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Antibacterial Evaluation of Plant Extracts: An Insight into Phytomedicine

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Abstract

This study was carried out to evaluate the antibacterial activity of petroleum ether, methanol and aqueous extract of the two plant Ocimum sanctum and pepper nigrum extract using agar well diffusion and broth dilution method against gram-positive bacterial strains (B. firmus, B. megaterium and B. cereus) and gram-negative bacterial strains (Escherichia coli, Enterobacter sp. and Klebsiella pneumoniae). The results indicate that petroleum ether extract compare to methanol and aqueous extract of O. sanctum and P. nigrum exhibited significant antibacterial activity against gram-positive bacteria with minimum inhibitory concentration (MIC) ranging from 0.13 to 0.21x 10^{-4} mg/well concentration. Moreover, gram-negative bacteria were less susceptible against petroleum ether, methanol and aqueous extract of O. sanctum and P. nigrum and their MIC ranging from 0.13 to 0.21x 10^{-2}. The most susceptible organism to the organic extracts from both studied plants was B. firmus and the most resistant organism was Enterobacter sp. The result obtained with B. cereus and K. pneumoniae were particularly interesting, since it was inhibited by antibiotic ampicillin used and susceptibility was observed with the individual extracts, where higher antibacterial activity with petroleum ether and aqueous extracts of O. sanctum and P. nigrum respectively. The presence of phytochemicals such as alkaloids, tannins, saponin, triterpenoids, steroids and glycosides in the extracts of these plants supports their traditional uses as medicinal plants for the treatment of various ailments. The present study reveals potential use of these plants for developing new antibacterial herbal drugs against pathogenic microorganisms.

Keywords: Antibacterial activity, *O. sanctum, P. nigrum*, Phytomedicine.

Introduction

According to the World Health Organization (WHO), infectious diseases are the first cause of death worldwide with more that 50% of the death appearing in tropical countries. In the developing countries, treatment of such diseases is complicated not only because of the occurrence of resistant microorganisms to the commonly used antibiotics, but also because of the low income of the population, which drastically reduce their accessibilities to appropriate drugs [1]. The world health organization (WHO) has adopted a major policy change wherein most developing nations have to make use of more traditional practices for primary health care.

The uses of medicinal plants as a source for relief from illness can be traced back over five millennia. The potential of higher plants as a source for new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemicals and the fraction submitted to biological or pharmacological screening is even smaller. In the recent year, there has been renewed interest on plants as a source of antimicrobial agents. Plant based antimicrobials represent a vast untapped source. The use of plant extract for medicinal treatment has become popular when people realized that the effective life span of antibiotic is limited and over prescription and misuse of traditional antibiotics are causing microbial resistance [2]. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [3]. Today, World's laboratories have found literally thousands of phytochemicals that have inhibitory effects on all types of microorganisms in vitro [4]. Unfortunately, development of effective antibacterial agents has been accompanied by the emergence of drug-resistant organisms due to the irrational and overuse of antibiotics, failure to complete a course of treatment, genetic versatility of microbes and horizontal transfer of resistant genes among bacterial species. All the mentioned factors diminish the clinical effectiveness of antibiotics [5,6].

The genus Ocimum sanctum L. (Lamiaceae) comprises circa 30 species that are found in tropical and subtropical regions. It is used in some religious ceremonies and not highly suited for culinary uses. They are rich in essential oils and have been the subject of numerous chemical studies. In traditional Ayurvedic system of medicine, several medicinal properties have been attributed to this plant. Recent pharmacological studies have established the anabolic, hypoglycemic, smooth muscle relaxant, cardiac

depressant, antifertility, adaptogenic and immunomodulator properties of the plant.

The genus *Piper nigrum* is one of the oldest and most widely used spices of the world and aptly called "the king of spices". The compounds of pepper contributing to its value as a food additive are essential oil for aroma and alkaloid compounds for pungency. The perennial climbing vine *Piper nigrum* L is native to India and Malabar region of Kerala is considered as the place of origin. After harvest the fresh green pepper is dried to get the dry black pepper of commerce [7]. Both aqueous and ethanolic extracts of black pepper have been screened for antibacterial activity against a penicillin G resistant strain of *Staphylococcus aureus* [8], *Bacillus cereus* and *B. subtilis* [9].

The present work was therefore undertaken to evaluate the antimicrobial activities of Petroleum ether, Methanol and Aqueous extract of the plants i.e. *Ocium sanctum* and *Piper nigrum*.

Materials and Method

Plant Material

The medicinal plants used for the experiment were *Ocimum sanctum* and *Piper nigrum.* Collected plants were washed thoroughly and chopped into small pieces shade dried and grinded into powdered form.

Extraction

The extraction of soluble compounds from *O. sanctum* and *P. nigrum* by the soxhlet method was performed using water, Petroleum ether (ACE), ethanol (EtOH), as solvents. Aqueous solutions of 25% and 50% (v/v) of EtOH: water ratio was also used. The soxhlet procedure consisted of dried and powdered samples (95.0 g) placed inside a thimble loaded into the soxhlet extractor. The total extracting time was 6 hrs, and the total amount of solvent was 150 mL maintained continuously refluxing over the sample. The solvent assays were performed at solvent boiling temperature. After the extraction the solvent was removed from the solute mixture by reduced pressure with rotary evaporator and then stored at 4^0C until further use.

Standardized bacterial colony numbers

Pure strains of bacterial cultures like; *Bacillus firmus, Bacillus megaterium, Bacillus cereus*; *Escherichia coli, Enterobacter spp.* and *Klebsiella pneumoniae* were used for the antibacterial assay. In order to ensure that the same number of bacteria was always used, a set of bacterial growth curves was established in the laboratory using the method described by Cappuccino and Sherman [10] for each bacterial strain prior to the evaluation of antimicrobial activities. From these curves, we determined the optical density (OD) at 600 nm that corresponded to the desired number of colony forming units (CFU).

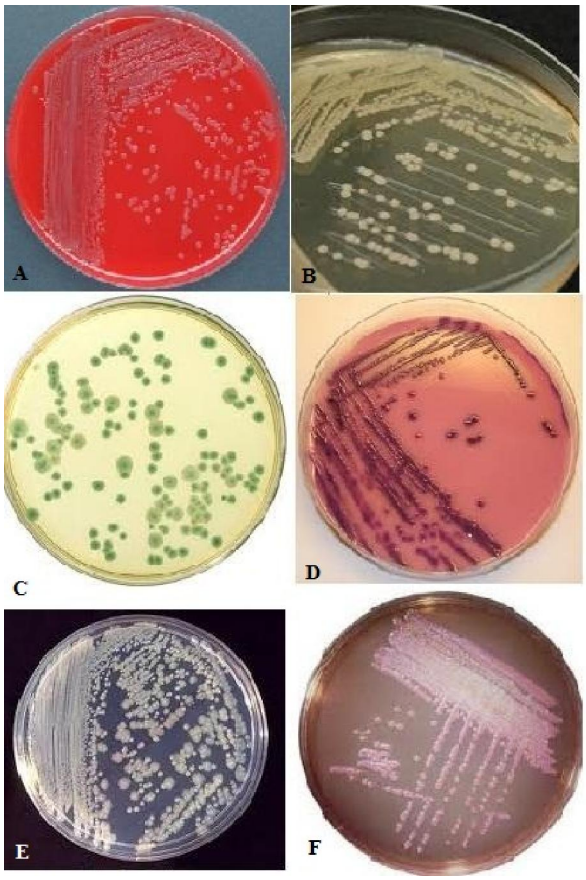

Figure 1. Bacterial strain used for antibacterial assay A) Bacillus firmus B) Bacillus megaterium C) Bacillus cereus D) E. Coli E) Enterobacter cloacae F) Klebsielaa Pnemoniae

Antibacterial Assay

Agar-well diffusion method

Well-in agar assay was carried out according to the method described by Mathabe et al. [11] with some modifications. Bacteria colonies (gram positive and gram negative) from plates were grown in MHB until they reached their specific OD at 600 nm to give a starting inoculum of $1x10^8$ bacteria/ml. Mueller-Hinton Agar plates were each divided into quadrants and labelled accordingly. 100µl of inoculum, equivalent to 107 cfu, was mixed with 6 ml of molten soft MHA (to ensure even distribution of bacteria) and poured immediately onto the base layer of MHA. The plates were left to solidify for 10 min. A sterilized 5 mm borer was used to make holes in the center of the divided areas. The bottom of the well was then sealed with molten soft agar. 10µl of each of the test samples i.e. plant extract (Petroleum ether, methanol and aqueous solution), ampicilin (positive control), DMSO (negative control) was then pipetted into the holes.

Plates with bacteria and test samples were incubated at 37°C for 16 to 18 hrs after which the inhibition diameter (ID) was measured using a vernier caliper. Antibacterial activity was recorded if the zone of inhibition was greater than 6mm. The antibacterial activity

results were expressed in term of diameter of zone of inhibition and <7mm zone was considered as inactive; 7-10mm as partially active; while11-15mm as active and >15mm as very active. Each experiment was carried out on at least three separate occasions.

Minimal Inhibitory Concentration (MIC)

MIC of the extracts *O. sanctum* and *P. nigrum* (Petroleum ether, Methanolic and Aqueous extract) was determined by broth dilution technique in which 12 test tubes with 3 ml of nutrient broth were autoclaved and cooled to room temperature. Two sets each containing 6 test tubes were made and the extract was serially diluted in both the tubes upto 10^{-5} dilution. One of the sets was inoculated with the pathogens and incubated at 37^0C for 24 hrs at 120rpm and the uninoculated set was preserved below 4^0C and used as blank. After the incubation period the growth of pathogens in the test tubes was detected by reading the absorbance at 600nm. Concentration of the test tube in which growth of pathogen increased suddenly was called as MIC. All experiments were performed in triplicates.

Statistical analysis

The results of the experiments were analyzed by two factorial analysis of variance (ANOVA). The Package programme Statistica (release 4.5, Copyright StatSoft, Inc. 1993) was used for statistical evaluation. Experiments on antimicrobial activity were replicated thrice on the same occasions. All analysis was done in triplicate for each replicate (n = 3x3).

Results

Strains of Bacillus firmus, Bacillus megaterium, Bacillus cereus; Escherichia coli, Enterobacter spp. and Klebsiella pneumoniae were selected to study the effect of antibacterial activity of O. sanctum and Piper nigrum extract. The antibacterial activity was analyzed by agar-well diffusion method. Results obtained in the present study relieved that some of the strain showed antibacterial activity while another strain showed very less inhibition or resistant using two different plant or six different extract (Petroleum ether, Methanol and Aqueous extract of each plant). When tested by agar-well diffusion method, the petroleum ether extract of O. sanctum showed significant activity against Bacillus firmus, Bacillus megaterium, Bacillus cereus recorded around 10mm while Escherichia coli and Enterobacter spp. showed very less or negligible antibacterial activity excepting Klebsiella pneumoniae. The highest antibacterial activity recorded of 12 mm in Bacillus firmus and least activity recorded in E. coli and Enterobacter sp. measured 7 and 8 mm excepting K. pneumonia recorded 9 mm at 1 mg/well concentration. Methanolic extract of O. sanctum exhibit highest activity against B. firmus measured 11 mm at 1 mg/well concentration and least activity against E. coli recorded 7 mm at 1 mg/well concentration. Aqueous extract of this plant showed highest inhibitory activity against B. firmus recorded 9 mm and least inhibitory activity against Enterobacter spp. recorded 7 mm at 1 mg/well concentration (Fig 2 A).

In the other hand, the extracts of *piper nigrum* showed least antimicrobial activity against most of the tested strain. In petroleum ether extract, the highest antimicrobial activity was recorded in *B. megaterium* measured 12mm and least inhibition was found in *K. pneumonia* measured 7 mm at 1 mg/well concentration. Moreover, in methanolic extract, strain of *B. firmus, B. megaterium, B. cereus, E. coli, Enterobacter sp.* and *K. pneumonia* showed inhibition 10, 10, 8.5, 8, 7 and 8 mm at 1 mg/well concentration used in which highest antimicrobial activity were recorded against *B. firmus* and *B. megaterium* and least recorded against *Enterobacter sp.* In the aqueous extract, one-gram positive and one-gram negative bacteria showed no inhibition while remaining strains *B. firmus, B. megaterium, E. coli* excepting K. *Pneumoniae* showed least antibacterial activity, recorded 8, 7, 7.5 and 10 mm at 1 mg/well concentration respectively. In this experiment DMSO used as a negative control and antibiotic ampicilin was used as a positive control (Fig 2 B).

Determination of Minimal Inhibitory Concentration (MIC)

1. MIC of petroleum ether, methanol and aqueous extract of *O. sanctum* against six pathogens was determined and results are summarized in Fig. 2 (C).
2. MIC of petroleum ether, methanol and aqueous extract of *P. nigrum* against six pathogens was determined and results are summarized in Fig. 2 (D).

Discussion

Herbal medicines are valuable and readily available resource for primary health care and complementary care system. They can be the best alternative for the available antibiotics against which the pathogens are adapting resistance. It is necessary to investigate those plants scientifically, which have been used, in traditional medicine to improve the quality of healthcare. Plant extracts are potential sources of novel antimicrobial compounds especially against bacterial pathogens. *In vitro* studies in this work showed that the plant extracts inhibited bacterial growth but their effectiveness varied. The antimicrobial activity of many plant extracts has been previously reviewed and classified as strong, medium or weak.

The medicinal plants like tulsi and black pepper are being used traditionally for the treatment of inflammation, cough, toothache, asthma, chronic indigestion, colon toxins, obesity, sinus, congestion, fever [12], intermittent fever, cold extremities, colic, gastric ailments and diarrhea [13]. The antibacterial activity has been attributed to the presence of some active constituents in the extracts. An important characteristic of plant extracts and their

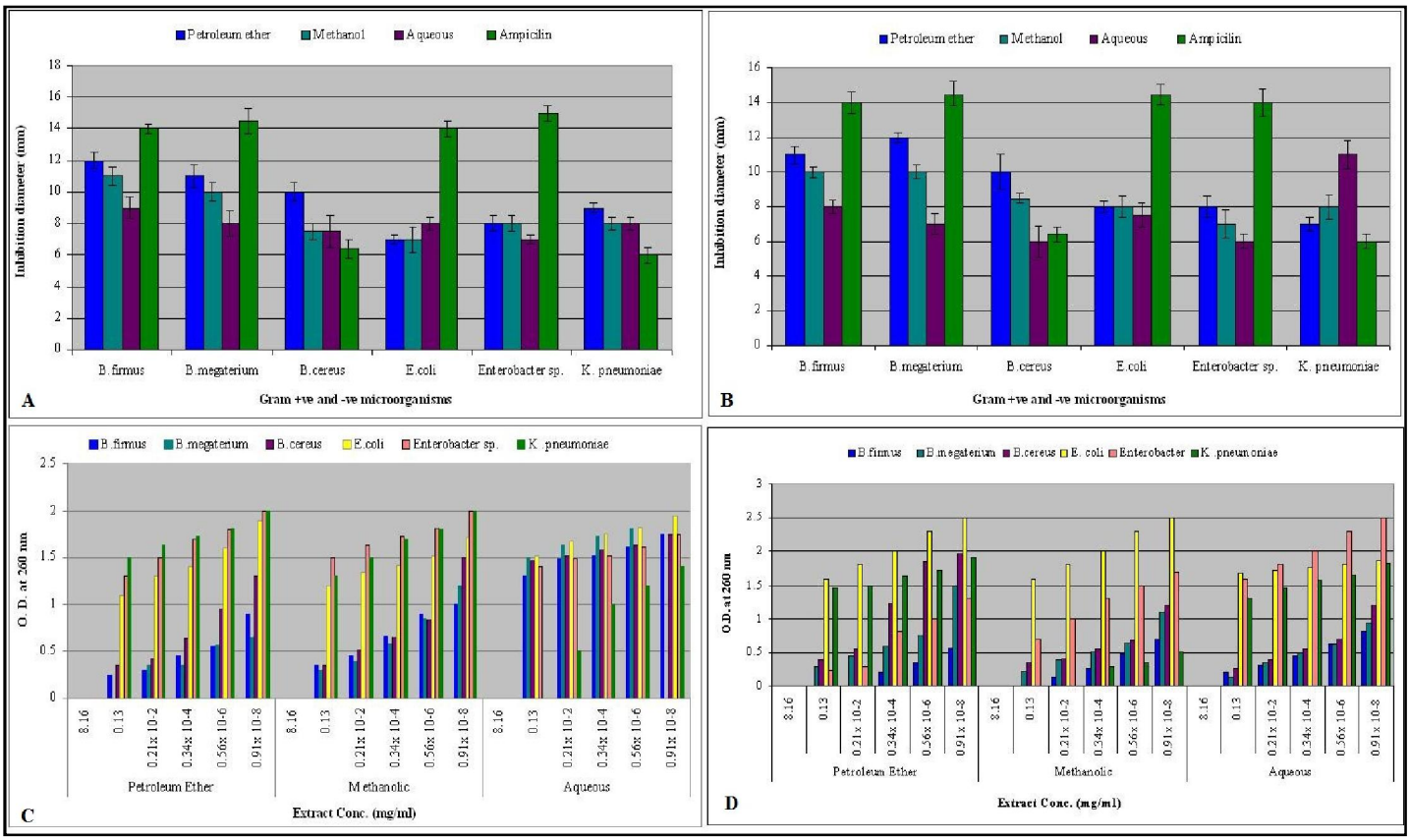

Figure 2 Antimicrobial Activity of the *O. sanctum* and *P. nigrum* extracts on different strain of bacteria and their MIC; DMSO (10 µl) did not affect the growth of any of the strain; Values shown are Mean±SE, well size is 6 mm. (A) *Ocimum sanctum* extracts and their activity against strains (B) *Pepper nigrum* extracts and their antibacterial activity against strains. MIC of six strains against different extract of *O. sanctum* (C) and *P. nigrum* (D).

components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death [14].

According to the antibacterial assay done for screening purpose, all the gram positive microorganisms viz., *B. firmus,* and *Bacillus megaterium* were the most susceptible bacteria to petroleum ether, methanolic and aqueous extract of *O. sanctum* plant extracts excepting *B. cereus*, that showed only susceptibility to petroleum ether extract, whereas all gram negative microorganisms, viz. *E. coli*, and *Enterobacter sp.* were least susceptible to petroleum ether, methanol and aqueous extract excepting *K. pneumoniae* that showed significant susceptibility with the petroleum ether extract. On the contrary, *Escherichia coli* was most resistant microorganism.

In case of black pepper, the Petroleum ether extracts showed more susceptibility against all gram-positive microorganism viz. *B. firmus, B. megaterium, B. cereus* compare to methanolic and aqueous extract, whereas gram-negative microorganism viz. *K. pneumonia* showed its high susceptibility against aqueous extract.

On the contrary, the rest of the microorganism showed very less susceptibility to petroleum ether, methanol and aqueous extract excepting *E. coli* that showed resistance against aqueous extract of the plant.

These observations are likely to be the result of the differences in cell wall structure between Gram-positive and Gram-negative bacteria, with Gram-negative outer membrane acting as a barrier to many environmental substances including antibiotics [15]. Moreover, the observed difference between these plants extracts might be due to insolubility of active compounds in water or the presence of inhibitors to the antimicrobial components [16]. Previous research showed that inactivity of plant extracts might be due to age of plant, extracting solvent, method of extraction and time of harvesting of plant materials [17, 18,19]. Petroleum ether extracts of *O. sanctum* and *P. nigrum* showed more antibacterial activity against *B. firmus,* and *Bacillus megaterium* than other organisms tested. This result is in agreement to Agatemor [20] where it was reported that gram-negative bacteria are more resistant than gram-positive bacteria. Aqueous extract of *O. sanctum* and *P. nigrum* showing highest resistance against gram-

negative bacteria *Enterobacter sp* and *E. coli* excepting *K. Pneumoniae.* Furthermore, antibiotic ampicillin used in the study was inactive against two of the microorganism viz. *B. cereus* and *K. pneumonia* and surprisingly both plant extracts were active and showed antibacterial activity.

According to Sikkema et al. [21] hydrophobicity, is an important characteristic of plant extracts and their components that enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. The inhibition produced by the plant extracts against particular organism depends upon various extrinsic and intrinsic parameters. Due to variable diffusion ability in agar medium, the antibacterial property may not demonstrate as ZOI commensurate to its efficacy. Therefore MIC value has also been computed in this study. MIC is the lowest concentration of antibacterial substance required to inhibit the growth of strain [22]. MIC values of *O. sanctum* and *P. nigrum* with petrolium ether extract showed better results compare to methanolic and aqueous extract. *E.Coli* and *Enterbacter spe.* (Gram-negative bacterium) were observed as most resistant bacterium as high growth observed at initial concentration i.e. 0.13 mg/ml of petroleum ether and methanol and aqueous extract of *O. sanctum*. However, *K. pneumoniae* showed less growth in aqueous extract excepting petroleum ether and methanolic extract. Therefore, our results revealed the importance of plant extracts when associated with antibiotics, to control resistant bacteria, which are becoming a threat to human health. Furthermore, in a few cases, these plant extracts were active against antibiotic resistant bacteria under very low concentration, thus minimizing the possible toxic effects.

Intensive use of antibiotics often resulted in the development of resistant strains [23], these create a problem in treatment of infectious diseases, furthermore antibiotics sometimes associated with side effects [24] whereas there are some advantages of using antimicrobial compounds of medicinal plants such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature [25]. Because of this, the search for new antibiotics continues unabated. These findings support the traditional knowledge of local users and it is a preliminary, scientific, validation for the use of these plants for antibacterial activity to promote proper conservation and sustainable use of such plant resources.

Awareness of local community should be enhanced incorporating the traditional knowledge with scientific findings. In conclusion, the results of the present study support the folkloric usage of the studied plants and suggest that some of the plant extracts possess compounds with antimicrobial properties that can be further explored for antimicrobial activity. This antibacterial study of the plant extracts demonstrated that folk medicine could be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use of these plants in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases.

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