and followed by methanol flowers extract of *Calendula officinalis (Linn.)* had higher activity compared to other extracts. Therefore this extracts were the most effective and showed a strong antibacterial activity. (Refer Fig.11, Fig.12, Fig.13, and Fig.14)

## CONCLUSION

A. The method was found to be suitable for qualitative and simultaneous identification of caryophyllene ,lupeol ,stigmasterol and gamma.-sitosterol markers from flower powder of *Calendula Officinalis (Linn.)* using Gas Chromatography-Mass Spectroscopy (GC-MS). The proposed method is simple, rapid, precise and accurate and can be further use for routine quality control analysis. Evaluation of potential antibacterial activity showed interesting results of *Calendula officinalis (Linn.)* against *Bacillus subtilis* (Gram-positive), *Enterococcus faecalis* (Gram-positive), *Staphylococcus aureus* (Gram-positive), *Klebsiella pneumonia* (Gram-negative). The purified components may have even more potency with respect to inhibition of microbes. The proposed method can be further use for purification of individual groups of bioactive components which may further reveal the exact potential of the plant to inhibit several pathogenic microbes encourage the development.

Peak Report TIC

| Peak# | R.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 2.079 | 399496 | 2.55 | 107312 | Dimethylsulfoxonium formylmethylide |
| 2 | 2.799 | 236681 | 1.51 | 123668 | Butane, 1,1-diethoxy-3-methyl- |
| 3 | 3.387 | 42297 | 0.27 | 28350 | Cyclohexanol, 4-methyl-1-(1-methylethyl)- |
| 4 | 4.324 | 32715 | 0.21 | 24336 | .alpha.-Methyl-.alpha.-[4-methyl-3-pentenyl]oxirane |
| 5 | 4.540 | 29947 | 0.19 | 22203 | Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propa |
| 6 | 4.706 | 477005 | 3.04 | 321704 | (Methoxymethyl)trimethylsilane |
| 7 | 5.950 | 541536 | 3.46 | 178062 | Naphthalene |
| 8 | 6.055 | 108278 | 0.69 | 54955 | Dodecane |
| 9 | 8.855 | 123563 | 0.79 | 74806 | Tetradecane |
| 10 | 9.455 | 68691 | 0.44 | 31192 | Cycloheptasiloxane, tetradecamethyl- |
| 11 | 11.427 | 111918 | 0.71 | 69871 | Hexadecane |
| 12 | 12.048 | 25454 | 0.16 | 14171 | Cyclooctasiloxane, hexadecamethyl- |
| 13 | 13.611 | 28766 | 0.18 | 13445 | Methoprene |
| 14 | 13.752 | 73816 | 0.47 | 39133 | Heneicosane |
| 15 | 14.195 | 707892 | 4.52 | 410669 | Phytol, acetate |
| 16 | 14.270 | 158905 | 1.01 | 50376 | 2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E |
| 17 | 14.463 | 137168 | 0.88 | 76858 | Phytol, acetate |
| 18 | 14.658 | 200548 | 1.28 | 123896 | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol |
| 19 | 15.027 | 243801 | 1.56 | 100737 | Lidocaine |
| 20 | 15.515 | 1470035 | 9.38 | 447593 | Stigmasterol |
| 21 | 15.821 | 287675 | 1.84 | 137711 | Hexadecanoic acid, ethyl ester |
| 22 | 17.164 | 322748 | 2.06 | 100441 | Phytol |
| 23 | 17.523 | 279202 | 1.78 | 45596 | (Z)6,(Z)9-Pentadecadien-1-ol |
| 24 | 17.782 | 18866 | 0.12 | 16690 | Octadecanoic acid |
| 25 | 17.855 | 77783 | 0.50 | 25473 | 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl |
| 26 | 18.201 | 24719 | 0.16 | 11092 | Octadecanoic acid, ethyl ester |
| 27 | 21.210 | 47386 | 0.30 | 19709 | Hexadecane, 2,6,10,14-tetramethyl- |
| 28 | 21.728 | 33791 | 0.22 | 15406 | Phenol, 2,4-bis(1-phenylethyl)- |
| 29 | 22.410 | 509340 | 3.25 | 215918 | Pentacosane |
| 30 | 22.801 | 37394 | 0.24 | 16826 | Tetradecanal |
| 31 | 22.887 | 66173 | 0.42 | 24452 | Benzyldiethyl-(2,6-xylylcarbamoylmethyl)-ammoni |
| 32 | 23.017 | 27257 | 0.17 | 16659 | Diisooctyl phthalate |
| 33 | 23.474 | 67504 | 0.43 | 33946 | Eicosane |
| 34 | 24.439 | 1506046 | 9.61 | 722960 | Hexatriacontane |
| 35 | 25.319 | 159452 | 1.02 | 66323 | Tetratetracontane |
| 36 | 25.629 | 83550 | 0.53 | 30831 | Cyclononasiloxane, octadecamethyl- |
| 37 | 26.142 | 2032372 | 12.97 | 954826 | Caryophyllene |
| 38 | 26.736 | 58103 | 0.37 | 24453 | Cyclononasiloxane, octadecamethyl- |
| 39 | 26.996 | 87257 | 0.56 | 29891 | Disulfide, di-tert-dodecyl |
| 40 | 27.554 | 178718 | 1.14 | 41489 | Silane, diethyl(2-phenylethoxy)tetradecyloxy- |
| 41 | 27.742 | 215421 | 1.37 | 44512 | 5,5',8,8'-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthal |
| 42 | 27.984 | 252244 | 1.61 | 67499 | Tetratriacontane |
| 43 | 28.092 | 345793 | 2.21 | 74030 | 1-Heptacosanol |
| 44 | 28.245 | 634968 | 4.05 | 106052 | Stigmast-5-en-3-ol, oleate |
| 45 | 28.583 | 509342 | 3.25 | 144387 | Vitamin E |
| 46 | 30.677 | 1480211 | 9.45 | 265912 | Lupeol |
| 47 | 31.229 | 793596 | 5.07 | 149075 | .gamma.-Sitosterol |
| 48 | 31.514 | 312462 | 1.99 | 60938 | Fucosterol |
| | | 15667885 | 100.00 | 5776434 | |

Fig. 9. Phytochemicals identified by GC-MS in flower extract of Calendula Officinalis (Linn)

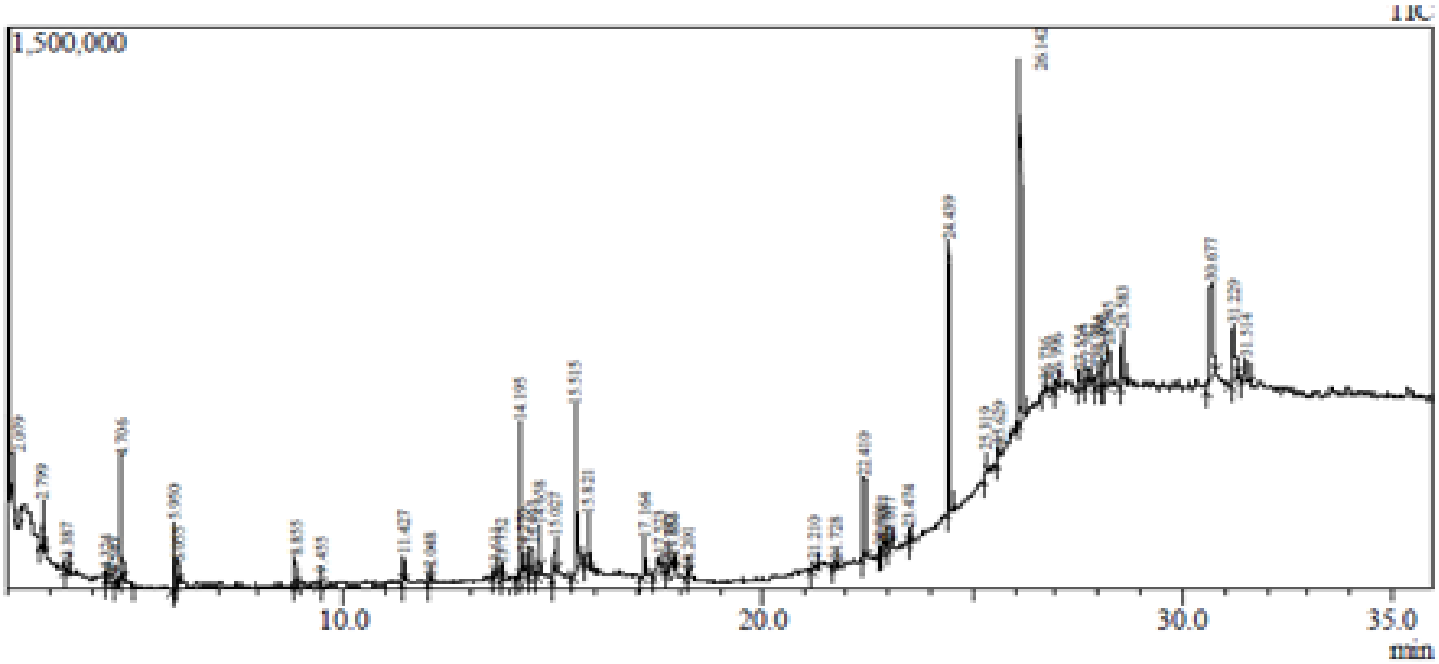


Fig. 10. Chromatogram of Phytochemicals identified by GC-MS in flower extract of *Calendula Officinalis (Linn).*

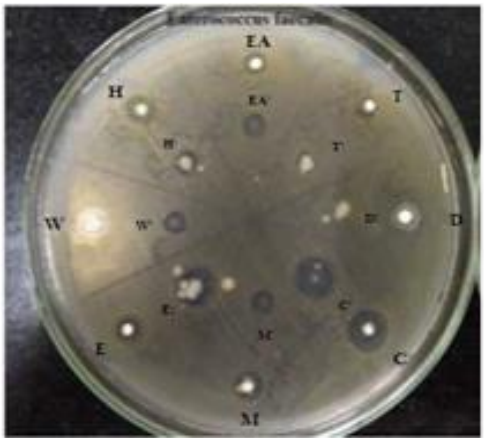


Fig. 11.

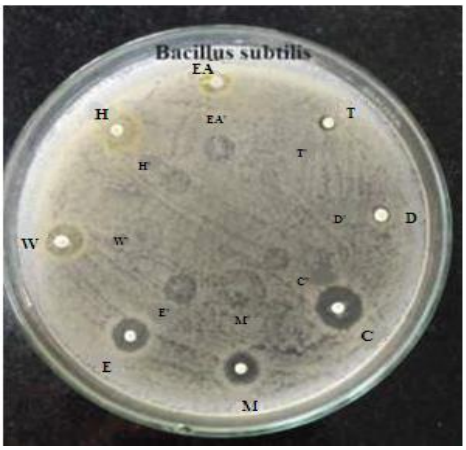


Fig. 12.

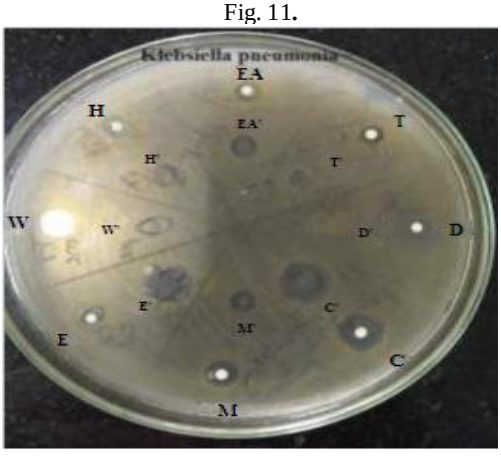


Fig. 13.

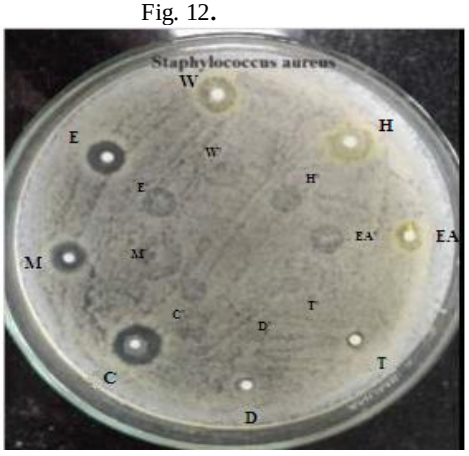


Fig. 14.

Antibacterial Activity Assay Using Disc Diffusion Method

REFERENCES


Agrawal, O.P., Raju, P.S., Abdin, M.Z., & Abroi, Y.P. (2006). Global market of herbal products: Opportunities for Indian Traditional System of Medicine. Narosa Publishing House, New Delhi, India, 5-10.

Ali, E.M., & Abd El-Moaty, H. (2017). Antifungal activity of Achillea santolina L. and Calendula officinalis L. Essential oils and their constituents against fungal infection of liver as complication of cyclophosphamide therapy. *Journal of Essential Oil Bearing Plants*, 20(4), 1030-1043.

Al-Mussawi, Z.K., & Al-Hussani, I.M. (2019). Phytochemical Study of Calendula officinalis Plant by Used Gc-Ms and FTIR Techniques. *Plant Archives*, 19(1), 845-851.

Bogdanova, N.S., Nikolaeva, I.S., Shcherbakova, L.I., Tolstova, T.I., NIu, M., & Pershin, G.N. (1970). Study of antiviral properties of Calendula officinalis. *Farmakologiia i toksikologiia*, 33(3), 349-355.

Chalchat, J.C., Garry, R.P., & Michet, A. (1991). Chemical composition of essential oil of Calendula officinalis L.(Pot Marigold). *Flavour and Fragrance Journal*, 6(3), 189-192.

Hamza, L.F., Sahi, N.M., & Hameed, I.H. (2018). Analysis of Methanolic extract of Secondary Metabolites Released by Candida glabratus using GC-MS and Evaluation of Its Antimicrobial Activity. *Indian Journal of Public Health*, 9(3).

Jalill, A., Dh, R., & Jalill, A. (2014). GC-MS analysis of Calendula officinalis and cytotoxic effects of its flower crude extract on human epidermoid larynx carcinoma (HEP-2). *World Journal of Pharmacy & Pharmaceutical Science*, 3(4), 237-275.

James, A.T., & Martin, A.J. (1952). Gas-liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. *Biochemical Journal*, 50(5), 679.

Jan, A.K., Hazrat, A., Ahmad, S., Jan, T., & Jan, G. (2019). In vitro antifungal, antibacterial, phytotoxic, brine shrimp, insecticidal activities and composition of essential oil of tagetes minuta from dir-kohistan, pakistan. *Pakistan Journal of Botany*, 51(1), 201-204.

Khalid, K.A., & Da Silva, J.T. (2012). Biology of Calendula officinalis Linn.: focus on pharmacology, biological activities and agronomic practices. *Medicinal and Aromatic Plant Science and Biotechnology*, 6(1), 12-27.

Markham, K.R., & Mabry, T.J. (1975). The flavonoids. Harborne, JB, Mabry, T, J and Mabry, H. editions Chapman and Hall, London, 45.

Moghtader, M., Salari, H., Mozafari, H., & Farahmand, A. (2016). Evaluation the qualitative and quantitative essential oil of Calendula officinalis and its antibacterial effects. *Journal of Cellular and Molecular Research (Iranian Journal of Biology)*, 29(3), 331-339.

Mohammed, G.J., Al-Rubaye, A.F., & Hameed, I.H. (2018). Using GC-MS Technique for Analysis of Bioactive Chemical Compounds of Penicillium Italicum and Determination of its Anti-Microbial Activity. *Indian Journal of Public Health*, 9(3), 353.

Ourabia, I., Djebbar, R., Tata, S., Sabaou, N., & Fouial-Djebbar, D. (2019). determination of essential oil composition, phenolic content, and antioxidant, antibacterial and antifungal activities of marigold (calendula officinalis l.) cultivated in algeria. *Carpathian Journal of Food Science & Technology*, 11(2).

Pandey, A., & Tripathi, S. (2014). Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. *Journal of Pharmacognosy and Phytochemistry*, 2(5).

Prasad, S., Kalra, N., Singh, M., & Shukla, Y. (2008). Protective effects of lupeol and mango extract against androgen induced oxidative stress in Swiss albino mice. *Asian Journal of Andrology*, 10(2), 313-318.

Sahingil, D. (2019). GC/MS-olfactometric characterization of the volatile compounds, determination antimicrobial and antioxidant activity of essential oil from flowers of calendula (Calendula officinalis L.). *Journal of Essential Oil Bearing Plants*, 22(6), 1571-1580.

Sivananthan, M. (2013). Antibacterial activity of 50 medicinal plants used in folk medicine. *International Journal of Biosciences*, 3(4), 104-21.

Tewari, D., Sah, A.N., Pandey, H.K., Meena, H.S., Meena, R., Ramaswamy, R.S & Murthy, P.H. (2012). A review on phytoconstituents of Ocimum (Tulsi). *International Journal of Ayurvedic Medicine*, 3(1), 1-9.

Tosun, G., Yayli, B., Arslan, T., Yasar, A., Karaoglu, S.A., & Yayli, N. (2012). Comparative essential oil analysis of Calendula arvensis L. Extracted by hydrodistillation and microwave distillation and antimicrobial activities. *Asian Journal of Chemistry*, 24(5), 1955.

Wilken, J.N. (2016). The effect of environmental conditions on the antibacterial properties of Calendula officinalis (Doctoral dissertation, University of Johannesburg).


***