MULTIPLE DRUG RESISTANCE ACTIVITY OF CLOVE EXTRACT BY EXTRACTION SOLVENTS

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A thesis submitted to the **Department of Mathematics & Natural Sciences** in partial fulfillment of the requirements for the degree of **Bachelor of Science in Biotechnology**

Department of Mathematics & Natural Sciences

BRAC University

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Declaration:

It is hereby declared that_

1. The thesis submitted is my original work during completion of the bacherlor degree at BRAC University.
2. The thesis does not contain material formerly published, neither it is written by a third party, excluding where it is correctly cited through complete and precise referencing.
3. The thesis does not comprise any material that has been submitted or acknowledged for any other degree or diploma at a university or other institution.
4. I have accurately acknowledged all the core sources of help.

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Of consecutively Fall, 2015 and Spring, 2016 has been accepted as satisfactory in partial fulfillment of requirement for the degree of Bachelor of Science in Biotechnology on 03.10.2021.

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MULTIPLE DRUG RESISTANCE ACTIVITY OF CLOVE EXTRACT BY EXTRACTION SOLVENTS

Abstract

Clove extraction is produced from dried clove flower buds, *Eugenia caryophyllata* L. Merr. & Perry (Myrtaceae), is utilized in the fragrance and flavoring industries, as well as as a topical therapy to reduce pain and promote healing. The extraction's major components are phenylpropanoids such as carvacrol, thymol, eugenol and cinnamaldehyde. The biological activity of *Eugenia caryophyllata* has been investigated on several microorganisms and parasites, including pathogenic bacteria, *Herpes simplex* and hepatitis C viruses. In addition to its antimicrobial, antioxidant, antifungal and antiviral activity, clove extractions possesses antiinflammatory, cytotoxic, insect repellent and anaesthetic properties.The antibacterial activity of clove extracts was examined using different extraction solvents in this study. *Klebsiella spp.*, *Escherichia coli* and *Pseudomonas spp.* were investigated for antibacterial activity. The antibacterial activity of the methanol extract was higher than that of the water extract. The clove water acetone extract inhibited the development of Escherichia coli, but had no effect on *Klebsiella spp., Pseudomonas spp.*. In Gram (-) bacteria, the acetone extract of clove extracts demonstrated more sensitive antibacterial action than in Gram (+) bacteria. The antibacterial activity of the clove extract increased as the concentration was raised.

Keywords:

Clove, Extract, *Syzygium aromaticum*, Methanol, Acetone, Ethanol, Bacteria, Antibacterial, Multidrug Resistant, *E. coli, Pseudomonas spp., Klebsiella spp.,* Antibiogram, Gram negative, Concentration, Zone of inhibition, MDR, XDR, Antimicrobial.

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List of Abbreviations & Codes:

NC = Negative Control

PC = Positive Control

Ipm = Imipenem

Crude = Undiluted concentration organic solvent extracts

1:2 = Diluted concentration ratio of the organic solvent extracts

1:4 = Diluted concentration of the organic solvent extracts

BIH-19-176 = E. coli Strain

BIH-19-002 = Pseudomonas spp. Strain 1

BIH-19-064 = Pseudomonas spp. Strain 2

BIH-19-068 = Klebsiella spp. Strain 1

BIH-19-177 = Klebsiella spp. Strain 2

BIH-19-130 = Klebsiella spp. Strain 3

Introduction

Infectious diseases remain an important cause of morbidity and mortality in developing and developed nations (Lewis et al., 2006). They are responsible for over half of all deaths in tropical nations, with bacterial infections appearing to be the most common (Iwu et al.,1999). (Lewis et al., 2006). They are responsible for over half of all deaths in tropical nations, with bacterial infections appearing to be the most common (Iwu et al.,1999). Antibiotic discovery and development has resulted in a significant improvement in the ability to treat infectious diseases, and is considered one of the most significant achievements of the twentieth century. Unfortunately, due to 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, the development of effective antibacterial agents has been accompanied by the emergence of drug-resistant organisms. All the mentioned factors diminish the clinical effectiveness of antibiotics (Amit et al., 2006; Aibinu, 2007). As a result, there is a constant demand for innovative, effective, and economical antimicrobial agents. (Cowan, 1999). In recent times, there has been renewed interest on plants being sources of antimicrobial agents due to their use historically and the fact that a good portion of the world's population, particularly in developing countries, rely on plants for the treatment of infectious and non-infectious diseases. Antiseptic properties of plant volatile extraction have been recognized since antiquity (Dorman et al., 1999). Indeed, clove extraction is commonly used as an anesthetic in the relieve of toothache in dentistry. It is also used as a carminative, rubefacient and serves as a preservative in herbal recipes, signifying possible antimicrobial properties (Odugbemi, 2006). Clove extraction can be obtained from the flower buds of Syzigium aromaticum, family Myrtaceae. Syzygium aromaticum (L.) Merr. & L.M.Perry (Syn. Eugenia caryophyllus) is a tree in the family Myrtaceae, native to Indonesia. The aromatic flower buds of the plant are known as cloves and are commonly used as a spice. Cloves are commercially harvested in Indonesia, India, Pakistan, Sri Lanka, as well as in African countries, such as Comoro Islands, Madagascar, Seychelles, and Tanzania. Several therapeutic uses of S. aromaticum have been recognized. The clove plant is used as a medicine in China and Western countries against many diseases, such as oral diseases or dental complaints (Cai and Wu, 1996; Wankhede, 2015). The plant is also used to control nausea and vomiting, cough, diarrhea, dyspepsia, flatulence, stomach distension, and gastrointestinal spasm; relieve pain; cause uterine contractions; and stimulate the nerves

(Shrivastava et al., 2014). The cloves are also used in folk medicine as a diuretic, odontalgic, stomachic, tonicardiac, and condiment with carminative and stimulant effects (Pandey and Singh, 2011). Essential oil derived from this aromatic plant not only serves as a fragrance and flavor agent, but also as a dietary antioxidant expected to prevent several diseases caused by free radicals (Cai and Wu, 1996; Halliwell, 1999). It has been reported that the majority of cloves are used by kretek cigarette manufacturers in Indonesia and only about 10% for other purposes, such as folk medicine, food flavoring, food preservation, fragrance, and pharmaceuticals (Nurdjannah and Bermawie, 2012). In the present chapter, we will discuss the antimicrobial activity and potency of gram-negative bacteria with emphasis on the aromatic part of the clove and report the extraction and analysis of the chemical constituents of clove extraction with the elucidation of its major component attributed to its acclaimed antimicrobial activities.

Materials & Methods

Collection of plant materials: Flower buds of clove collected from local market for antimicrobial study.

Test Organisms: Six strains of three gram negative bacteria with sample identification numbers such as *Klebsiella spp.* (BIH-19-177, BIH-19-068, BIH-19-130); *Pseudomonas spp.* (BIH-19-002, BIH-19-064); *E. coli* (BIH-19-176) were collected from institute for developing Science & Health initiatives, Mohakhali, Dhaka.

Standardisation of inoculum:

The test organisms were sub-cultured onto fresh plates of McConkey agar and Mueller Hinton for 24 h for 5 - 7 days at 37°C for bacteria respectively. Colonies from these plates were suspended in Mueller-Hint on broth to a turbidity matching 0.5 McFarland standard (10^8 colony forming units (cfu)/ml).

Preparation of clove extracts: Clove buds were washed using distilled water and dried in a 60℃ oven overnight. Then the dried clove buds were made into a fine powder using an electric blender (Phillips, Taiwan). 20g of powdered clove was macerated into 200ml (1:10 (w/v) ratio) of different organic solvents (absolute ethanol, methanol and acetone) in separate conical flasks (Mostafizur et al, 2011). The conical flasks were fully covered with aluminum foil and kept in a cool, dry place at room temperature for overnight. After soaking overnight, each of the solutions were filtered into a beaker using Double Rings 102 11cm qualitative filter papers. The filtered solutions were evaporated using a 60℃ oven. It took 3 days for acetone and methanol extracts to evaporate and ethanol took almost 5 days. Following the evaporation process, a sticky, oily crystal-like product was found at respectively 12.718g, 3.655g and 6.8g for ethanol, acetone and methanol. All of the extracts were diluted with DMSO (3ml for ethanol and methanol, 4.5 ml for acetone) and stored at +4℃ freezer for future use. The extracts dissolved in DMSO could be usable up to two months in this process.

Antibacterial Assay: In vitro antibacterial activity of the different clove extracts were tested against six strains of three multi drug-resistant gram negative bacteria using agar well diffusion method (Parekh and Chanda, 2007). Under aseptic conditions, petri dishes were made using MH agar and they were pre-sterilized using the autoclave machine. In the MH agar media six wells were made around the plate using the wide side of yellow-tips. Each of the wells was 2mm in diameter. The extract solutions were diluted twice using DMSO so that we could use three concentrations of the extract to determine the level of the antimicrobial strength. There were three concentrations: crude, 1:2 and 1:4 (v/v) for each of the extracts to be used against all six different strains of bacteria. Three negative controls were also made using DMSO and further diluting it with distilled water. For a positive control, the antibiotic *Imipenem* (IMP) was used as antibiotic discs in the middle of the agar media. *Imipenem* was checked to be not resistant to 5 of the strains and resistant to one *Klebsiella spp.* strain in previous studies at ideSHi. The bacteria were taken from stored vials and streaked into McConkey agar plates. After overnight incubation one single colony of each bacterium were taken and inoculated into 5ml LB broth and were further incubated in shaker incubator at 37℃ 250 rpm overnight. The Muller Hinton agar petri-plates were marked and bacterial lawn was done on it with the designated cultured bacteria. Then the six wells were filled with three concentrations of the specific extract and three concentration

of the negative control for each plate. The plates were incubated at 37°C overnight. Following this incubation, the plates were checked for antimicrobial activity by measuring zone of inhibition in mm scale for each concentration of different extracts against each bacterium. Each assay was carried out in duplicate to assure accuracy.

Literature Review

1. Isolation of essential oils and flavor compounds by dense Carbon dioxide

Kaisli Kerrola Food Reviews International 11 (4), 547-573, 1995

Procedures used to isolate flavor components can have profound effects on the composition of the final product. Distillation, extraction, adsorption, and other methods are frequently applied to the isolation of flavor constituents from plant material. Loss of volatile compounds and formation of artifacts by enzymatic reactions, oxidation, isomerization, and chemical and thermal decomposition have been observed (reviewed, eg, by Drawert et al., 1981; Sugisawa, 1984; Ohloff et al., 1985).

Extraction with dense carbon dioxide offers the advantages of low-temperature processing, recovery of solvent-free extract, rapid extraction resulting from high mass transfer due to higher diffusivity, and lower viscosity in comparison to organic solvents (Gouw and Jentoft, 1972). Carbon dioxide extraction has been used for the isolation of flavor components in two separate applications: a large scale process and an analytical-scale separation method. In both applications the aim is to separate either a single component or a class of compounds from complex matrices. The selectivity and solvent power depend on the density, which can be varied during the extraction by controlling pressure and temperature. By changing the extraction conditions, class-selective extractions and fractionation of the extract can be achieved.

2. Safety assessment of a standardized polyphenolic extract of clove buds: Subchronic toxicity and mutagenicity studies

Liju Vijayasteltar, Gopakumar Gopinathan Nair, Balu Maliakel, Ramadasan Kuttan, IM Krishnakumar

Toxicology reports 3, 439-449, 2016

Despite the various reports on the toxicity of clove oil and its major component eugenol, systematic evaluations on the safety of polyphenolic extracts of clove buds have not been reported. Considering the health beneficial pharmacological effects and recent use of clove polyphenols as dietary supplements, the present study investigated the safety of a standardized polyphenolic extract of clove buds (Clovinol), as assessed by oral acute (5 g/kg b.wt. for 14 days) and subchronic (0.25, 0.5 and 1 g/kg b.wt. for 90 days) toxicity studies on Wistar rats and mutagenicity studies employing Salmonella typhimurium strains. Administration of Clovinol did not result in any toxicologically significant changes in clinical/behavioural observations, ophthalmic examinations, body weights, organ weights, feed consumption, urinalysis, hematology and clinical biochemistry parameters when compared to the untreated control group of animals, indicating the no observed-adverse-effect level (NOAEL) as 1000 mg/kg b.wt./day; the highest dose tested. Terminal necropsy did not reveal any treatment-related histopathology changes. Clovinol did not show genotoxicity when tested on TA-98, TA-100 and TA-102 with or without metabolic activation; rather exhibited significant antimutagenic potential against the known mutagens, sodium azide, NPD and tobacco as well as against 2-acetamidoflourene, which needed metabolic activation for mutagenicity.

Clove buds are a spice of relevance in food, traditional medicine, pharmaceutics and cosmetics and, among the spices, they have the highest content of total polyphenols with exceptional antiviral and antimicrobial properties. Various approaches have been reported for the isolation of essential oil from clove buds. Nonetheless, the qualitative and quantitative analysis of hydrosoluble polyphenols and solid residues simultaneously yielded during the extraction process has not been explored yet. This work is focused on the analysis of some variables' effect on yield and composition of the clove buds essential oils on a green microwave assisted

extraction, and the characterization and quantification of the different compounds obtained from the extraction process. A versatile coaxial dipole antenna, to directly apply the electromagnetic energy inside the extraction medium, was used to thermally activate the hydrodistillation. The composition profiles of clove buds essential oil and hydrosoluble polyphenols obtained during in-situ microwave assisted extraction (IMWAE) were analysed and quantified by headspace gas chromatography mass spectrometry (HS-GC–MS) and liquid chromatography with UV/visible diode array/fluorescence detector (HPLC-DAD-FD). The solid residue was characterized by Fourier Transform Infrared (FTIR) spectroscopy and its composition in terms of lignin, cellulose and hemicellulose was predicted. The green IMWAE process was compared with the conventional hydrodistillation (CH) in terms of yield and quality of isolated products. Thermogravimetry coupled to FTIR analyses of the evolved gases from the solid residue evidenced that the solid residue obtained from IMWAE of clove buds is richer in cellulose-hemicellulose than the residue obtained from CH. This can be because of microwaves that allow to remove a higher amount of phenolic compounds/lignin oligomers. The enthalpy of combustion values (ΔcH) (kJ/g) of IMWAE and CH residues were determined by colorimetric combustion and were compared with the –ΔcH (kJ/g) values calculated using the hemicellulose, cellulose and lignin compositions predicted by partial least square chemometrics. The ΔcH highlighted the energetic features of solid residues from IMWAE and CH for their potential uses as alternative biomass for fuel production and here firstly reported for this kind of biomass. The extraction approach here presented is environmentally friendly, highly flexible, easily controllable, time saving, and enables to break the scale-up barrier in microwave assisted industrial processes aimed to valorise aromatic herbs and eventually to exploit vegetable wasting materials. This leads to a lowering of production costs and, therefore, of the market price of isolated extracts from aromatic herbs.

For the first time, extracts from clove buds obtained by supercritical carbon dioxide extraction were screened for antioxidant and antibacterial activities. Additionally, antioxidant and antibacterial activities of extracts obtained by the supercritical extraction of the clove bud–oregano leaf mixtures were studied. Supercritical extract of pure clove had the highest eugenol (64%) and total phenolic content (530.56 mg GAE/gextract). All extracts had antioxidant activity comparable to synthetic antioxidants against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and formation of peroxides. Presence of 0.6% and 5% of oregano extract in the clove extracts

obtained from the clove–oregano plant mixtures improved their antioxidant activity with respect to the extract from pure clove. Clove extract showed moderate antibacterial activities against selected Staphylococcus and Enterococcus bacterial strains. Presence of 50% of the oregano extract improved antibacterial activity of clove extract against all tested strains and resulted in a synergistic antibacterial activity against Methicillin-resistant Staphylococcus haemolyticus strain (MIC $\leqslant$ 1.25 μg/mL). Study demonstrated great potential of supercritical clove extract as a natural functional ingredient and the possibility of increasing its antioxidant and antibacterial efficiencies in order to apply lower concentrations and to reduce undesirable flavour notes and toxicological effects in final products.

The essential oils of Syzygium aromaticum (clove bud) were obtained by hydro-distillation. The antimicrobial activity of clove bud oil and was investigated by agar well diffusion method against four multidrug resistant strains namely Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus and Enterococcus faecalis as well as two standard strains, StaphylococcusaureusATCC29213 and PseudomonasaeruginosaATCC27853. These essential oils exhibited inhibitory effects towards all the test organisms, clove essential oil had antibacterial activity little higher. MICs ranged from 0.312%(v/v) to 1.25%(v/v) for all tested bacteria. Based on this finding, it may be suggested that this essential oil may be used as natural antibacterial agents to treat infections caused by multidrug resistant bacteria.

Result

The result of this study denominates the antibacterial activities of the spice Clove on six strains of three different pathogenic bacteria. The antibacterial activities were marked in accordance with the measurement of the zone of inhibition on the Muller-Hinton agar plates by agar well diffusion method. The results depict that differently processed clove extracts have promising effects on different pathogenic bacteria. The zone of inhibition was ranged from 15mm (Klebsiella spp. BIH-19-068) to 7mm (Klebsiella Spp. BIH-19-177) at 20µl extract. Best results were given by Acetone extract (15mm to 8mm), followed by Methanol (14mm to 7mm) and then ethanol extract (12mm to 7.5mm). In case of the positive control (Imipenem), the zone of inhibition ranged from 19mm (Klebsiella Spp. BIH-19-177) to 30mm (*E. coli* BIH-19-176) at disc strength. Negative controls done using DMSO showed no zone of inhibition indicating it's no significant effect on the bacteria.

***E. coli* (BIH-19-176) Antibiogram Result**

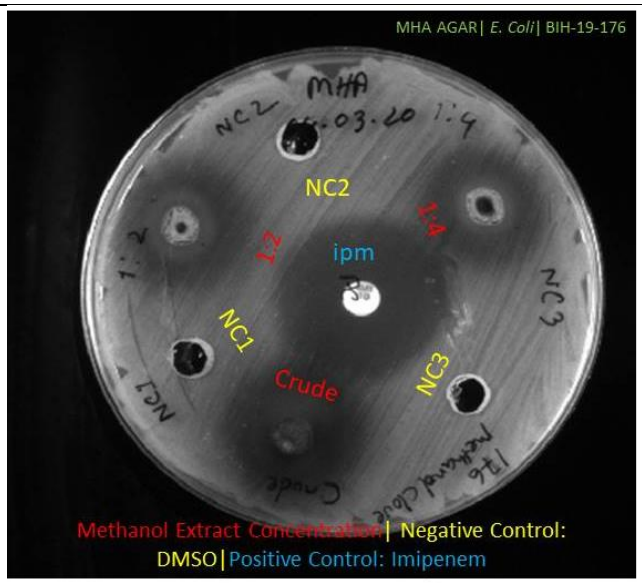


Fig.1: Antibiogram of the Methanol extract of clove against *E. coli* (BIH-19-176)

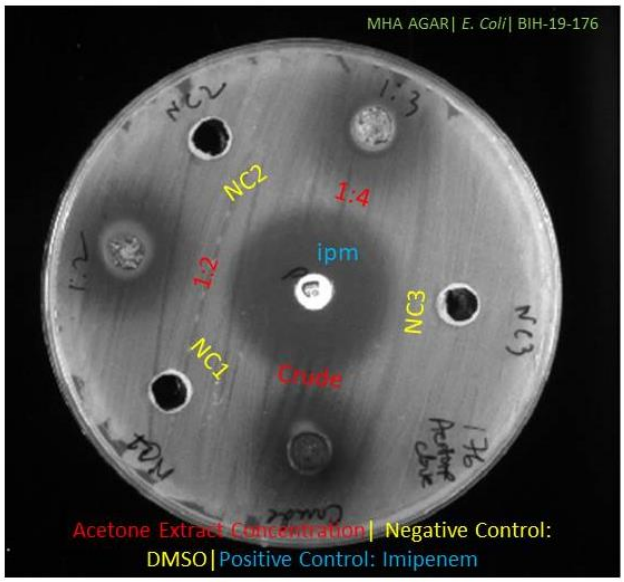


Fig.2: Antibiogram of the Acetone extract of clove against *E. coli* (BIH-19-176)

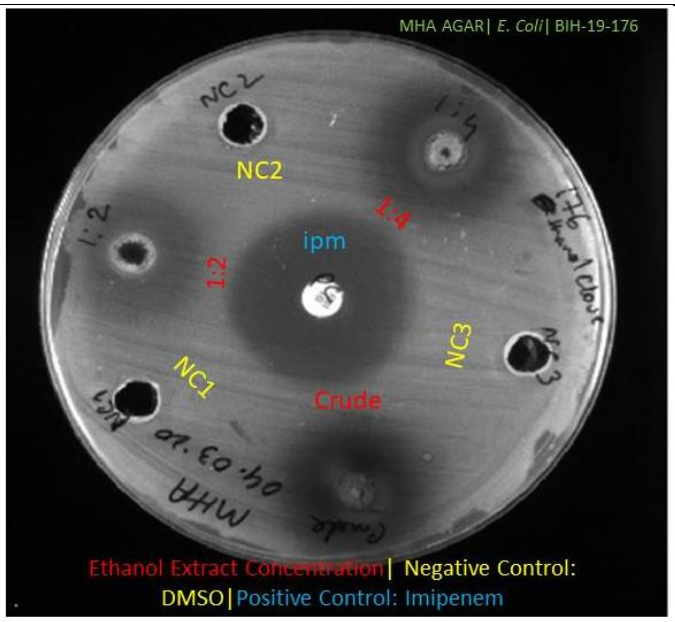
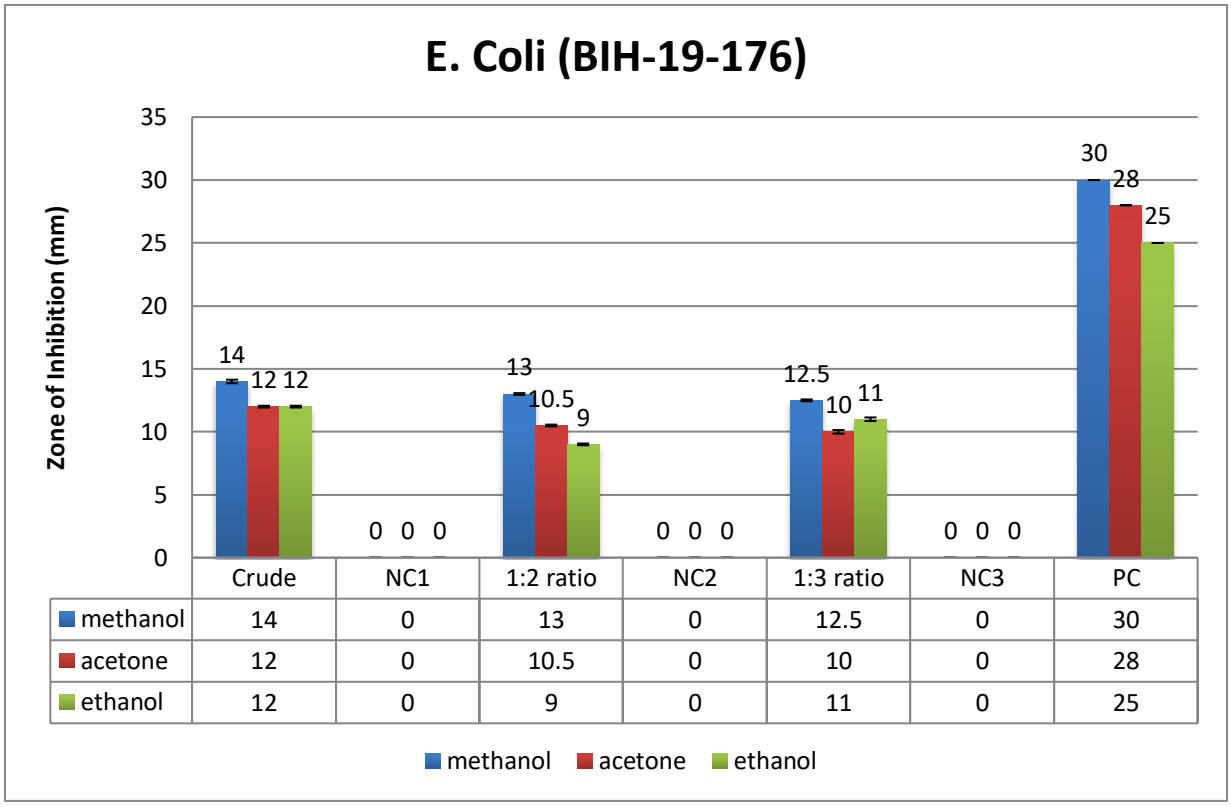


Fig.3: Antibiogram of the Ethanol extract of clove against *E. coli* (BIH-19-176)

(Agar well diffusion method was used to determine the zone of inhibition by three organic extracts of clove (methanol, acetone, ethanol) at three different concentrations (crude, 1:2, 1:4) against this multidrug resistant E. coli strain. Three concentrations of negative controls (crude DMSO, DMSO diluted by 1:2 and 1:3) and one positive control (Imipenem) was used as standard procedure.)

The *E. coli* strain used in this experiment was a low drug resistant (LDR) bacterium with resistance to 3 antibiotics. In case of *E. coli*, the methanol extract showed better results than Acetone and Ethanol extracts. The crude concentration of methanol extract presented better zone of inhibition. (Chart: 2)

In the following bar graphs, NC stands for "Negative Control", which was also diluted maintaining the same ratio as the extracts. DMSO was diluted down using water as water has no antimicrobial activities. Again, PC stands for "Positive Control" as which Imipenem (10µg) was used.



	Crude	NC1	1:2 ratio	NC2	1:3 ratio	NC3	PC
methanol	14	0	13	0	12.5	0	30
acetone	12	0	10.5	0	10	0	28
ethanol	12	0	9	0	11	0	25

Chart.1: Comparative graph of the E. coli zones of inhibition vs. the organic extracts

Pseudomonas spp. (BIH-19-002) Antibiogram Result

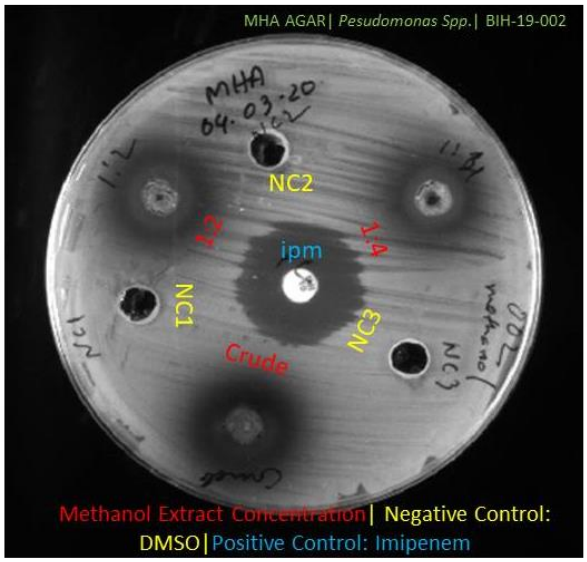


Fig.4: Antibiogram of the Methanol extract of clove against *Pseudomonas spp.* (BIH-19-002)

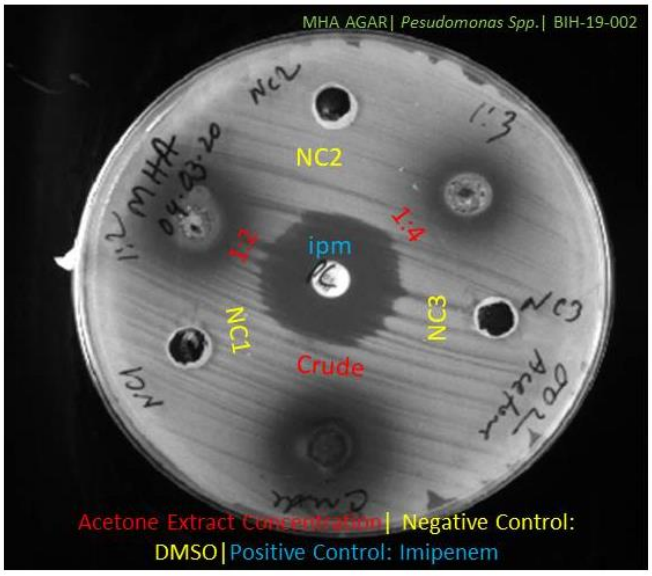


Fig.5: Antibiogram of the Acetone extract of clove against *Pseudomonas spp.* (BIH-19-002)

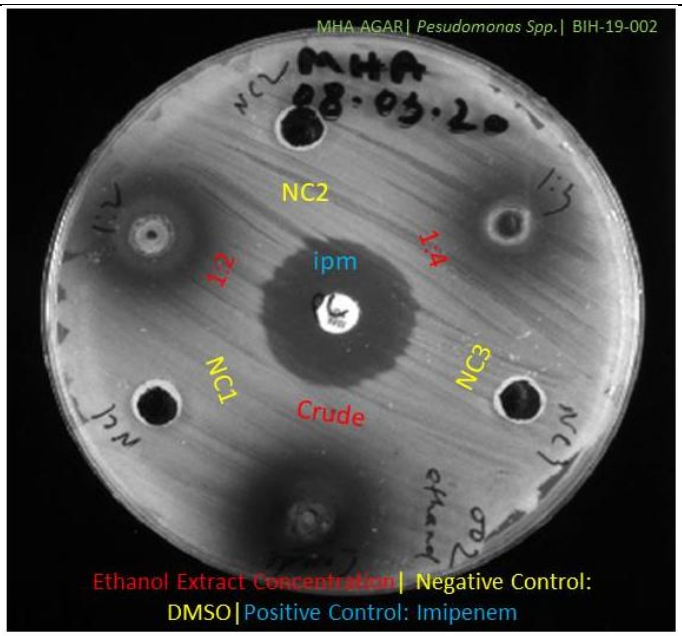


Fig.6: Antibiogram of the Ethanol extract of clove against *Pseudomonas spp.* (BIH-19-002)

(Agar well diffusion method was used to determine the zone of inhibition by three organic extracts of clove (methanol, acetone, ethanol) at three different concentrations (crude, 1:2, 1:4) against this multidrug resistant Pseudomonas spp. strain. Three concentrations of negative controls (crude DMSO, DMSO diluted by 1:2 and 1:3) and one positive control (Imipenem) was used as standard procedure.)

For this strain of Pseudomonas spp., acetone extract performed best in it's crude concentration. It was a low drug resistant bacteria or LDR having resistance to one antibiotic.

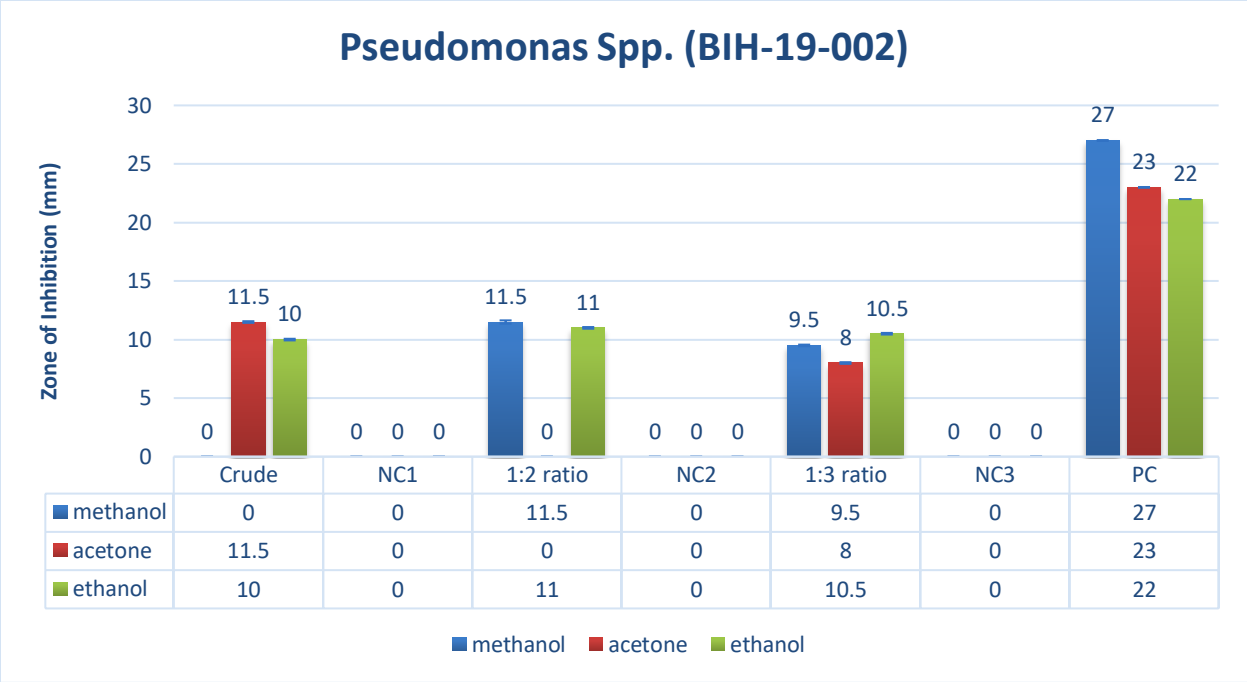


Chart.2: Comparative graph of the Pseudomonas spp. (BIH-19-002) zones of inhibition vs. the organic extracts

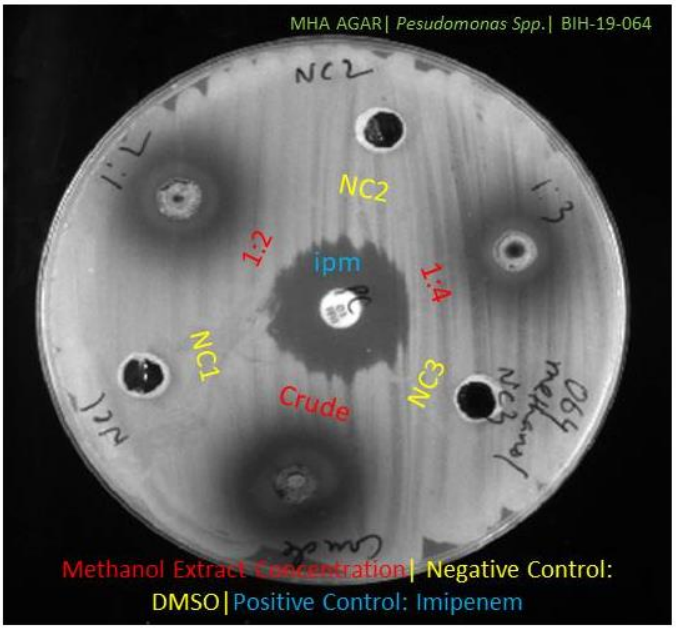
***Pseudomonas spp.* (BIH-19-064) Antibiogram Result**


Fig.7: Antibiogram of the Methanol extract of clove against *Pseudomonas spp.* (BIH-19-064)

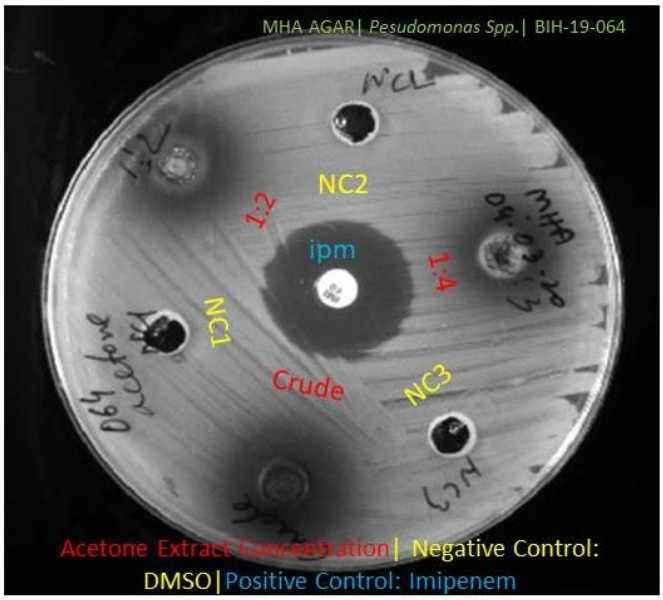


Fig.8: Antibiogram of the Acetone extract of clove against *Pseudomonas spp.* (BIH-19-064)

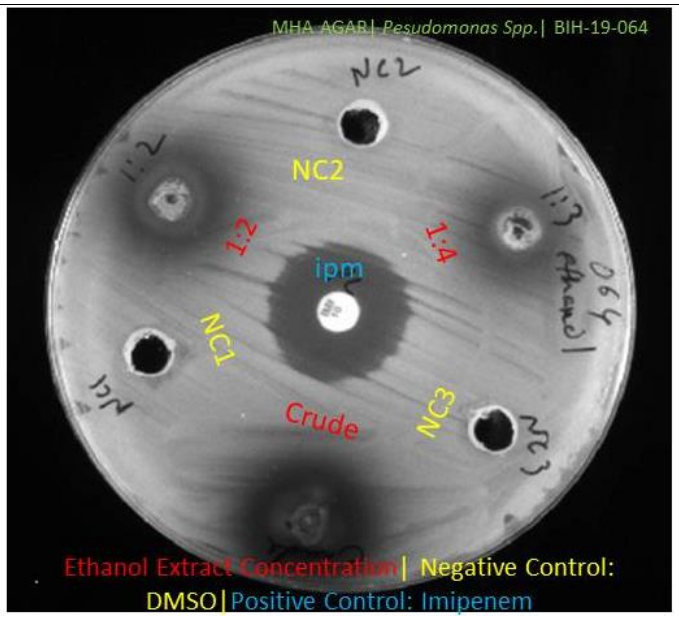


Fig.9: Antibiogram of the Ethanol extract of clove against *Pseudomonas spp.* (BIH-19-064)

(Agar well diffusion method was used to determine the zone of inhibition by three organic extracts of clove (methanol, acetone, ethanol) at three different concentrations (crude, 1:2, 1:4) against this second multidrug resistant Pseudomonas spp. strain. Three concentrations of negative controls (crude DMSO, DMSO diluted by 1:2 and 1:3) and one positive control (Imipenem) was used as standard procedure.)

Pseudomonas Spp. (BIH-19-068) strain was best responsive to the acetone extract in the concentration of 1:3 ratio. This strain is a multidrug resistant bacteria. It is resistant towards 5 antibiotics.

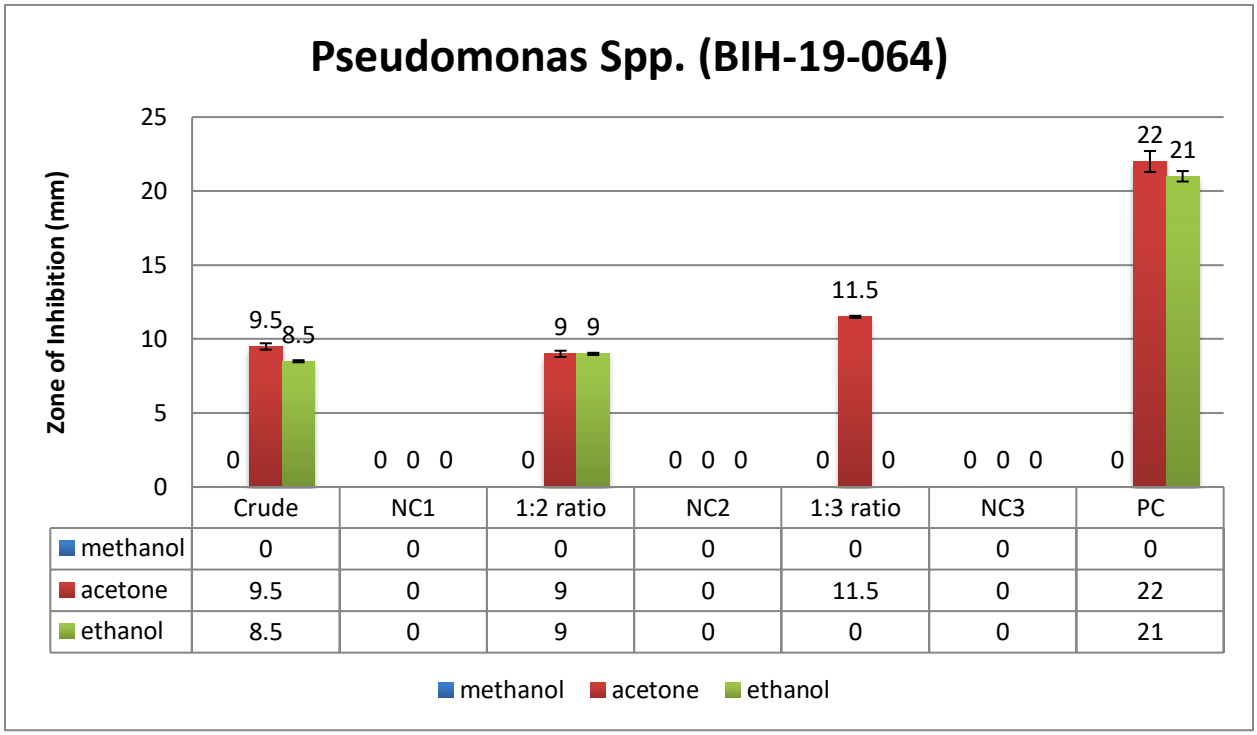


Chart.3: Comparative graph of the Pseudomonas Spp. (BIH-19-064) zones of inhibition vs. the organic clove extracts

Klebsiella spp. (BIH-19-068) Antibiogram Result

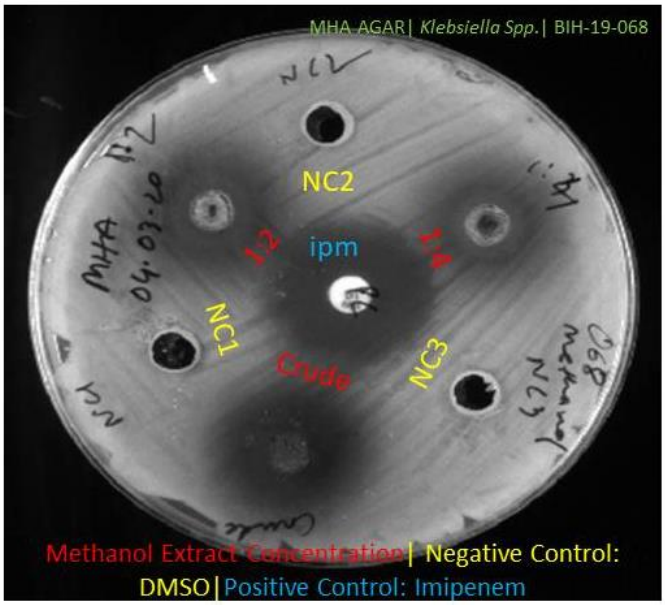


Fig.10: Antibiogram of the Methanol extract of clove against *Klebsiella spp.* (BIH-19-068)

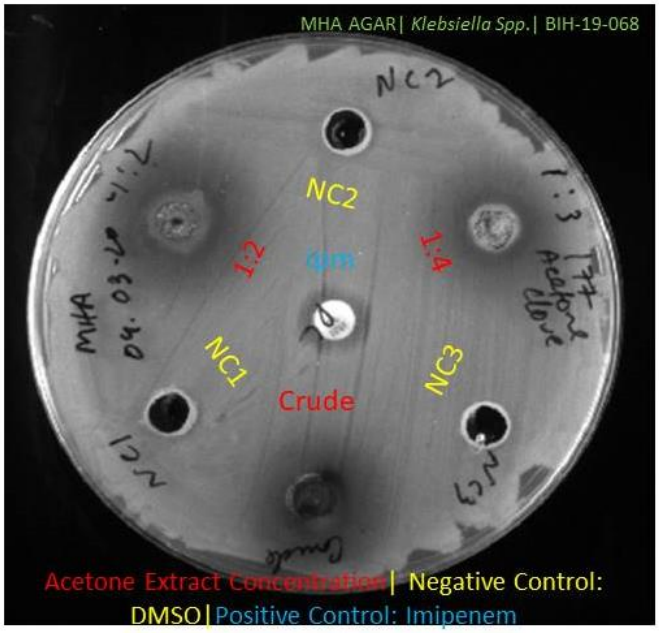


Fig.11: Antibiogram of the Acetone extract of clove against *Klebsiella spp.* (BIH-19-068)

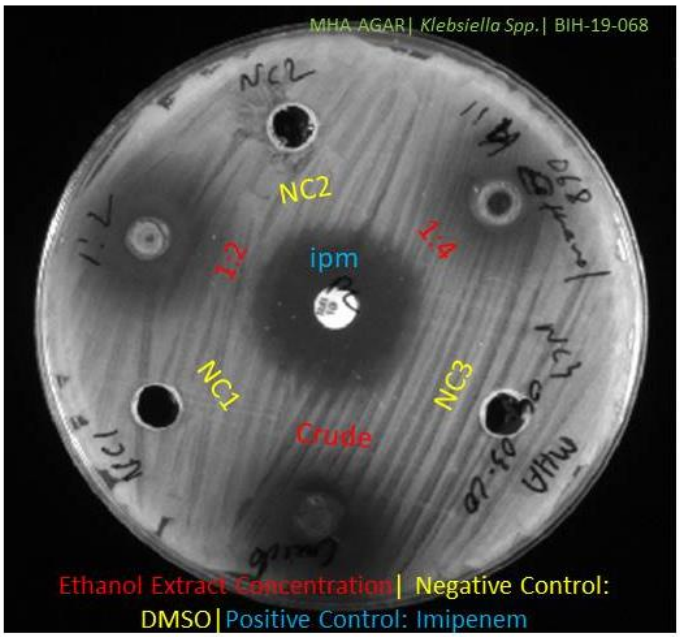


Fig.12: Antibiogram of the Ethanol extract of clove against *Klebsiella spp.* (BIH-19-068)

(Agar well diffusion method was used to determine the zone of inhibition by three organic extracts of clove (methanol, acetone, ethanol) at three different concentrations (crude, 1:2, 1:4) against this multidrug resistant Klebsiella spp. strain. Three concentrations of negative controls (crude DMSO, DMSO diluted by 1:2 and 1:3) and one positive control (Imipenem) was used as standard procedure.)

This *Klebsiella spp.* (BIH-19-068) strain is a multidrug resistant bacterium with resistance to 6 antibiotics and also, in the intermediate stage of being resistant to 3 other antibiotics. The extract that displayed better result was the acetone extract in crude concentration.

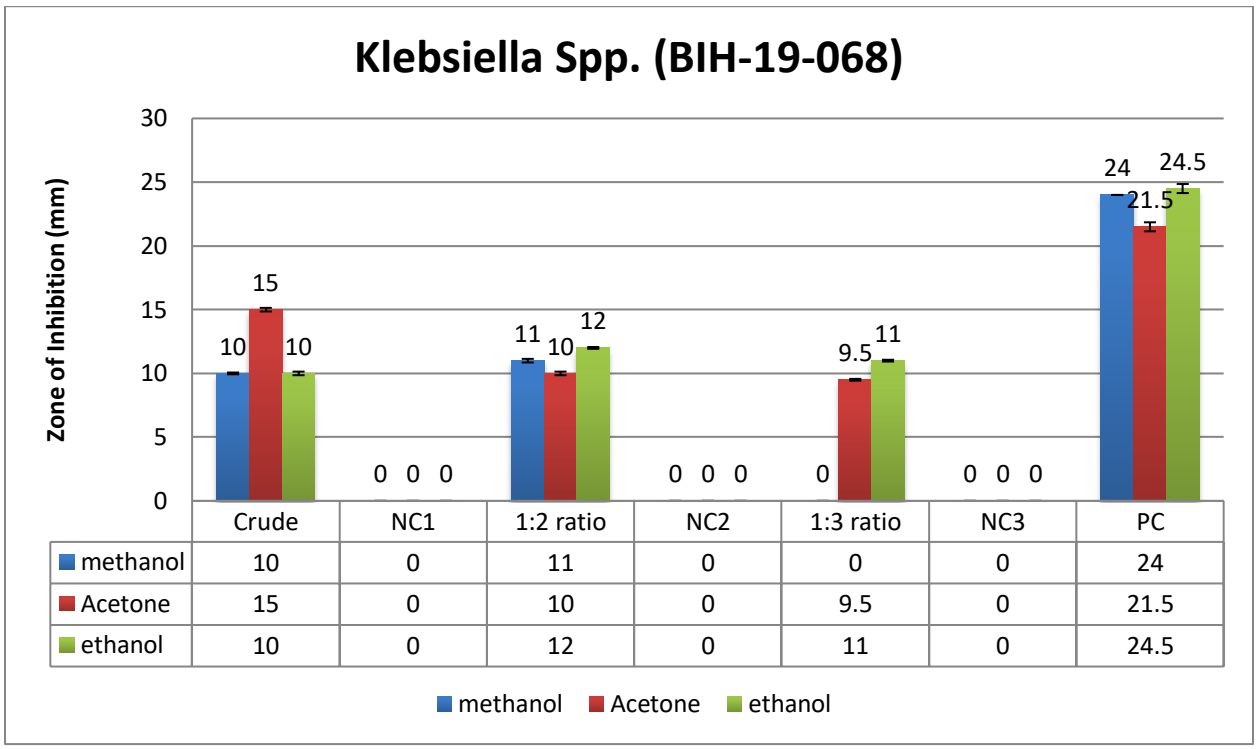


Chart.4: Comparative graph of the Klebsiella Spp. (BIH-19-068) zones of inhibition vs. the organic clove extracts

Klebsiella spp. (BIH-19-177) Antibiogram Result

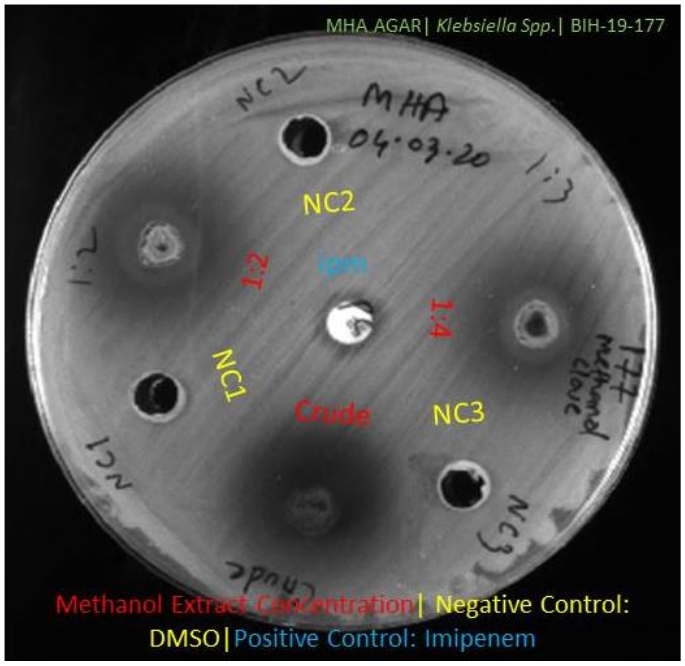


Fig.13: Antibiogram of the Methanol extract of clove against *Klebsiella spp.* (BIH-19-177)

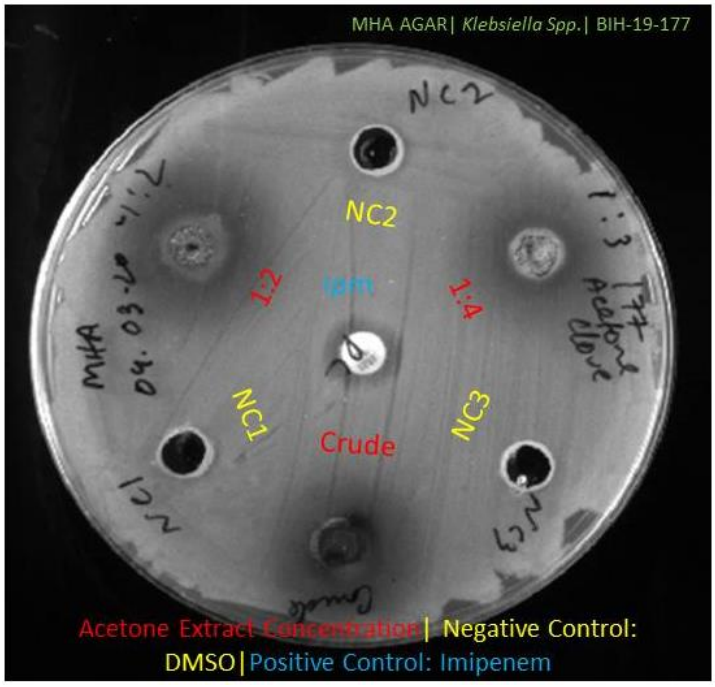


Fig.14: Antibiogram of the Acetone extract of clove against *Klebsiella spp.* (BIH-19-177)

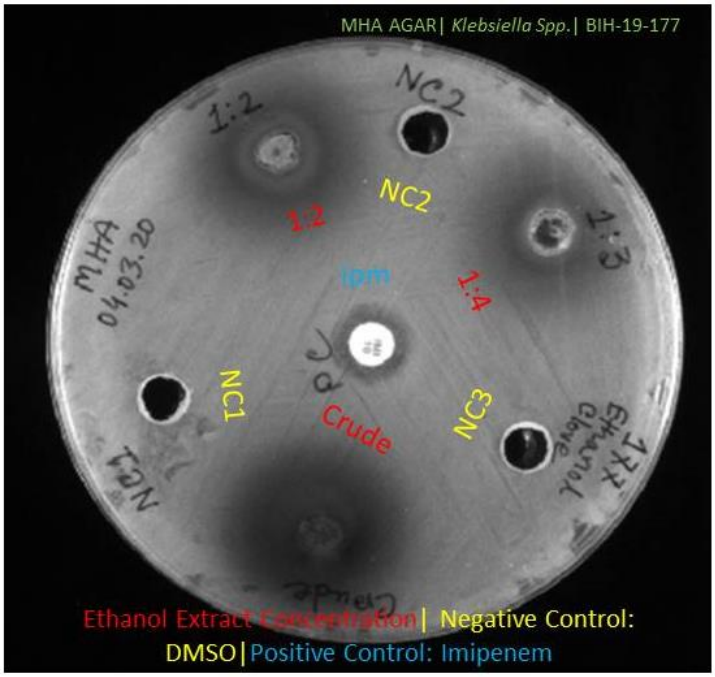


Fig.15: Antibiogram of the Ethanol extract of clove against *Klebsiella spp.* (BIH-19-177)

(Agar well diffusion method was used to determine the zone of inhibition by three organic extracts of clove (methanol, acetone, ethanol) at three different concentrations (crude, 1:2, 1:4) against this second multidrug resistant Klebsiella spp. strain. Three concentrations of negative controls (crude DMSO, DMSO diluted by 1:2 and 1:3) and one positive control (Imipenem) was used as standard procedure.)

In case of this extremely drug resistant (XDR) bacteria having resistance to 12 antibiotics, where Imipenem showed poor results in comparison, acetone extract in the crude concentration showed promisingly good results.

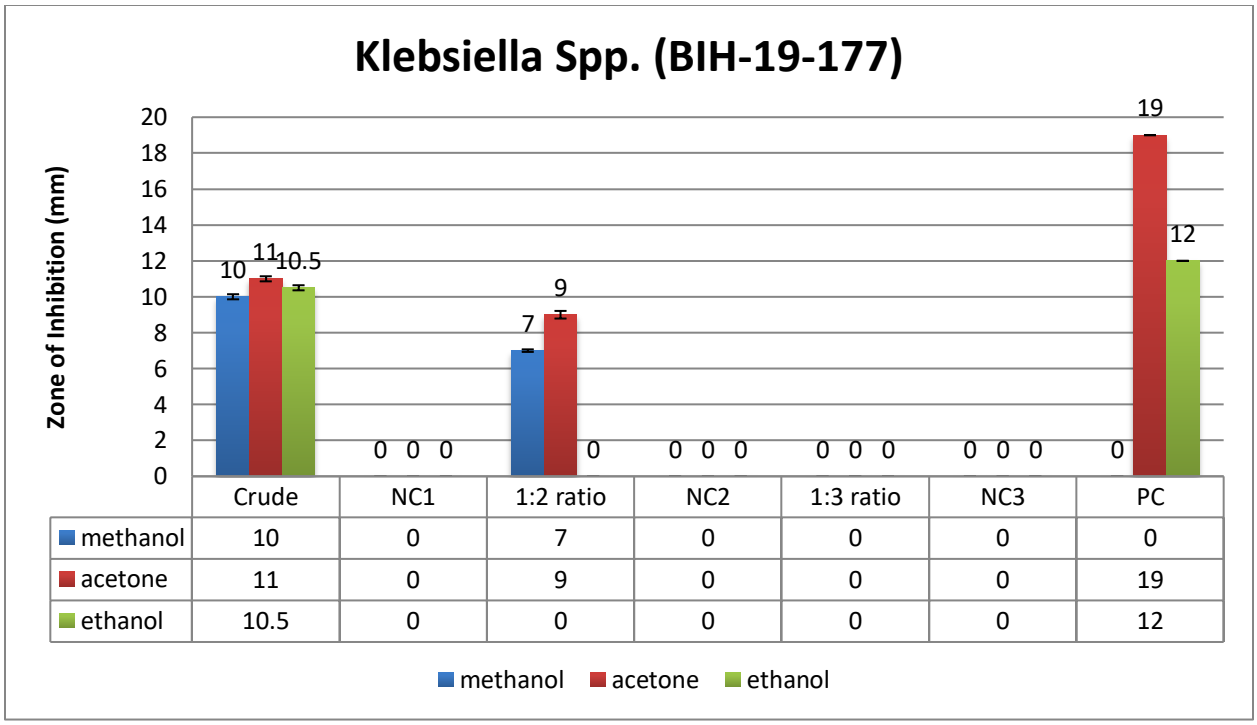


Chart.5: Comparative graph of the Klebsiella Spp. (BIH-19-177) zones of inhibition vs. the organic clove extracts

Klebsiella spp. (BIH-19-130) Antibiogram Result

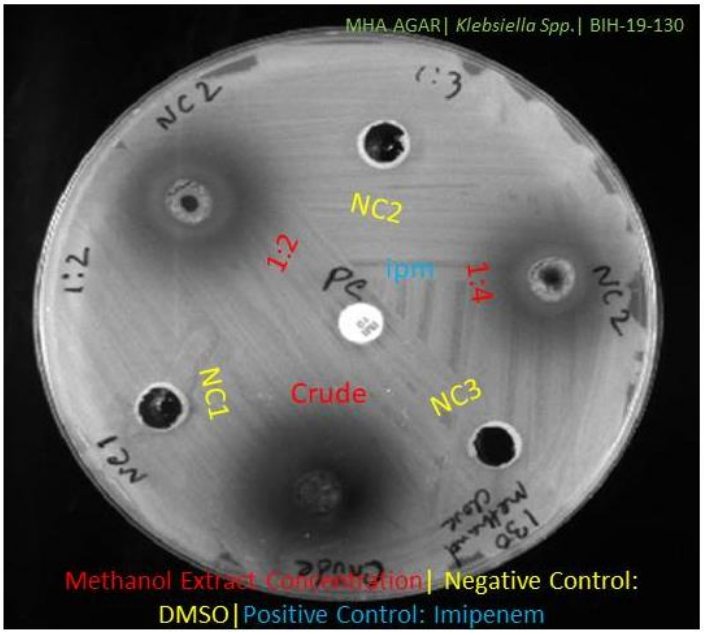


Fig.16: Antibiogram of the Methanol extract of clove against *Klebsiella spp.* (BIH-19-130)

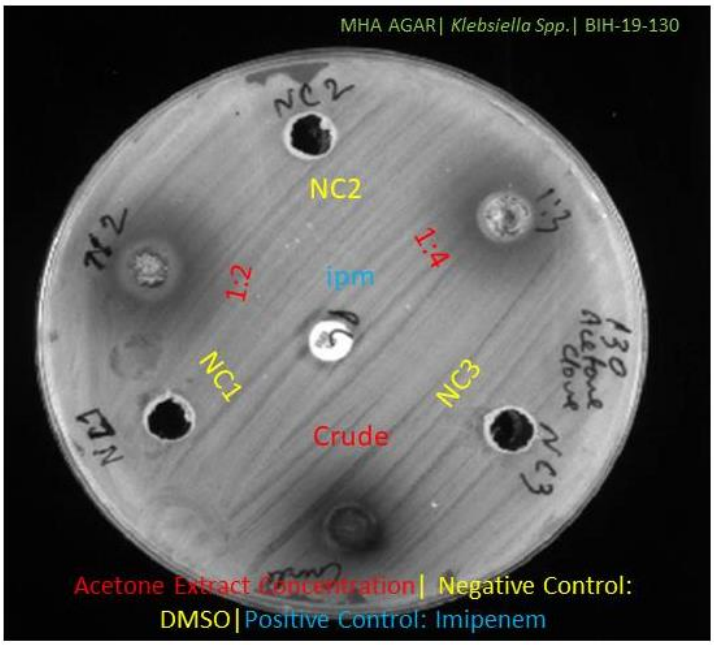


Fig.17: Antibiogram of the Acetone extract of clove against *Klebsiella spp.* (BIH-19-130)

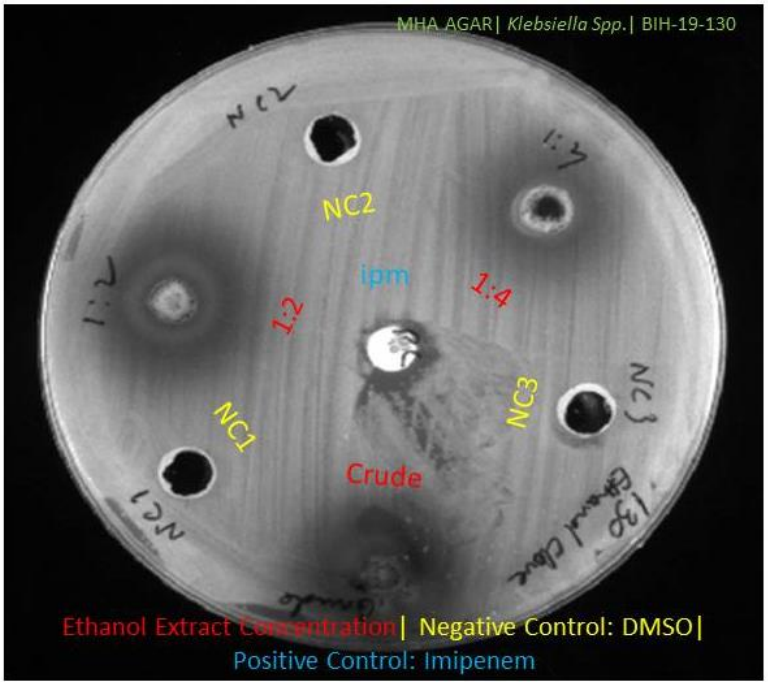


Fig.18: Antibiogram of the Ethanol extract of clove against *Klebsiella spp.* (BIH-19-130)

(Agar well diffusion method was used to determine the zone of inhibition by three organic extracts of clove (methanol, acetone, ethanol) at three different concentrations (crude, 1:2, 1:4) against this third and extreme drug resistant Klebsiella spp. strain. Three concentrations of negative controls (crude DMSO, DMSO diluted by 1:2 and 1:3) was used. Even though we could not find any viable antibiotic for this strain to be used as positive control, Imipenem was used to maintain standard procedure.)

This particular strain of *Klebsiella Spp.* is the most drug resistant bacteria from our study. It is resistant to 12 antibiotics and in the intermediate stage of resistance to another. Where imipenem showed zero zones of inhibition, the methanol extract displayed good outcome in the crude concentration.

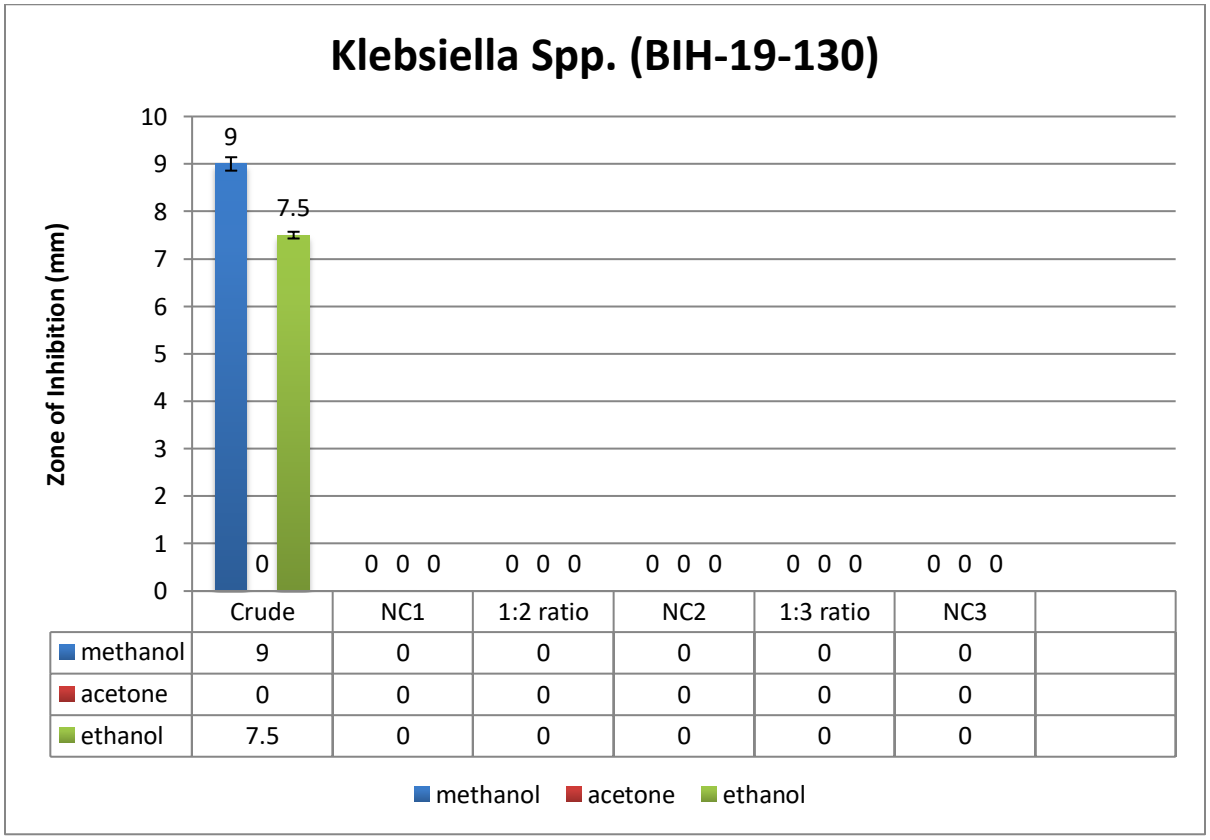


Chart.6: Comparative graph of the Klebsiella Spp. (BIH-19-130) zones of inhibition vs. the organic clove extracts

The acetone and methanol extracts of clove exhibited overall good bactericidal effect in comparison to the selected antibiotic (Imipenem) against all three kinds of human pathogenic bacteria. The maximum effect of clove extract was displayed on with maximum zone of inhibition in Kebsilla spp. (MDR strain) and the minimum effect was shown with minimum zone of inhibition, also in Klebsiella spp. (XDR strain). The results indicate that that using the clove spice can be a substantial move against diseases caused by these organisms. It is evident by the results that plants contain a wide spectrum of antimicrobial compounds or metabolic toxins inside them which can be processed to be used as sustainable antibiotics.

Discussion:

In this study, we analyze the viability of various organic solvent concentrates of the clove extract in vitro against some normal pathogenic microscopic organisms. Since ancient times, spices and plant extricates have been utilized in Ayurveda, Homeopathy and common households as remedies to various diseases. Clove being perhaps the most widely recognized extract utilized for common influenza, toothache, etc. crested our advantage to be experimented in laboratories for explicit outcomes. The results were recorded by considering the zones of inhibition using agar well diffusion method. We found that, among different organic solvents, acetone performed impressively against the chosen strains of bacteria in harmony to the results of (Rahman et al., 2011). In the group of sample strains we chose, there were two from each of the MDR (Multiple Drug Resistant), XDR (Extensively Drug Resistant) and LR (Low Resistant) category. A study performed in the Institute for developing Science & Health initiatives (ideSHi), collected, isolated and categorized these bacteria as having resistance to more than 3 antimicrobial agents is MDR, more than 6 is XDR and resistance to 1-3 is LR. In previous studies, solvent extracts showed greater potential than aqueous extracts (Saeed et al., 2013) and so in our study, we compared three organic solvent extracts side by side to find out which produces better outcome. As we worked as an understudy project at ideSHi, we had six strains of three gram negative bacteria in our sample group from a previous study. However, these bacteria being the most common human pathogens and highly evolving to MDRs and XDRs in the context of our country, were perfect set of test subjects. The extraction method we followed was in consistency to that of (Rahman et al., 2011) where they used three typed of organic solvent and mixed them with powdered clove.

Previous studies using clove as an antimicrobial agent used different sophisticated extraction methods that basically isolated the essential oil of clove. However, studies similar to ours used disc diffusion method (Rahman et al., 2011) for more tidy results in contrast to ours where we used agar well diffusion method in aim to get more direct contact of the antimicrobial agent with the targeted bacteria.

A MDR strain of *Klebsiella spp.* (BIH-19-068) was inhibited best by Acetone extract of clove. The lowest inhibition (7mm), thus the highest resistance was shown by an XDR strain of *Klebsiella spp.* The zones of inhibition prove clove's credibility as an antimicrobial agent. For an

XDR strain of Klebsiella spp. (BIH-19-130) from our group of test organisms, our control antibiotic (Imipenem) failed to inhibit the bacteria's growth, where methanol and ethanol extracts of clove killed the bacteria in a relatively small zone around the crude concentration. Even if the zone of inhibition was not overwhelmingly good as established antibiotics, it shows promising results that it can still work as a bacteriostatic agent when the bacteria becomes a pan drug resistant or PDR bacteria. In case of *E. coli*, the strain at our hands was a low resistant or LR bacteria having resistance to three antibiotics. However, with the alarming rate of increase at bacterial resistance and *E. coli* being a common test subject in laboratories, it can soon become an XDR bacterial strain. Whereas, Acetone extract overall has shown best inhibition in the experiment (for *E. coli*), Methanol extract has shown almost as good result as Acetone extract.

However contrarily, in case of the MDR strain of Pseudomonas spp. (BIH-19-064), the methanol extract could not inhibit the bacteria at all, whereas the acetone extract worked best at its concentration of 1:3. The low resistant strain of Pseudomonas spp. (BIH-19-002) also reacted better to Acetone extracts (crude concentration) in terms of bacterial inhibition.

Our sampling method basically aimed to experiment the bactericidal effectiveness of three different organic solvent extracts against three different categories (MDR, XDR, LR) of the same genre (gram negative) of bacteria. So, six different strains in consistency to the categories of three different bacteria were sampled against Methanol, Acetone and Ethanol extracts of the clove spice. The methodology of extraction and antibacterial effect detection was intentionally kept to the basic structure of laboratories for the scope of easier duplication and future improvisation. As COVID-19 hit all the other sectors of Bangladesh, this study was too affected by the pandemic. As for future scope of work on this study, we are farseeing the HPLC (full form) analysis of the extracts to find out the basic chemicals working behind inhibition of each of the different type of bacteria. A mammalian cell culture with the extracts used as antibiotics followed by MIC, MBC of the extracts by using McFarland standard bacterial culture can show the exact concentration at which the extracts can be used as drugs for human.

Conclusion:

In the final analysis, clove has shown to contain pharmaceutically active constituents with antibacterial properties. Even though, different studies have shown the efficacy of clove as an antibacterial agent on diverse variables, in our study, clove has proven to be effective on multidrug resistant (MDR) bacteria and even on one extremely drug resistant (XDR) one. Correspondingly, we investigated three different organic solvents (Methanol, Acetone & Ethanol) to extract the best antibacterial potential of clove and paralleled their results to the agar well diffusion test results to discover the best solvent(s) for the purpose. The outcome of this thesis is a groundwork for further experimenting with other pathogenic drug resistant bacteria associated with human diseases; leading to establishment of some components which are promising for newer and more potent drug development. We hope to elaborate this project in future following the worldwide search for antimicrobial agents from the nature and formulate a prospective new drug from clove.

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