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Antibacterial activity of selected plants extract against pathogenic bacteria and detection of phytochemicals

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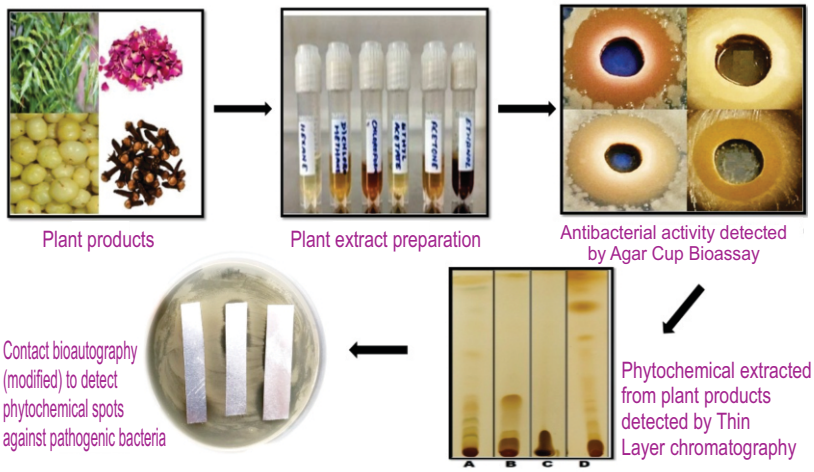
Abstract

Aim: The aim of the present study was to assess the antibacterial activity of selected plants extract against Gram- positive and Gram- negative pathogenic bacteria.

Methodology: Extracts of neem leaf (*Azadirachta indica*), rose petal (*Rosa indica*), amla fruit (*Emblica officinalis*) and clove buds (*Syzygium aromaticum*) were prepared in different solvents. Antibacterial activity against selected microorganisms was assayed using agar cup diffusion method, thereby measuring the zone of inhibition. The extracts with higher zone of inhibition were run on Thin Layer Chromatography (TLC) plates. The presence of phytochemicals was detected using iodine fumigation method followed by contact bioautography.



Results: The extracts of neem leaf, rose petal, amla fruit and clove bud prepared in solvents like acetone, ethanol and ethyl acetate showed higher antibacterial effect against *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhimurium* in agar cup diffusion assay. TLC separation of the extract of plant products showed dark brown bands of phytochemicals on silica-gel G 60 plate. Contact bioautography results showed maximum antibacterial activity against *Klebsiella pneumoniae.*

Interpretation: The results of the present study provide scientific evidence for the traditional uses of *Azadirachta indica, Rosa indica, Emblica officinalis* and *Syzygium aromaticum.*

Key words: Agar cup diffusion assay, Antibacterial activity, Contact bioautography, Pathogenic bacteria, Phytochemicals

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Introduction

Interest in the study of various medicinal plants has increased globally during last few decades, may be due to their antibacterial and antioxidant activities, low toxicity and comparatively cheaper to synthetic drugs (Chew *et al.,* 2012). The determination of antibacterial activities of different medicinal plants is of special interest due to the current global issue of increasing antibiotic resistance of microorganisms (Farjana *et al.,* 2014). The most important of these bioactive compounds are alkaloids, flavonoids, tannins, and phenolic compounds. Plants produce secondary metabolites that possess effective pharmacological activity (Raja and Sreenivasulu, 2015). Flavonoids with dominant antimicrobial activities act via microbial cell membranes and interacts with bacterial cell membrane protein of both Gram-positive and Gram-negative organisms (Upadhyay *et al.,* 2014). Medicinal importance of plants has been used in various countries all over the globe. These medicinal plants have been a source of many powerful, effective and potent drugs (Srivastava *et al.,* 1996).

Pathogenic bacteria such as *Staphyllococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhimurium* are the basic cause of many diseases in animals and humans. It is elaborated that death provoking pathogens are often unidentified or arise in mixed infections with more than one pathogenic agent (Hessling *et al.,* 2017). A list of globally most important pathogens was generated based on the causes of death, and this statistics was published in the Global Burden Disease Study 2015 (Wang *et al.,* 2016). *S. aureus,* a Gram-positive bacterium is usually found on the skin and in the upper respiratory tract. It commonly causes bacteremia and infected endocarditis (Tong *et al.,* 2015). *S. pyogens* is another Gram-positive bacterium that colonizes throat, rectum, skin and genital mucosa causing mild skin infections to fatal systemic diseases (Ryan and Ray, 2004). *E. coli* is a Gram-negative bacterium that inhabit the lower abdomen of warm-blooded animals. The majority of the *E. coli* strains do not cause any disease, but the virulent strains cause neonatal meningitis, urinary tract infection, and Crohn's disease (Todar, 2007; Lim *et al.,* 2010). *K. pneumonia,* a Gram-negative bacterium is found in the normal flora of skin, mouth and intestine (Ryan and Ray, 2004). It commonly causes of pneumonia. *S. typhimurium* is another Gram-negative bacterium, mainly found in the intestinal lumen, causing gastroenteritis in humans and other mammals (Everest, 1999).

According to World Health Organization, 80% of the world population depends on herbal medicines for their primary health care needs International Union for Conservation of Nature & World Wildlife Fund (1993). Globally over 40% of the population is dependent directly on plant-based medicines (Jagatheeswari *et al.,* 2013). *Azadirachta indica* commonly known as neem is a potent medicinal plant with multiple biological activities of its various parts against many pathogenic strains that possess antibacterial activities, antiviral and antifungal properties (Atawodi and Atawodi, 2009). The neem plant has been reported having more than 135 bioactive compounds isolated (Girish and Bhat, 2008). Recently Uzzaman 2019 has reviewed wide range of medicinal activities of neem (*Azadirachta indica*) plant. The leaves and petals *Rosa indica* or Rose are found effective against fever, diuretic, bronchial congestion, cold and sore throat.

There have been very few reports on the antibacterial activity of rose, but rose water and oil have been found effective against eye and skin irritation (Koday *et al.* 2010; Sahoo *et al.,* 2011). Numerous beneficial biological properties make *Emblica officinalis* or Amla, an important plant for herbal drugs. Amla fruit a super-rich source of vitamin C is used against a variety of clinical manifestations (Majeed *et al.,* 2009). *Syzygium aromaticum* or Clove is reported having properties against bacterial diseases and also as fragrance and flavor (Saeed and Tariq, 2008). The determination of antibacterial activities of different medicinal plants is of special interest these days due to the current global issue of increasing antibiotic resistance of microorganisms. The objective of the present study therefore was to assess the antibacterial activities of neem leaf (*Azadirachta indica*), rose petal (*Rosa indica*), amla fruit (*Emblica officinalis*) and clove buds (*Syzygium aromaticum*) against selected Gram-positive and Gram-negative pathogens.

Materials and Methods

Plant materials: Fresh leaves of *A. indica*, petals of *R. indica*, and fruits of *E. officinalis* were collected from University of Agriculture and Technology, Faizabad. *S. aromaticum* buds were purchased from the local vendor in Ghaziabad The leaves of neem and petals of rose were separated from the stem and air-dried for 3-4 days till constant weight was obtained. They were regularly examined to check any fungal growth or rotting. The fruit pulp of *E. officinalis* was also dried at room temperature. Dried plant materials were stored in paper bags and labelled properly.

Extraction of plant materials: Dried plant materials were crushed and soaked in hexane in a sterile conical flask and placed in an incubator at 37°C with continuous shaking for 24 hr. The content was filtered using Whatman filter paper No. 1 and the residues were re-soaked in dichloromethane for the next 24 hr followed by chloroform, ethyl acetate, acetone and ethanol. Filtrates were concentrated to dryness; weight was taken, dissolved in respective solvents, and then stored in a sterile glass vial at 4°C until used.

Microorganisms and maintenance: Gram-positive bacteria used in this study were *Streptococcus pyogenes* (ATCC 19615) and *Staphylococcus aureus* (MTCC 96), while the Gram-negative bacteria were *Escherichia coli* (MTCC 739), *Klebsiella pneumoniae* and *Salmonella typhimuriam* (ATCC 14028). Luria Bertani (LB) agar (HiMedia) was used to maintain all the bacteria other than *Streptococcus pyogenes,* for which Trypticase Soy Agar (TSA- HiMedia) was used. The bacteria were maintained at 4°C and cultured in broth at 37°C.

Agar Cup Assay: The LB agar plates for *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae* were prepared and TSA for *Streptococcus pyogenes* was also prepared. The bacteria were cultured in their respective broths, labelled carefully and kept in an incubator-shaker at 37°C overnight. In an aseptic environment, the inoculum was spread on the agar plate of each bacteria. With the help of a sterile cork borer cups were made in each petri plate. The concentrated plant extract was pipetted in each cup.The same amount of solvent was also pipetted in one of the cups and was treated as control. These plates were then kept in an incubator for 24 hr at 37°C. After 24 hr, the plates were observed for the zone of inhibition that was calculated in mm (Bauer *et al.,* 1966).

Thin Layer Chromatography: TLC Silica gel 60 F_{254} Aluminium sheet 20*20 cm (Merck, Germany) was used. Concentrated plant extracts (50 µl each) were pipetted on this sheet. The sheet was left to air-dry overnight. The next day this sheet was dipped in Toluene:Chloroform:Acetone (40:25:35) solution. Faint bands appeared on TLC plate after the solvent run. TLC sheet was left to air-dry. Dry TLC sheets were put in a chamber containing Iodine globules. Pink fumes started to emit. TLC sheet turned reddish-brown and the bands appeared (Lewis and Moody, 1989).

Contact Bioautography: The TLC sheet was cut into separate strips after Iodine fumigation. The LB and TSA agar plates were prepared. The bacteria were cultured in their respective broths, labelled carefully and kept in an incubator-shaker at 37°C overnight. In an aseptic environment, the inoculum was spread on the agar plate of each bacterium. TLC chromatogram was carefully placed with face down onto the inoculated plates. These plates were left in an incubator at 37°C for 24 hr. The zone of inhibition was thereafter calculated in mm.

Statistical Analysis: The experiment was conducted in triplicates and statistically analyzed for standard error.

Results and Discussion

The subcontinent of India is centre of possessing about 45000 plant species, out of which about 15000 have been

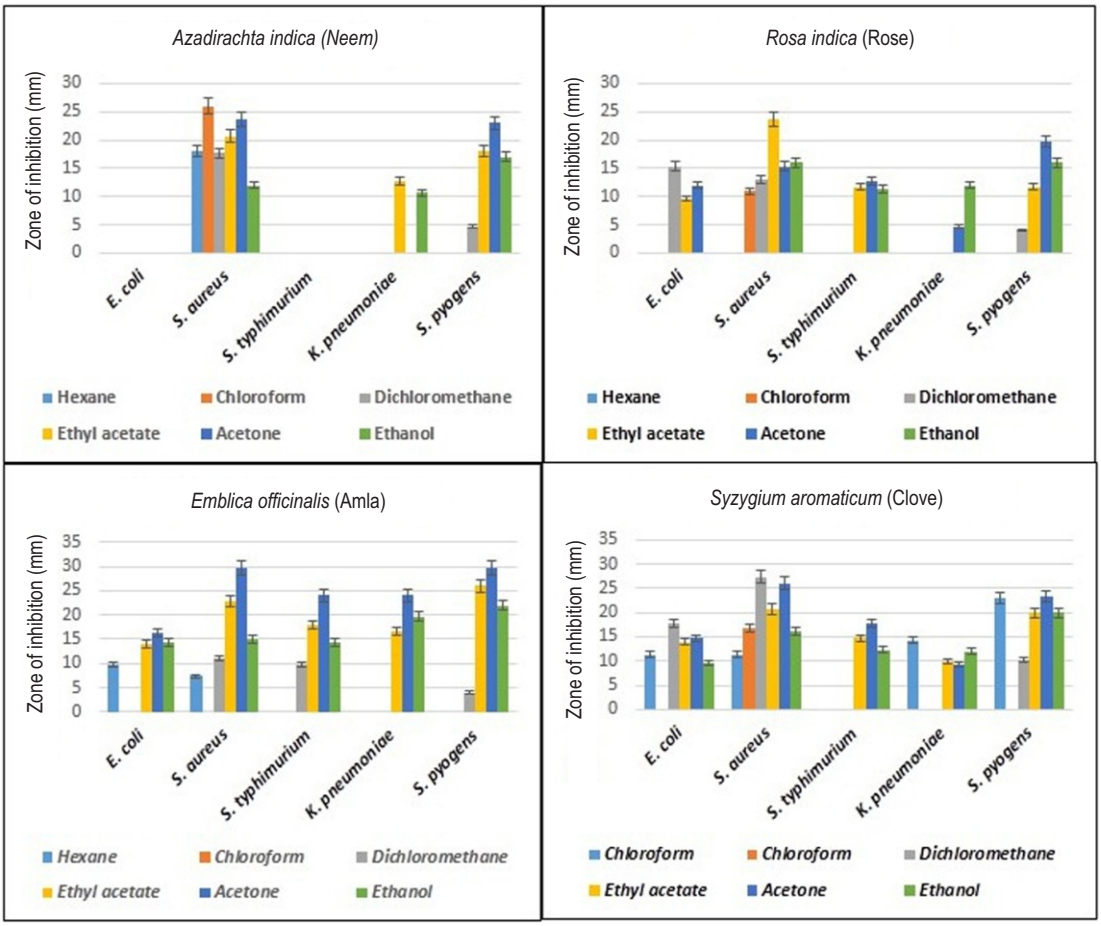

Fig. 1: Antibacterial activity of (A) *Azadirachta indica* (Neem);(B) *Rosa indica* (Rose); (C) *Emblica officinalis* (Amla) and (D) *Syzygium aromaticum* (Clove) solvent extracts against pathogenic bacteria, *E. coli*, S. aureus, *S. typhimurium*, *K. pneumonia* and *S. pyogenes.*

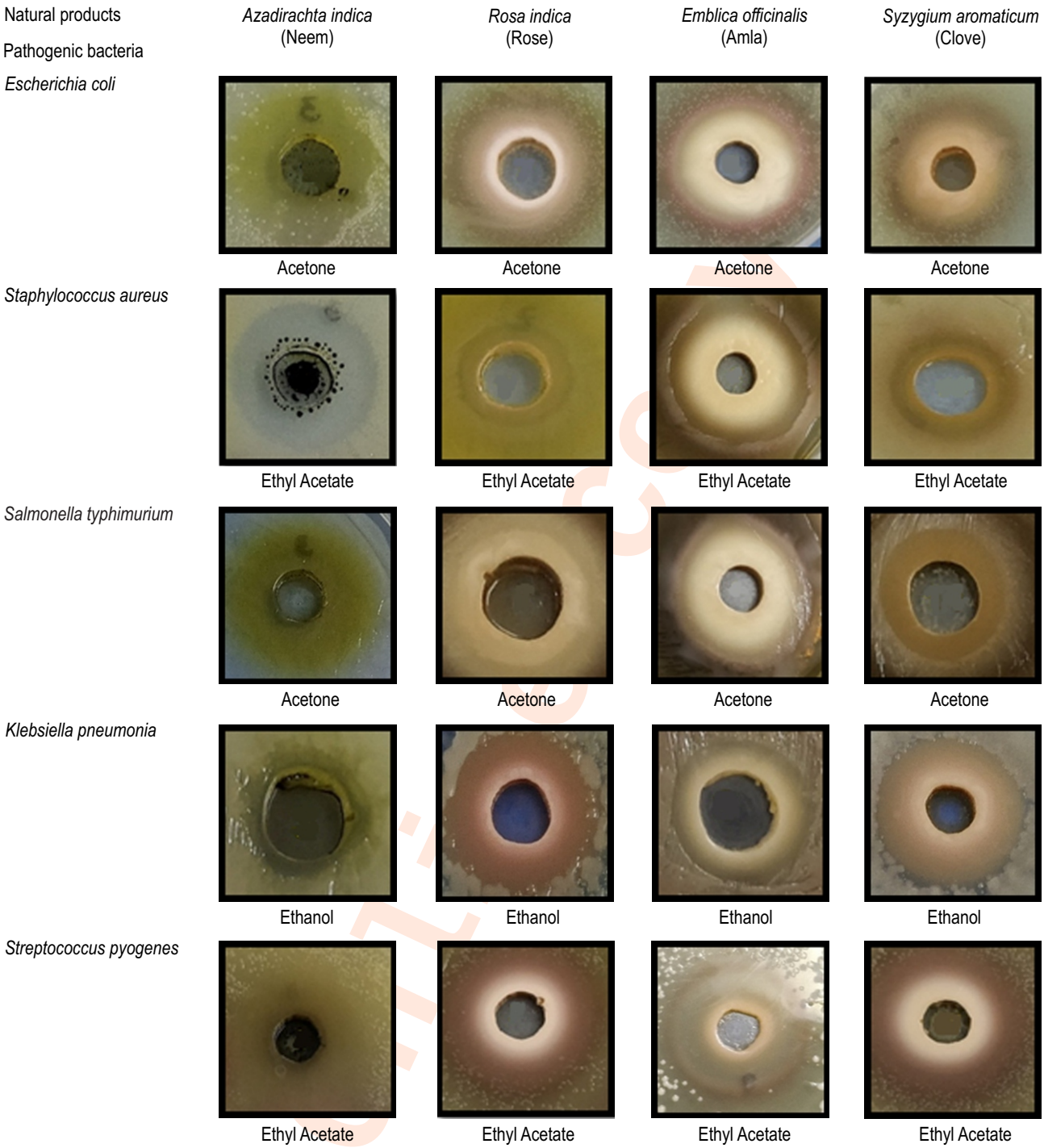

Fig. 2: Observation on Zone of Inhibition (ZoI) as measured by agar cup diffusion assay of *Azadirachta indica* (Neem), *Rosa indica* (Rose), *Emblica officinalis* (Amla) and *Syzygium aromaticum* (Clove) extract prepared in ethanol, acetone and ethyl acetate extracts giving best results against pathogenic bacteria, *E. coli, S. aureus, S. typhimurium, K. pneumonia* and *S. pyogenes.*

recorded as medicinal plants (Parasuraman *et al.,* 2014). In the present study, neem leaf extracts showed zone of inhibition (Fig. 1 A) against bacterial strains *S. aureus* in all the solvent used (12-26 mm), *K. pneumoniae* in ethyl acetate and ethanol (10.67-12.67mm) and *S. pyogenes* in dichloromethane, ethyl acetate, acetone and ethanol (4.67 -23 mm), but it failed to produce zone

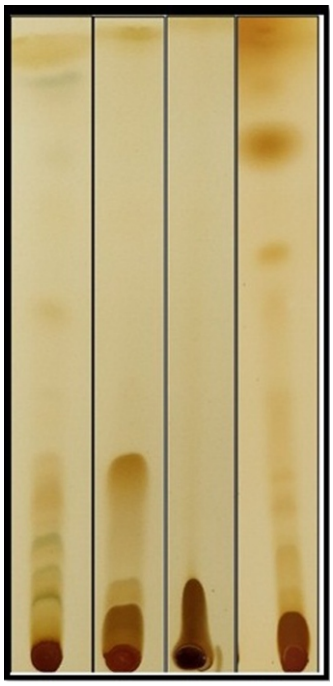

Fig. 3: Separation of the phytochemicals extracted from (A) *Azadiracta indica* (neem), (B) *Rosa indica* (rose), (C) *Emblica officinalis* (amla) and (D) *Syzygium aromaticum* (clove), TLC containing silica-gel G 60 plate using toluene: chloroform: acetone (40:25:35) solution using iodine fumes.

of inhibition in *E. coli* and *S. typhimurium* irrespective of solvent. A study similar to the present study, reported that ethanolic neem leaves extract had growth hindrance effect on *Salmonella* and *Klebsiella* at concentrations of 6.2 mg ml^{-1} and 12.5 mg ml^{-1} respectively. The average diameter of zone of inhibition was 18 mm for *Klebsiella* and 20 mm for *Salmonella* (Banna *et al.,* 2014). It however failed to inhibit the growth of *S. aureus* at the highest concentration used that is 50 mg ml^{-1}. The study thus suggested that, neem can be tried against *Klebsiella* and *Salmonella.* The ethyl acetate extractable fraction of neem leaves was inhibitory to the growth of *E. coli* O157 in LB broth whereas Azadirachtin, a neem product having insect anti-feedant properties, failed to inhibit *E. coli* O157 (Ravva and Korn 2015). Phytochemicals derived from the neem have also been reviewed for pharmacological effects such as anti-pyretic, anti-viral, analgesic, anti-bacterial, contraceptive and hepatoprotective activities (Nishan and Subramanian, 2014). Ethyl acetate, acetone and ethanol extracts of rose petals exhibited zone of inhibition to tested pathogens (Fig. 1B) in order *S. aureus* (11-23.67 mm), *S. pyogenes* (11.67-19.67 mm), *E. coli* (9.67-15.33 mm) *S. typhimurium* (11.33-12.67 mm) and *K. pneumoniae* (4.67-12 mm). The methanolic, ethanolic and distilled water extract of rose petals inhibited the common human pathogens.

The zone of inhibition was similar in the present study for the rose petal extracts prepared in ethyl acetate, acetone and ethanol (Fig. 1B) against *E. coli*, *S. typhimurium*, *S. aureus*, *S. pyogenes* and *K. pneumonia* (Laxmi *et al.,* 2017). *Rosa damascena* petals extract in water, hexane and ethanol and further fractionation with chloroform, ethyl acetate and butanol exerted broad spectrum antimicrobial activities against the tested organisms for example *S. aureus*, *Bacillus subtilis*, *S. pyogenes*, *Acinetobacter baumannii* and *K. pneumonia* (Shohayeb *et al.,* 2014). Amla extract prepared in ethyl acetate, acetone and ethanol had shown zone of inhibition (14 – 29.67 mm) against all the tested pathogens for ex. *E. coli*, *S. aureus*, *S. typhimurium, K. pneumoniae* and *S. pyogenes* (Fig. 1C). The inhibition was however not uniform in extracts prepared in hexane, dichloromethane and chloroform (0-11 mm). It has also been noted that amla extract prepared in acetone produced maximum inhibition (29.67 mm) in *S. aureus* and *S. pyogenes.* There are several reports on different degree of antimicrobial activity against some Gram negative and Gram positive pathogenic bacteria of the chloroform soluble fraction of the methanolic extract, aqueous extracts and aqueous infusion and decoction of amla (Rahman *et al.,* 2009; Vijayalakshmi *et al.,* 2007 and Saeed and Tariq 2007). Chloroform extract of amla in the present study has not shown inhibition against tested pathogens (Fig. 1C). Extracts of clove prepared in ethyl acetate, acetone and ethanol also induced zone of inhibition (9.33-26.00 mm) in all the tested pathogens (Fig. 1D) for example *E. coli*, *S. aureus*, *S. typhimurium*, *K. pneumoniae* and *S. pyogenes*. It is interesting to note that zone of inhibition was maximum (27.33 mm) against *S. aureus* with extract in dichloromethane followed by acetone (26 mm).

The antimicrobial activities of clove against several bacterial strains have been proved. Absolute bactericidal effect was seen when an aqueous extract of clove at 3% was tested against all the food-borne pathogens tested *E. coli, S. aureus* and *Bacillus cereus*. Good inhibitory action at the concentration of 1% clove extract was also reported (Sofia *et al.,* 2007). According to a study suggested, clove oil inhibits the growth of *S. aureus* at molecular level. The authors of this study hypothesised that the mechanism involved was inhibition of DNA synthesis by clove oil entering inside the cell by damaging cell wall or membrane causing loss of intracellular material, finally resulting into bacterial death (Xu *et al.,* 2016). The results of present study have shown higher zone of inhibition when the plant extracts were tested against the pathogenic bacteria. The results where compared with the available literature. In a study, the antimicrobial activity of standard Ciprofloxacin against *E. coli* and *S. aureus* was 10 mm and 7mm respectively (Balogun and Tunde 2020).

The antibacterial activity of neem leaf extract was almost 20 mm when tested against *E. coli* and 24 mm when Gentamycin was used against *E. coli* (Panchal *et al.,* 2013). The methanol extract of Amla pulp gave a zone of inhibition of 19 mm and 16 mm against *E. coli* and *S. aureus* respectively. The standard of Amoxicillin and Amphotericin B gave a zone of inhibition of 23 mm and 21 mm respectively, when tested against *E. coli* (Vijayalakshmi *et al.,* 2007). In the present study, the ethanol extract of Amla pulp has given a zone of inhibition of 15 mm and 14 mm against *S. aureus* and *E. coli* respectively. A zone of inhibition of 20 and 22 mm found when the ethanol extract of Amla

pulp was tested against *K. pneumonia* and *S. pyogens* respectively. The available literature when compared to the present study clearly indicated that not much difference is seen when compared with the standard antibiotics. It is interesting to note that higher antibacterial activity was found in extract of plants prepared in ethanol, acetone and ethyl acetate (Fig.2), when assayed against *S. aureus*, *S. pyogenes*, *E. coli*, *K. pneumonia* and *S. typhimurium*. The plants extracts studied qualitatively through TLC have shown several bands representing presence of phytochemicals (Fig. 3). It has also been noted that the degree of band colour on TLC plates as a measure varies indicating broad range of biological activities. TLC is considered a cost-effective, simple, and easy-to-operate technique having many applications used in the development of new drugs from plant resources (Kumar *et al.,* 2013). Several workers have reported presence of high content of varied bioactive compounds in amla, clove, neem and rose (Khan 2009, Rojas *et al.,* 1992, Girish and Bhat 2008, Cendrowski *et al.,* 2017, and Laxmi *et al.,* 2017).

In the present study the results of contact bioautography have shown antibacterial activity of phytochemicals against *K. pneumonia*, suggesting that the selective extraction by appropriate solvents is very important for obtaining bioactive fractions of significance from natural sources (Boakye-Yiadom and Konning 1975). The contact bioautography gave a 16 mm zone of inhibition of ethanol extract of *E. officinalis* against *K. pneumonia*. The ethanol extracts of *A. indica* and *R. indica* gave a zone of 12 mm and 13 mm respectively against *K. pneumonia.* The results of contact bioautography of acetone extract of *E. officinalis* against *S. aureus*, *S. typhimurium*, *K. pneumonia* and *S. pyogens* were 16 mm, 13 mm, 18 mm and 20 mm respectively. These results suggest the choice of solvent for extraction of the bioactive fraction is very important. Various reports showed that alkaloids and flavonoids, phenolics and tannins act as active antimicrobial compounds by precipitating microbial protein, which act as an inhibitor against pathogens (Chithrashree *et al.,* 2014; Prasad *et al.,* 2008). The antibacterial and antioxidant activities of the extract of *E. officinalis* and *Terminalia bellirica* which were found to be firmly associated with the total phenolic and flavanoid content of the extract have been reported (Badoni *et al.,* 2016). Alkaloids are also important group of differently distributed chemicals of biologically and commercially powerful natural products. The need for extensive research on alkaloids for drug development and discovery has been suggested (Cordell *et al.,* 2001). The present study based on the results has suggested that ethanol, acetone and ethyl acetate extracts of plants extract had better antibacterial activity than hexane, chloroform and dichloromethane against tested Gram-positive and Gram-negative pathogens. The phytochemicals present in the extract also confirmed antibacterial activity against *K. pneumoniae*. The presence of phytochemicals can be valuable substance for the production of pro health preparations.

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