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Bioactive agents of *Origanum vulgare* against *Staphylococcus haemolyticus*

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Abstract

There are several bacterial infections that are associated with the use of medical devices in patients. Compromised immune system tend to play an important role in the pathogenesis of disease. In this research work, the microbial flora of medical devices of the patients being treated at OPD (Out Patient Door) wards of three local tertiary care hospitals was investigated. After the morphological, biochemical, and molecular characterization of the most common bacterial isolate, it was found to be *Staphylococcus haemolyticus*. For the determination of antibacterial and bioactive potential of *O. vulgare*, its extracts were prepared and examined for their antibacterial potential. The results demonstrated that the methanolic extract was the most effective against *S. haemolyticus*. It was selected on this basis for further analysis. The suppression of proteins in case of both extracts was also observed during the quantitative and qualitative analysis of proteins by Bradford assay and SDS-PAGE, respectively. The phytochemical analysis of various compounds of the methanolic extract comprised of the Thin Layer Chromatography (TLC) and GC/MS analysis, respectively. TLC demonstrated the presence of compounds of R_f values of 0.9 and 0.81, respectively. The GC/MS analysis of *O. vulgare* extract revealed the presence of 12 phytochemical compounds in the methanolic extracts.

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Introduction

The coagulase negative Staphylococci (CoNS) are the part of microbial flora which resides on skin and in mucous membranes, ear canals and respiratory passage of humans. The coagulase-negative Staphylococci (CoNS) are a heterogeneous group of staphylococcal species classified clinically by the absence of the blood-clotting enzyme coagulase (Nguyen *et al*., 2017). In 1961, the first strain of CoNS was isolated in hospital laboratory in UK, but at that time, it was not considered as pathogen to humans (Czekaj *et al*., 2015). The commensal bacterium changes into a pathogen due to acquisition of genes. According to a report, CoNS are considered as the fifth most common pathogen causing hospital acquired infections (Deyno *et al*., 2018). In the group of CoNS, many species are involved such as *S. haemolyticus, S. simulans, S. epidermidis, S. lugdunensis, S. caprae. S. homini* and *S. xylosus*. The methicillin resistant CoNS are the major cause behind the infectious diseases. It was found after the analysis of *mecA* gene that the sequence similarities among the three strains of *Staphylococcus* species including *S. aureus, S. haemolyticus* and *S. epidermidis* were about 99 % (Gilling *et al*., 2014).

Origanum vulgare belongs to Labiatae family which is widely used in pharmaceuticals, cosmetics and food industry. The Mediterranean countries are the main producers of aromatic plants. The Egyptians have been using oregano as a preservative and as a pharmaceutical for a long time. Turkey exports oregano leaves and its oils all over the world in the markets to meet the basic needs of life. Oregano is a perennial herb belonging to the tropical and mountainous regions, having white and purple flowers with angular stem. The aerial parts of the oregano plant are covered by the glandular trichomes which has some major components of oregano which give it a unique taste. The chemical composition of the medicinal plants varies because of their diverse environmental conditions. The height and yield are dependent on the oregano cultivation conditions (Manandhar *et al*., 2019). The presence of different volatile compounds in plants increasing the aroma of a plant, for which these herbs are also used in food. These volatile compounds are present in different parts of plant such as stems, flowers, resins, buds, seeds, roots and leaves (Butnariu and Sarac, 2018). It is also used for food preservation. When oregano is combined with other natural extracts, it extends the shelf life of food products (Arambula *et al*., 2019). The antioxidant properties of the medical herbs used in food products, when compared with each other showed that it has more efficient properties than butylated hydroxy anisole (BHA) and butylated hydroxytoluene (BHT) (Ibanez and Blazquez, 2017).

The plant has the antibacterial activity against *S. aureus, B. subtilis, E. coli* and *P. aeruginosa*. This is due to the two main secondary metabolites which are present in the plant including carvacrol and thymol constituting about 75-86 % of oregano (Perez *et al*., 2015). The antimicrobial activity of oregano is caused by the disturbance of cell homeostasis which leads to cell death and growth inhibition. The rosmarinic acid, quercetin and gallic acid are present in the leaves of oregano which plays an important role in inhibiting different microbes and in decreasing inflammation (Rodríguez-Calleja *et al*., 2015). The antioxidant properties of *O. vulgare* are due to the presence of phenolic content. These compounds are present abundantly in this plant and their concentration gets increased with change in weather conditions. They damage the cells and tissues of the body. The consumption of these herbs can provide a defence mechanism against invasive diseases (Özer *et al*., 2020).

This research work was carried out to determine the antibacterial potential of *O. vulgare* against *S. haemolyticus* which was isolated from the medical devices used in OPD wards of the tertiary care hospitals. The phytochemical compounds and their presence were also evaluated in the later part of the study.

Materials and methods

Sample collection from OPD wards

The samples were collected from fifty subjects of OPD

(Out-Patient Door) wards of three local tertiary care hospitals in Lahore city, Punjab. By using sterile swabs, the samples were collected from the medical devices used on patients who were being treated there.

Isolation of bacteria from patient samples and their characterization

For the isolation of bacteria from patient samples, the swabs were processed on basic media like Luria Bertani (LB) as well as selective media like mannitol salt agar (MSA), and blood agar (Cheesbrough, 2001). The bacterial culture was then streaked onto the plates which were then incubated overnight.

The results were observed the next day. The identification of the selected bacterial isolate was carried out at morphological (size, shape, pigmentation, margin, elevation, texture) and biochemical level. For the molecular characterization (16s rRNA sequencing), the bacterial DNA was isolated by the CTAB (Cetyltrimethylammonium bromide) method (Wilson, 2001) followed by horizontal gel electrophoresis.

Preparation of Origanum vulgare extract

The leaves were purchased, washed and ground into a fine powder. It (50 g) was macerated in 1 L of respective solvents (ethanol, methanol, chloroform, n-hexane, petroleum ether, and distilled water) and left to stand at room temperature for seven days.

The extracts were then filtered using Whatman filter paper and were then subjected to rotary evaporation for extract preparation. The prepared extracts were lyophilized, transferred to sterile falcon tubes and were labelled properly prior to storing them in the refrigerator (Ishnava *et al.*, 2013).

Determination of the antibacterial potential of O. vulgare extract

The antibacterial potential of *O. vulgare* extract was carried out according to the method of Okeke *et al.* (2001), with slight changes. The LB agar was prepared, autoclaved and poured into sterile Petri plates and were left to solidify. The plates were then streaked with pure bacterial culture in the form of a mat. The holes were made by using the back end of a sterile Pasteur pipette, and 100 µl of respective extracts of *O. vulgare* were poured into the wells.

The plates were then incubated at 37 ºC for 24 hours. The results were observed the following day, and the zones of inhibition, if any, were measured using a calibrated ruler.

Protein analysis

The quantitative protein estimation was carried out by Bradford assay (Bradford, 1976).

TLC (Thin-layer chromatography)

For TLC, the margin was drawn on a silica gel plate where the extract was placed and dried. The solvent mixture was placed in the beaker which comprised of butanol: methanol: water (1:1:1). The silica gel plate containing the samples was placed in the solvent mixture beaker and was covered. The samples were allowed to travel upwards the plate. Once the solvent mixture had covered enough distance, the plate was removed from the beaker and the compounds were immediately visualized. The R_f value of the compounds was then calculated (Dwivedi and Mehta, 2011).

Phytochemical analysis using Gas chromatography-Mass spectrometry (GC/MS) (3.9 is deleted from the beginning of this heading)

The evaluation of the phytochemical compounds of *O. vulgare* extract was performed using GC/MS. The GC equipment (GC 7890 A) and mass spectrometer (MS 5975 A) was coupled with a capillary column (HP-5MS) where the gas used was Helium with specified velocity and flow rate.

The injection volume was specified at 1 µl of extract sample, with a mass scan of low and high mass of 40-700 *m/z,* respectively. The total run time of the process was 28 minutes. The results of the mass spectra were obtained in the form of peaks which were then analyzed (Rukhsana *et al.,* 2017).

Results

(Both figures 1 and 2 are deleted)

Isolation, morphological, biochemical and molecular characterization of the selected bacterial isolate

Out of fifty, thirty-five samples were culture positive (Table 1) as revealed by the growth on Luria Bertani agar, blood agar and mannitol salt agar medium (Table 2). The Gram staining revealed the morphology to be positive, with purple hued cocci shaped bacteria appearing to be in clusters.

The different series of biochemical tests were carried out for the determination of the bacterial isolate KR-8. The results are summarized in Table 3. The 16s rRNA sequencing elucidated the bacterial isolate to be *Staphylococcus haemolyticus*.

Table 1. Sample collection from different hospitals in Lahore.

Sr. No.	Hospitals	No. of samples collected	No. of positive samples
1.	Hospital 1	16	12
2.	Hospital 2	17	13
3.	Hospital 3	17	10
Total no. of samples		50	35

Evaluation of the antibacterial potency of O. vulgare

Among different extracts of *O. vulgare* checked against *S. haemolyticus*, the methanolic extract was found to be most effective exhibiting the zone of inhibition of 3.1 mm.

Quantitative estimation of protein

The protein concentration was found to be less in the methanolic extract as compared to the control (Table 4).

TLC

The R_f values in TLC were 0.9 and 0.81.

GC-MS analysis of methanolic extract of O. vulgare

GC-MS analysis of the methanolic extract is demonstrated in Fig 1. It yielded the presence of 12 compounds in the methanolic extract, namely 1, 3, 5-Cycloheptatriene, 3,7,7-trimethyl-, Thymol, Phenol, 4-methoxy-2,3,6-trimethyl-, Hexadecanoic acid, methyl ester, n-Hexadecanoic acid, 9, 12-Octadecanoic acid (Z, Z)-, methyl ester, 6-Octadecanoic acid, methyl ester, (Z)-, Oleic acid, Ethyl oleate, Lupeol, Piperine, and Lup-20(29)-en-3-ol, acetate, (3β)-, respectively.

Discussion

In humans, *S. haemolyticus* is most commonly isolated pathogen from hospital acquired infections.

It causes infection mainly in immunocompromised patients (Szemraj *et al.*, 2019).

Table 2. Colony morphology of *S. haemolyticus* on different growth media.

Sr. No.	Growth medium	Colony morphology
1.	Luria Bertani agar	Small, circular colonies with regular boundaries and yellow sheen colonies
2.	Blood agar	Beta hemolysis, small size and dull white pigmented colonies
3.	Mannitol salt agar	Beige colored colonies, circular, entire and firm boundary, smooth surface, flat elevation and mucoid texture

The pathway crossed by the microbes in causing infections has following steps: colonization of microbes, host response, antibiotic resistance and disease development. Among all the CoNS species, the most resistant species to antibiotics is *S. haemolyticus* which prolongs the recovery time of the illnesses caused by this bacterium. When the whole genome sequence of *S. haemolyticus* was performed, it was revealed that many resistance genes are present (Takeuchi *et al.*, 2005).

The ability of CoNS to survive in hospital environment is higher than *S. aureus* (Ahmed *et al.*, 2019). A great number of CoNS were isolated from blood cultures and medical devices, it was because they have the ability to penetrate deeply into the skin, which also helped other opportunistic organisms to cause infections. It penetrated into the deep tissues and membrane barriers through which it entered into the bloodstream. Children were reported to be more prone to these infections (Salguiro *et al.*, 2019).

Table 3. Biochemical tests of the selected bacterial isolate.

Sr. No.	Parameters	Characteristics
1.	Gram staining	Gram +ve
2.	Catalase	+ve
3.	Coagulase	-ve
4.	Oxidase	-ve
5.	Indole	-ve
6.	Urease	-ve
7.	Citrate	+ve
8.	MR	-ve
9.	VP	-ve
10.	Motility	-ve

Among all nosocomial pathogens, it is also known as the second most frequently isolated pathogen from blood samples after *S. epidermidis*. It was found earlier that intravenous catheters and medical devices are the leading cause of bloodstream infections. Less than half of the bloodstream infections, especially in intensive care unit (ICU), are caused by CoNS. The patients undergoing dialysis treatments have a higher chance of contracting the infection and about more than half of infections are caused by CoNS (Pinder and Viau, 2018). It is really important to solve this problem by removing medical devices when not needed and not keeping them intact for a longer period time. Some superantigen toxins are present in CoNS which are the cause of sepsis in humans. CoNS are less contagious as compared to *S. aureus*. In CoNS, phenol soluble modulin (PSM) are the toxins responsible for sepsis and biofilm formation. The sepsis occurs more commonly in neonates (Da *et al.*, 2017). Due to the presence of a wide range of virulence factors and its expression at the main parts of the innate immune system turns as a success for the bacterium to act as an infectious agent (Pietrocola *et al.*, 2017). The virulence factors which play a significant role in the pathogenicity of the *Staphylococcus* species are toxins, bacterial adhesins, biofilm formation, enzyme formation and immune evasion (Chew *et al.*, 2018).

The formation of biofilms in *S. haemolyticus* increases its resistant to antibiotics. The medical devices implicated in hospitals are the main reason in development of biofilms which leads to infections. The bacterial biofilm develops on the medical devices due to the adhesion process involving cell wall proteins and enzymes. It is really important to control the development of biofilms (Seng *et al.*, 2017). Being an opportunistic pathogen, it has become a major health concern because of its high prevalence of resistance species to antibiotics.

It has been now a great challenge to eradicate these infections due to high prevalence of resistance to numerous antibiotics (Czekaj *et al.*, 2015). They are usually resistant to antibiotics such as rifampin, β-lactams, aminoglycosides, daptomycin, macrolides and vancomycin (Soumya *et al.*, 2017). Now there is a need of some new and novel therapies to be introduced for the treatment using antimicrobial agents. Because when inflammation occurs, it

damages body tissues and causes infections. Many mediators such as cytokines, prostaglandins, serotonin and histamine are produced which causes inflammation. Some medicinal plants such as rosemary, thyme and oregano have some anti-inflammation properties which can inhibit the mediators (Lopez *et al.*, 2017). In ancient years, medicinal herbs because of their antimicrobial activity are being used as an alternate to the conventional medicines (Yuan *et al.*, 2016).

Table 4. Estimation of protein by Bradford assay.

Samples	Protein concentration (µg/ml)
Control	0.272
Bacteria + methanolic extract	0.134

In our study, the antibacterial assay of the ethanolic and methanolic extract of *O. vulgare* was carried out, where the results demonstrated the inhibition of the growth of *S. epidermidis*, thereby demonstrating visible zones of inhibition in both extracts. The findings of Fournomiti *et al.* (2015) agreed with the results of our study, where the ethanolic extract of *O. vulgare* was observed to be effective against various Gram negative bacteria, with mean minimum inhibitory concentrations of 1.0 and 10.0 µg/mL, respectively. The results of the study conducted to examine the ethanolic extract of *O. vulgare* L. agreed with the conclusions of our study, where the ethanolic extract was found to inhibit the bacterial growth of many Gram positive and negative bacterial isolates (Coccimiglio *et al.,* 2016). In different studies, the antibacterial effects of oregano have been reported against *C. perfringens, S. typhimurium, S. aureus* and *P. aeruginosa*. According to the findings of Chouhan *et al.* (2017), it was proved that these compounds inhibited some bacteria such as *Salmonella*, *L. monocytogenes* and *S. aureus* and antibacterial activity was observed in *S. aureus* as well as *S. epidermidis* due to the presence of compound thymol. Liu *et al.* (2017) reported antibacterial property of oregano essential oil against *Enterobacter gergoviae*, *S. carnosus, Enterobacter lactobacilli, Lactobacillus sakei, Enterobacter gergoviae, Lactobacillus carvatus, Enterobacter amnigenus* and *S. xylosus*. When the study was performed to check the antibacterial action of decoction of oregano against some clinical bacteria, it was found that the highest inhibitory activity was observed against *S. aureus* followed by *E. coli* and *K. pneumoniae* (Swamy *et al.*, 2016; Kandasamy *et al.*, 2017). According to a report by Dahiya and Sharmishta, (2012), the oregano inhibited *S. aureus* and *E. coli* completely and was antifungal for *A. niger*. The oregano showed strong antimicrobial activity against pathogens *S. aureus*, *E. coli*, *L. monocytogenes*, *S. typhi* and *P. fluorescens* which in result suggested that in food formulation, oregano can be used as an antimicrobial (Mith *et al.*, 2014).

A study conducted by Rostro-Alanis *et al.* (2019) demonstrated the presence of volatile compounds o-cymene and thymol in the essential oil of *O. vulgare* L., as well as highlighting the antioxidant, and antimicrobial activities of the herb oil. Similar results were reported by Kocić-Tanackov *et al.* (2012). Generally herbal plants belonging to Laminaceae family are acknowledged for having antimicrobial effects because of the phenolics present in the plants which are known to have strong antioxidant activity due to which numerous medical plants have attract attention to use in organic medicine (Othman *et al.*, 2019). In plants, polyphenols contain about 10,000 different types of compounds but the most popular among them are thymol and carvacrol and their biogenetic precursor's γ-terpinene, saponins, cymenene, terpene, linalol, terpenoids, p-cymene, gammacariofilene, tannins and limonene.

All these have antimicrobial activity against a wide range of pathogens with various spectrums of activity (Guimarães *et al.*, 2019). Both thymol and carvacol make membrane permeable, although the structure for both of them is similar but they differ in the

position of the hydroxyl group in phenolic ring. They make the cell membrane permeable. According to a study, carvacol and menthol were more proficient to work on *S. aureus* infection as compared to thymol (O'Bryan *et al.*, 2015). The carvacrol and terpinenol-4 are the active compounds present in the oregano which plays an important role as an antiseptic and create a source for making new medicines increasing the effects of conventional antimicrobials which will improve the quality of treatment and possibly will decrease its cost (Marchese *et al.*, 2018). It was found that among all the components present in the essential oils of spices, carvacol is the most significant component having various pharmacological actions and in oregano it has a great impact in having aroma in food (Bhavaniramya *et al.*, 2019).

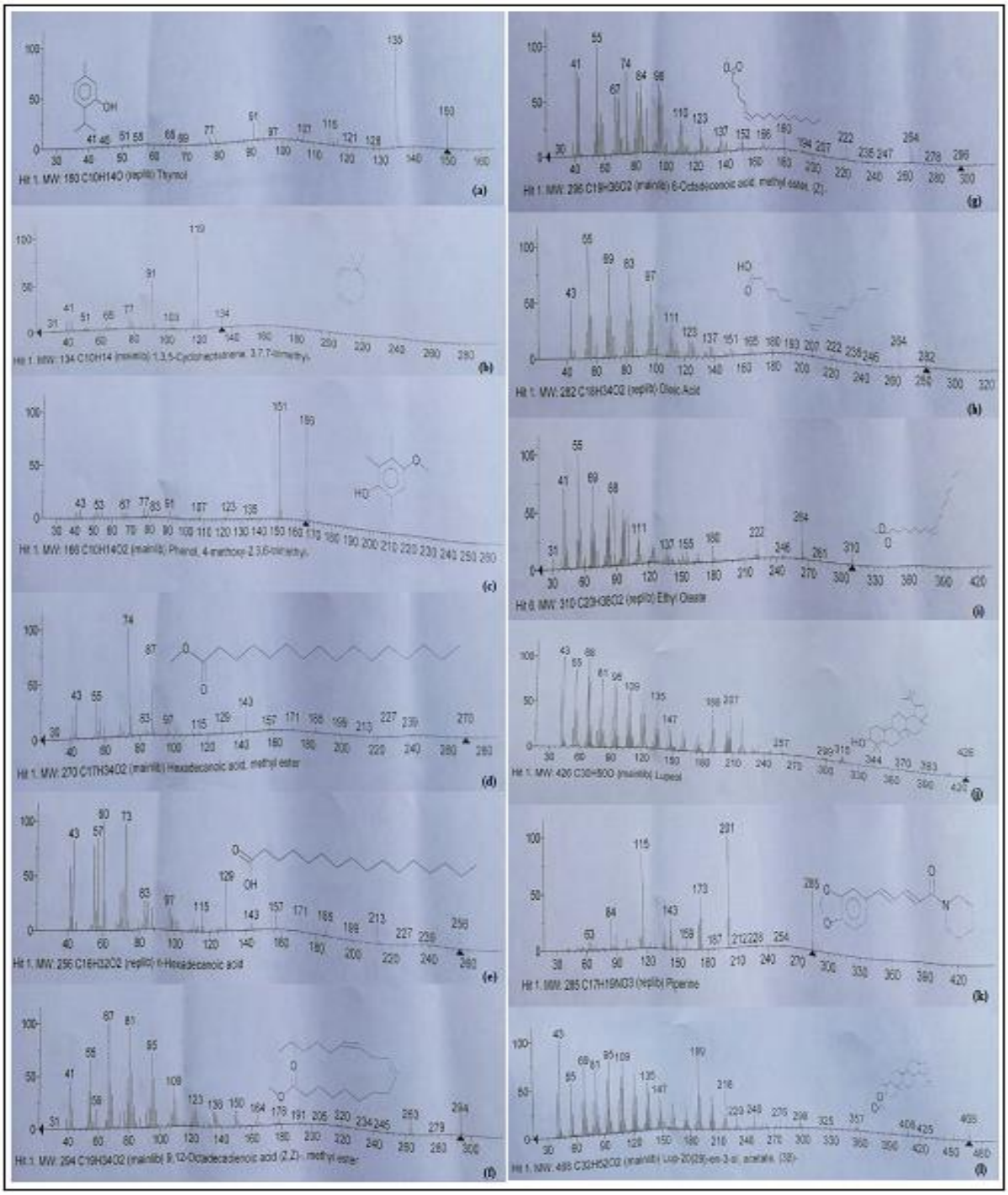

Fig. 1. Compounds detected in the methanolic extract of *Origanum vulgare,* as shown in the figure from (a) to (l).

The Turkish oregano has the highest number of phenolic compounds among all species of oregano, involved in several pharmacological roles. In oregano, the highest quantity of compound monoterpene is present with a range of 93.05 % (Shaaban, 2020). Nowadays, there is a huge expansion of developing medicines made of herbs and many phyto-chemists are showing their interest in order to substitute the antibiotics with plant-based medicine, especially because of the ban imposed in 2006 in European Union countries on antibiotics and the restriction on their use outside the Europe (Lillehoj *et al.*, 2018). The phytoncides which are derived from different plant substances showed several biostatic activities relating to *Staphylococci* (Abd El-Aziz and Sohaila, 2013). These biostatic activities helped in fighting the threat caused from *Staphylococci* (Steinka, 2018).

These compounds are present in different types of aromatic plants. The phytochemicals are present in different parts of plants such as seeds, leaves, tubers, stems, fruit, roots and leaves. One research reported that oregano is the source of phytochemicals that are active inhibitors of the *Staphylococci* (Maqbul *et al.*, 2020). These polyphenols play an important role against different types of stress providing defence against parasites, UV light, nitrogen species, pathogens and reactive oxygen (Mozjer *et al.*, 2016).

Conclusion

The study was conducted to examine the bioactive compounds of *O. vulgare* against *S. haemolyticus* which was isolated from the medical devices of OPD patients of local tertiary care hospitals. There are many diseases and medical ailments that require the use of antibiotics. These antibiotics are becoming weaker day by day against the resilient bacteria that are also becoming resistant and stronger. From this standpoint, the medicinal plants and herbs are much more significant than antibiotics. Their bioactive potential helps to treat various benign as well as life-threatening infections that are known menaces to men. Furthermore, recent advances in science and computational sciences help to enlighten man more about the mode of action of these traditionally used plants and search more about new ones, respectively. *In silico* studies can help to ascertain the structures of the bioactive compounds, in order to purify them and use them for molecular studies which can lead to the formulations of various medical concoctions.

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