

Antimicrobial and Phytochemical Studies of Methanolic Bark Extract of *Psidium guajava* L. and *Punica granatum* L.

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Abstract

Psidium guajava L. and *Punica granatum* L., commonly known as guava and pomegranate respectively are popular for their edible fruits. In addition, they also have medicinal value. Local people use their bark and leaves to cure many ailments. The aim of this study was to determine the antimicrobial and phytochemical properties of bark samples of guava and collected from Tinpatan-3, Bagthala, Sindhuli, Nepal during March, 2017. Methanolic extracts of the collected bark samples were evaluated for their antimicrobial activities against 12 microorganisms by agar well diffusion method. Pomegranate bark extract was found most active against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Proteus vulgaris* showing zone of inhibitions (ZOIs) 25mm and 22mm respectively, and minimum microbicidal concentrations (MMCs) 6.25 mg.ml⁻¹ and 0.78125 mg.ml⁻¹ respectively. Guava bark extract was found most active against *Proteus vulgaris* with ZOI value 18mm and MMC value 1.5625 mg.ml⁻¹. Phytochemical screening of guava bark extract confirmed the presence of alkaloids, flavonoids, reducing sugar and steroids, and absence of volatile oils, terpenoids, tannins, saponins and proteins. Pomegranate bark extract showed presence of alkaloids, terpenoids, flavonoids, tannins, saponins, reducing sugar and steroids, and absence of volatile oils and proteins.

Keywords: Extraction, Medicinal plants, Microorganism, Minimum inhibitory concentration (MIC), Zone of inhibition (ZOI)

Introduction

Medicinal plants are in use to treat different diseases since ancient time. Increasing resistance pattern and associated side effects of antibiotics have evolved the importance of medicinal plants to be used as an antibacterial agent (Sajjad et al., 2015). An antimicrobial is the physical or chemical agent that kills or inhibits the growth of micro-organisms such as bacteria, fungi, protozoa. Medicinal plants constitute several bioactive compounds that show antimicrobial and antioxidant activities (Kundal, 2013). Phyto constituents employed by plants to protect themselves against pathogenic insects, bacteria, fungi or protozoa have found applications in human medicine (Nascimento et al., 2000). Plant diversity serves the humankind as renewable natural resources for a variety of biologically active chemicals. These chemicals bear a variety of properties viz antibacterial, antifungal, antiviral, anthelmintic, anticancer, sedative, laxative,

cardiotonic, diuretic and others (Parajuli et al., 1998). Medicinal plants represent a rich source of antimicrobial agents (Abi Beaulah et al., 2011).

Psidium guajava L., commonly known as guava, belongs to Myrtaceae family. It is locally called ambaa in Nepali and is popular for its edible fruits. The plant is about 4-6m tall with peeling, reddish brown bark on young branches. Leaves are very short petioled, ovate or oblong, 7-10cm long, veins prominent, coriaceous, old leaves are reddish brown. Flowers are white, peduncled and axillary. Fruits are globose or pear-shaped, yellow and many seeded. *P. guajava* plant is native of Brazil and it is cultivated in Nepal. (Department of Plant Resources [DPR], 2016).

Punica granatum L., commonly known as pomegranate, belongs to Lythraceae family. It is a deciduous spiny tree of 5-10m tall. Its leaves are simple, glossy, opposite, oblong or obovate, 2-6cm long, narrowed to a short petiole. Flowers are bright

red, rarely white with thick fleshy petals. Fruits are ovoid, crowned with persistent calyx, outer surface woody, smooth, brownish red, 5-8cm in diameter and containing much red juice around the seed. Seeds are white, 1-2cm long. Flowering and fruiting time is May to June. (DPR, 2016).

Rokaya et al. (2014) found that bark of 152 plant species were used to cure gastrointestinal disorder in Nepal. Pomegranate and guava are popular not only for their edible fruits, but also for their stem, bark and leaf which are used for various purposes by the different communities in remote areas of Nepal. Traditional practices of local people in Nepal shows that stem bark decoction of guava is used to treat fever, diarrhea and dysentery, and leaf juice is taken to treat bowels, cuts, wounds and ulcers. Leaf bud is chewed to treat headache (Malla & Chhetri, 2009; Acharya, R., 2012; Thapa, S., 2012). Stem bark of pomegranate is also used to get relief during diarrhea and dysentery. This is due to medicinal properties found in the bark and leaf of this plant. Antimicrobial studies support and provide scientific basis for the traditional medical practices of local communities. In this study methanolic bark extracts of pomegranate and guava were screened for phytochemical and antimicrobial properties.

Materials and Methods

Collection and processing of samples

The bark samples of guava and pomegranate were collected in March, 2017 from Tinpatan-4, Bagthala, Sindhuli, Nepal. The bark samples were collected from the branch in strips of 3 inches randomly along the length of the tree taking precautions to avoid girdling. The collected samples were washed thoroughly, chopped into small pieces, dried in hot air oven at 60°C for 24 hours and ground into powder.

Extraction of plant materials

Powder of bark of pomegranate (35.5gm) and guava (15.2gm) were loaded for the Soxhlet extraction with methanol for 72 hours till the colorless solvent appeared in the siphon to obtain crude methanol extract of respective plants. After complete

extraction, solvent, i.e. methanol, was evaporated with the help of rotary vacuum evaporator using the water bath below 65°C. Solvent was completely evaporated and condensed solvent was collected in the separate round bottom flask (Eloff, 1998; Tiwari et al., 2011).

Percentage yield of the extract was calculated by using the following formula:

$$\text{Percentage yield} = \frac{\text{Initial weight of the sample} - \text{final weight of the sample}}{\text{Initial weight of the sample}} \times 100\%$$

Antimicrobial activity

Test organisms: *Bacillus subtilis* (Ehrenberg) Cohn, *Enterococcus faecalis* (Andrewes & Horder) Schleifer & Kilpper-Balz, *Staphylococcus aureus* Rosenbach, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli* (Migula) Castellani & Chalmers, *Klebsiella pneumoniae* (Schroeter) Trevisan, *Proteus vulgaris* Hauser, *Pseudomonas aeruginosa* (Schröter) Migula, *Salmonella enterica* subsp. *enterica* (ex Kauffmann & Edwards) Le Minor & Popoff serovar. Typhii, *Shigella dysenteriae* (Shiga) Castellani & Chalmers, *Candida albicans* (C.P. Robin) Berkhout, *Saccharomyces cerevisiae* Meyen ex E.C. Hansen were used as test organisms.

Preparation of the working solutions: Sterilized screw-capped tubes were calibrated and marked for 10ml. About 1 gm of extract was transferred into the calibrated tube. Methanol (solvent used for extraction) was added to the tube making the final volume to 10ml. Mixture was then homogenized by vortexing.

Preparation of standard culture inoculums: Required numbers of 18-24 hours old colonies of test organisms were inoculated aseptically to separate sterilized vials containing 5 ml of sterilized nutrient broth and were homogenized by vortexing. The inoculum, so prepared, was compared with turbidity of 0.5 McFarland Nephelometer standard recommended by World Health Organization [WHO] (1991) for antimicrobial susceptibility test.

Screening and evaluation of antimicrobial activity: The extract samples were screened for

antimicrobial activity using agar well diffusion methods as described by Perez et al. (1990).

A sterile swab was used to evenly distribute inoculums over Muller-Hinton Agar (MHA) for bacteria and Muller-Hinton Agar with Glucose and Methylene Blue (MHA, GMB) for fungi. The plate was rotated through an angle of 60° after each swabbing. The swabbing was done three times. The inoculated plates were allowed to dry for maximum 15 minutes. Four wells, each of 6 mm diameter, were created in the inoculated plates using a sterile cork borer (three wells for test samples and one well for the solvent as negative control). Micropipettes were used to dispense 50µl of the test solution of the extract samples and solvent as negative control into each of the four wells. The plates were left in the upright condition with lids closed for half an hour so that the test solutions diffused into the media. The inoculated plates were then incubated in inverted position at suitable temperature (35±2°C for bacteria and 25±2°C for fungi). After proper incubation (18-24 hours for bacteria, 24-48 hrs for fungi) the plates were examined for zone of inhibition (ZOI) around the well which is suggested by clear area with no growth of organisms. Diameter of each ZOI was measured using digital vernier caliper to the nearest whole millimeter (Rana et al., 2017).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Microbicidal Concentration (MMC): MIC was determined by observing the visible growth of the test microorganism in two-fold serial diluted antimicrobial substances in broth culture medium while MBC was determined by sub culturing the MIC cultures on suitable agar plates (Forbes et al., 2007).

The crude extract of medicinal plants, which showed zone of inhibition (ZOI), were subjected to two-fold serial dilution method to determine the MIC and further MMC. A set of 12 screw-capped vials, each containing 1 ml Muller Hinton Broth (MHB) for bacterium or 1 ml Sabouraud Dextrose Broth (SDB) for fungus, were prepared. The vials were then numbered from 0 to 11. MHB/SDB was discarded from vial no. 0 and 1 ml test solution was added.

Then, 1 ml of test solution was added to vial no. 1 and was homogenized by vortexing. From it, 1 ml content was transferred aseptically to vial no. 2 followed by homogenization. This process was repeated till two-fold serial dilution was done up to vial no. 10. Finally, 1 ml of the content was discarded from vial no. 10 after homogenization. Hence, all the vials contain equal volume i.e. 1 ml with gradually decreasing concentration. Now with the help of micropipette, 20µl of inoculums (a 1:100 dilution of a suspension of turbidity equal volume to McFarland Standard 0.5 supposed to have organism 1.5×10^6 CFU.ml⁻¹) was added to all vials except vial no. 0. All the tubes were incubated at 37±2°C for 18-24 hours for bacteria and 25±2°C for 24-48 hours for fungi. The tubes were then observed for turbidity and MIC of an extract was determined as the lowest concentration of antimicrobial agent in the two-fold dilution series which inhibited the growth of the test organisms (i.e. the lowest concentration in two-fold dilution series without turbidity). The vials were sub-cultured on nutrient agar plates and incubated at 35±2°C for 18-24 hrs (for bacteria) or potato dextrose agar plates at 25±2°C for 24-48 hrs (for fungi). Then, plates were examined for the growth of microorganisms. The tubes with minimum concentration of extract in which the growth was completely checked was noted as the MBC of the plant extract (Grumachhan, 2018; Gurmachhan et al., 2019).

Phytochemical screening

The phytochemical screenings of the plant extracts were carried out according to the standard protocol (Ciulei, 1982; Harborne, 1998). The barks of the plants were dried and extracted with methanol. Different phytochemicals in the extracts were identified by color reactions with different reagents.

Results and Discussion

Among the bark samples subjected to Soxhlet extraction with methanol, guava bark showed higher extract yield of 22%, while that of pomegranate showed comparatively lower yield of 19.7% (Table 1).

Antimicrobial activities of bark extracts were tested against twelve test organisms, amongst which pomegranate bark extract showed antimicrobial activities against *Bacillus subtilis* (Ehrenberg) Cohn, *Enterococcus faecalis* (Andrewes & Horder) Schleifer & Kilpper-Balz, *Staphylococcus aureus* Rosenbach, methicillin resistant *S. aureus* (MRSA),

Proteus vulgaris Hauser and *Candida albicans* (C.P. Robin) Berkhout. Similarly, guava bark extract showed antimicrobial activities against *B. subtilis*, *E. faecalis*, *S. aureus*, MRSA and *P. vulgaris*. The ZOI values of methanolic bark extract of guava bark extract were found to be 14 mm, 18 mm, 13 mm, 18 mm and 18 mm against *B. subtilis*, *E. faecalis*,

Table 1: Percentage yields of bark extracts

S.N.	Plants	Parts used	Sample weight (gm)	Total thimble weight with sample			Percentage yield
				Before extraction (gm)	After extraction (gm)	Weight of extract (gm)	
1	<i>Psidium guajava</i> L.	Bark	35.5	39.4	31.6	7.8	22.0
2	<i>Punica granatum</i> L.	Bark	15.2	19.1	16.1	3.0	19.7

Table 2: Zones of inhibition (ZOIs) of bark extracts (mm)

S.N.	Name of microorganisms	Zones of inhibition (ZOIs) (mm)	
		<i>Psidium guajava</i> L.	<i>Punica granatum</i> L.
1	<i>Bacillus subtilis</i> (Ehrenberg) Cohn	14	18
2	<i>Enterococcus faecalis</i> (Andrewes & Horder) Schleifer & Kilpper-Balz	18	21
3	<i>Staphylococcus aureus</i> Rosenbach	13	16
4	Methicillin Resistant <i>S. aureus</i> (MRSA)	18	25
5	<i>Escherichia coli</i> (Migula) Castellani & Chalmers	0	0
6	<i>Klebsiella pneumoniae</i> (Schroeter) Trevisan	0	0
7	<i>Pseudomonas aeruginosa</i> (Schröter) Migula	0	0
8	<i>Proteus vulgaris</i> Hauser	18	22
9	<i>Salmonella enterica</i> subsp. <i>enterica</i> (ex Kauffmann & Edwards) Le Minor & Popoff serovar. <i>Typhi</i>	0	0
10	<i>Shigella dysenteriae</i> (Shiga) Castellani & Chalmers	0	0
11	<i>Candida albicans</i> (C.P. Robin) Berkhout	0	14
12	<i>Saccharomyces cerevisiae</i> Meyen ex E.C. Hansen	0	0

Table 3: Minimum microbicidal concentration of methanolic bark extract of plants

S.N.	Plants	Minimum Microbicidal Concentration (mg.ml ⁻¹)					
		<i>Bacillus subtilis</i>	<i>Enterococcus faecalis</i>	<i>Staphylococcus aureus</i>	Methicillin Resistant <i>S. aureus</i> (MRSA)	<i>Proteus vulgaris</i>	<i>Candida albicans</i>
1	<i>Psidium guajava</i> L.	>50	6.25	6.25	12.5	1.5625	ND
2	<i>Punica granatum</i> L.	>50	6.25	1.5625	6.25	0.78125	12.5

ND= not done due to 0 ZOI

Table 4: Results of phytochemical screening of bark extracts

Plants	Volatile oils	Alkaloids	Terpenoids	Flavonoids	Tannins	Saponins	Glycosides	Reducing sugar	Steroids	Protein
<i>Psidium guajava</i> L.	-	+	-	+	-	-	±	+	+	-
<i>Punica granatum</i> L.	-	+	+	+	+	+	±	+	+	-

Note: + indicate Presence, - indicate absence, ± indicate may or may not

S. aureus, MRSA and *P. vulgaris* respectively. Similarly, ZOI values of pomegranate bark extract were found to be 18 mm, 21 mm, 16 mm, 25 mm, 22 mm and 14 mm against *B. subtilis*, *E. faecalis*, *S. aureus*, MRSA, *P. vulgaris* and *C. albicans* respectively. Methanolic bark extract of pomegranate formed comparatively larger ZOIs against Methicillin Resistant *S. aureus* (MRSA) and *P. vulgaris* while that of guava did so against *P. vulgaris* and *S. aureus*. Among all the ZOIs formed by the two extracts, the largest ZOI observed was of 25 mm diameter which was formed by methanolic bark extract of pomegranate against MRSA (Table 2).

Minimum microbicidal concentration (MMC) values of guava bark extract were found 1.5625 mg.ml⁻¹, 6.25 mg.ml⁻¹, 6.25 mg.ml⁻¹ and 12.5 mg.ml⁻¹ against *P. vulgaris*, *E. faecalis*, *S. aureus* and MRSA respectively. Similarly, MMC values of pomegranate bark extract were found 0.78125 mg.ml⁻¹, 1.5625 mg.ml⁻¹, 6.25 mg.ml⁻¹, 6.25 mg.ml⁻¹ and 12.5 mg.ml⁻¹ against *P. vulgaris*, *S. aureus*, *E. faecalis*, MRSA and *C. albicans* respectively. MMC value of sample extract was found >50 mg.ml⁻¹ against *B. subtilis*. MMC value 0.78125 mg.ml⁻¹ of pomegranate bark extract against *P. vulgaris* was found as the lowest MMC value. (Table 3). Hamid et al. (2015) found the ZOI values of methanolic extract of bark of pomegranate 23.3 mm, 22 mm, 22 mm, 22 mm and 20.4 mm against *K. pneumonia*, *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* respectively. They reported minimum inhibitory concentration (MIC) values of 6.10 mg.ml⁻¹, 6.25 mg.ml⁻¹, 6.0 mg.ml⁻¹, 6.2 mg.ml⁻¹ and 6.8 mg.ml⁻¹ against *K. pneumonia*, *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* respectively. Kuber et al. (2013) found the ZOI values 1.8 cm, 1.1 cm, 2 cm and 1.5 cm at 10 mg.ml⁻¹ concentration of guava root bark extract against *S. aureus*, *E. coli*, *B. subtilis* and *P. vulgaris* respectively.

B. subtilis sometimes causes food poisoning. *E. faecalis* causes endocarditis & septicemia, urinary tract infection, meningitis and other infections in humans. *S. aureus* causes skin infection, boils, conjunctivitis, secondary infections, pneumonia, acute endocarditis toxic shock syndrome and food

poisoning (Collee et al., 1996). MRSA is responsible for several difficult to treat infections in human pathogens. MRSA is common in hospitals, prisons and nursing homes where the people with open wounds, invasive devices such as catheters. In human, *S. aureus* is part of the normal microbiota present in the upper respiratory tract, on skin and in the gut mucosa. *S. aureus* along with similar species that can colonize and act symbiotically but can cause disease if they begin to take over the tissues they have colonized or invade other tissues, have been called pathobionts. After 72 hours MRSA can take hold in human tissues and eventually become resistant to treatment. *P. vulgaris* occasionally causes urinary tract infections, wound infection and abscesses and form cases of otitis media, meningitis, septicemia (Cheesbrough, 2000).

Phytochemical screening of methanol extract of guava bark showed the presence of alkaloids, flavonoids, reducing sugar, steroids and absence of volatile oils, terpenoids, tannins, saponins, and protein. Similarly, the methanol extract of pomegranate bark indicated the presence of alkaloids, terpenoids, flavonoids, tannins, saponins, reducing sugar, steroids and absence of volatile oils and protein. The study could not confirm the presence or absence of glycosides (Table 4). Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are secondary metabolites of plants that serve as defenses against many microorganisms, insects and other herbivores (Bonjar et al., 2004; Shihabudeen et al., 2010). Sajjad (2015) found presence of glycosides, tannins, anthraquinones, carbohydrates, amino acid, alkaloids, steroids, flavonoids and absence of saponins in crude methanolic peel extract of pomegranate. Growth and Sukritha (2018) found the ethanol extract of bark of guava more active than methanol extract, minimum bactericidal concentration (MBC) of ethanol extract of bark being 16gm.ml⁻¹ with presence of active phytochemicals alkaloids, steroids, flavonoids, saponins, tannins. They concluded that the observed antimicrobial activity was due to the phytochemicals present in the bark samples of the plant.

Conclusion

Methanol extract of pomegranate stem bark exhibited a potent antimicrobial activity against *S.aureus*, *P. vulgaris* and MRSA while that of guava stem bark against the *P. vulgaris*. From this study, it can be concluded that stem barks of pomegranate and guava contain important phytochemicals which are responsible for antimicrobial activity. Identification and isolation of such active phytochemicals from extract play crucial role in the development of new biologically active products.

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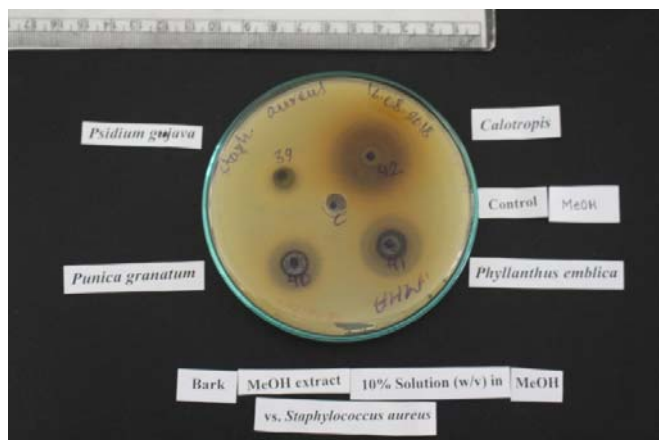


Plate 1: ZOIs of methanol extracts of *Psidium guajava* L. and *Punica granatum* L. barks against *Staphylococcus aureus* Rosenbach

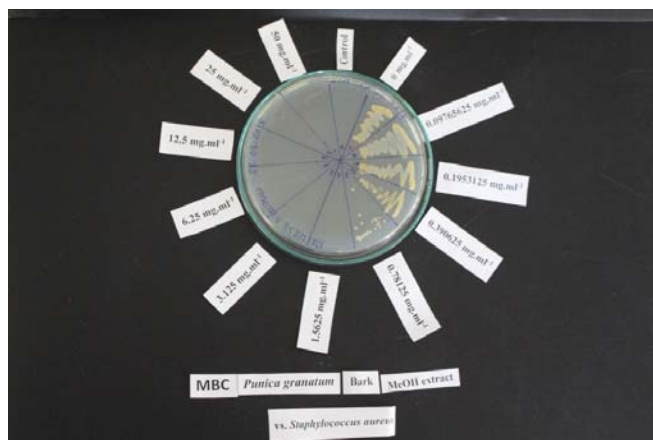


Plate 4: Determination of MBC of methanolic bark extract of *Punica granatum* L. against *Staphylococcus aureus* Rosenbach

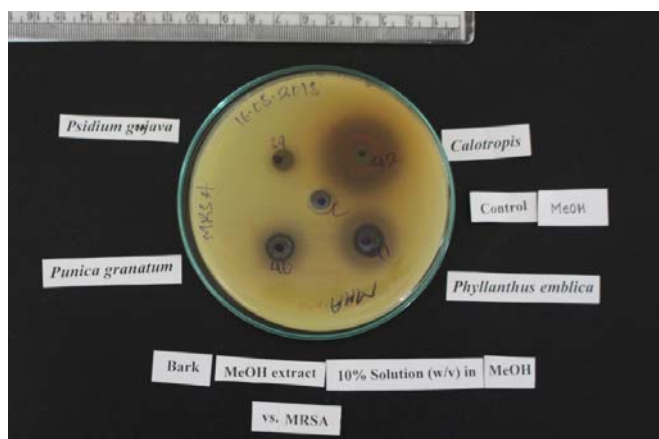


Plate 2: ZOIs of methanol extracts of *Psidium guajava* L. and *Punica granatum* L. barks against against MRSA

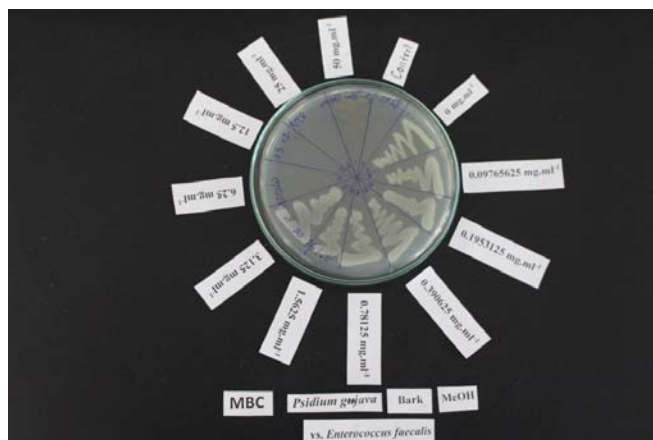


Plate 5: Determination of MBC of methanolic bark extract of *Psidium guajava* against *Enterococcus faecalis* (Andrewes & Horder) Schleifer & Kilpper-Balz

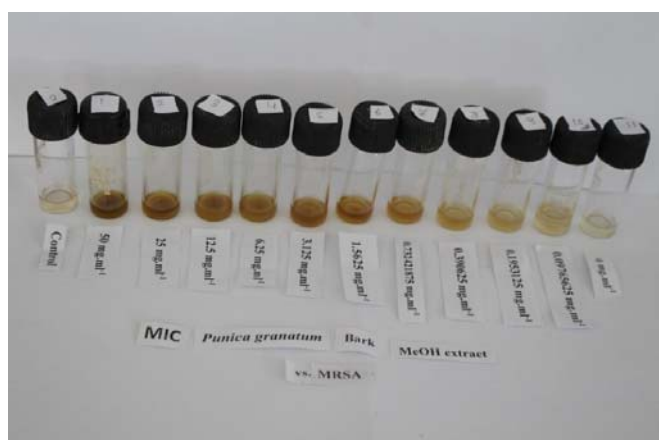


Plate 3: Determination of minimum inhibitory concentration (MIC) of methanolic bark extract of *Punica granatum* L. against MRSA

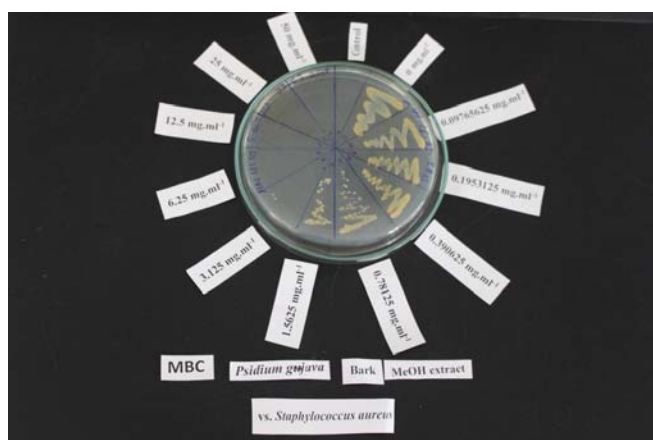


Plate 6: Determination of methanolic bark extract of *Psidium guajava* L. against *Staphylococcus aureus* Rosenbach