

**PHYTOCHEMICAL AND ANTIBACTERIAL ACTIVITY OF *PISTACIA
INTEGERMA* AGAINST MULTI DRUG RESISTANT BACTERIAL PATHOGENS
ISOLATED FROM URINARY TRACT INFECTIONS**

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farishta.shah@hotmail.com**Abstract****ABSTRACT**

Resistance against drugs has been developed as a result of the overuse and misuse of antimicrobial drugs. *Pistacia integerrima* is an important medicinal plant and a member of family Anacardiaceae and subfamily Anacardioideae. It is a dioecious tree that is native to Asia specifically Pakistan, East Afghanistan and North and West Himalaya to Kumaon. The present study aimed to evaluate the antibacterial activity of the galls of *P. integerrima* against bacteria isolated from

urinary tract infections. A total of 36 urine samples were collected from the infected individuals. They were processed according to the standard procedures. Gram staining and different biochemical tests were performed for confirmation of organisms i.e. Catalase, Oxidase, Coagulase, TSI (Triple Sugar Iron), Urease, Indole and Citrate Tests. It was observed that the *E. coli* (33%) was the most predominant specie isolated from UTI patients followed by *Proteus spp.* (22%). *Pseudomonas spp.* accounted for 19% whereas *Bacillus* for 13.8%. The phytochemical analysis of the plant was done and it was ascertained that alkaloids were present in galls-extracts, obtained with ethanol, methanol and water. Flavonoids and tannins were present in ethanolic and aqueous extract whereas absent in methanolic extract. However, saponins were not found in all the tested extracts. Well diffusion method was used for the evaluation of antibacterial activity of the extracts. *Bacillus spp.* showed highest zone of inhibition against

ethanolic, methanolic and aqueous extract i.e. 22 mm, 24 mm and 10 mm respectively. It was noticed that the extract showed best results at 100 µl concentration. Therefore, the extract of *Pistacia integerrima* is recommended to be used in case of Urinary tract infections.

INTRODUCTION

Drug resistant bacterial infections are emerging as a serious health related issue, worldwide, the antibiotic-resistant bacteria are spreading and increasing the morbidity and mortality rates. The development of antibiotic is a time consuming and lengthy process and it depends on the two strategies i.e. isolation of bioactive secondary metabolites from microorganisms and a target-based approach (Cheng *et al.*, 2018, Tacconelli *et al.*, 2018).

New classes of antibiotics need to be made as the old ones are getting resistant. Unfortunately, it will take years and decades to make such number of antibiotics available. As a solution, it can be considered that the resistant antibiotics can be used in combination with some non-antibiotic drugs to overcome resistance. Several drugs that currently donot have any antibacterial indications can be used to reduce the antibiotic MIC, kill bacteria, or modulate the defense through effects on host innate immunity, in particular by altering inflammation and autophagy when used along with the combination of an existing antibiotic (Brown *et al.*, 2015).

Plants are being used for the treatment of different infections since old time (Maciel *et al.*, 2002). From time immemorial, many herbs and plants are identified that can be used in relieving from pains in human population worldwide. Herbs are known to be cost effective and safest solution to be used as an alternative to drugs. Almost all the plants have therapeutic plants and their use is famous and well accepted. A large

number of medicinal plants are gifted to us by nature and different medicinal products have been made from them (Lai & Roy, 2004).

The human life in early time was very tough and it was difficult to treat infections because no proper medicines were available. It has been observed that people used plants as medicines and accidentally they were used as treatment for a certain disease. Nowadays with the help of the modern sciences, plants and extracts of plants have been discovered that can cure certain diseases. The records written on clay tablets in 3000-5000 Before Common Era (BCE) by Sumerians supported that the humans were able to understand the diseases along with the concept that plants can be used for the maintenance and restoration of good health. It has also been noticed that the selection of using any part of plant can be selective as the utilization of some parts can be more beneficial as compared to others. Therefore, from ancient times those plants that have medicinal values were grown more and remained healthy (Inoue *et al.*, 2019).

The demand and use of the medicinal plants is increasing day by day. Undoubtedly, ecosystem has been provided with essential services by the plants. Humans and other living organisms cannot live without plants. More specifically, the medicinal herbs are acting as an indicator for a healthy ecosystem. It has been shown that the humans were aware of plant properties (more or less) before even the plants were used globally for clothing, shelter, fuel and food. In many countries like Egypt, China, Greece and India these medicinal plants have been transformed. Plants were used as a disinfectant, drug and as an aromatic agent in Persia. The human beings can use medicinal plants as a treatment option (Jamshidi *et al.*, 2018).

Pistacia integerrima is an important medicinal plant and a member of family Anacardiaceae and subfamily Anacardioideae. It is a dioecious tree that is native to Asia

specifically Pakistan, East Afghanistan and North and West Himalaya to Kumaon. The tree grows at a height of 800-1900 m (Pant and Samant, 2010, Jeet *et al.*, 2019). The plant is commonly called Zebrawood or Crab's claw but still the plant has different local names in India (kakring, kakroi, kakkarsinghi, kakar, kakarsinghi) and Pakistan (khanjar, shnai and thoak) (Orwa *et al.*, 2009). The plant has a single stem containing many branches. The plant contains compound leaves. The fruits of the plant are globular having purple to blue color with a diameter of 4-6 mm (Padulosi *et al.*, 2002). The galls are considered as a store house for the secondary metabolites (Chopra *et al.*, 1982, Vashit and Anil, 2012).

Pistacia integerrima is used against many diseases which has a very wide range of curative and protective therapeutic efficacy. Now a day's different parts of the plant are still in use in the formulation of herbal medicines. This plant is being used in treating various diseases like gastrointestinal tract (GIT) and respiratory tract disorders (Jeet *et al.*, 2019). It has also been used as analgesic and anti-inflammatory, blood purifier, gastrointestinal disorders such as vomiting and diarrhea, expectorant, cough, asthma, fever and as antidiabetic etc. (Rauf *et al.*, 2017, Ahmad *et al.*, 2008 and Uddin *et al.*, 2012).

The galls of the plant have pistacienoic acid, resins, camphene, luteolin, amino acids, tannins, tetracyclic triterpenes, pistancin and dihydromavalic acids (Warrier *et al.*, 1995). The galls of the plant have medicinal importance and can be used in the treatment of asthma, cough, bronchitis, dysentery, ulcer, psoriasis, skin diseases, inflammation, pharyngitis, hiccough, fever, ulcer, dyspepsia, leprosy, anorexia, leucorrhoea, and general debility. The stem resins of *P. integerrima* are used for wound

healing, (Hussain *et al.*, 2007) while the stem are also used for fuel, ornamental wood, construction (Hussain *et al.*, 2007) while leaves are used as cattle food (Jan *et al.*, 2009).

MATERIALS AND METHOD

Plant Collection

P. integerrima galls were brought from local market of Mansehra Division. The plants were then identified at laboratory of Botany, Hazara University Mansehra and were subjected to shade drying by the method used by Islam *et al.*, 2015.

Soaking and Extraction

After shade drying, the plant material was chopped and grinded. The sample was extracted using distilled water, methanol and ethanol and left for 7 days (Islam *et al.*, 2015). The residues were separated by filtration. This process was repeated 3 to 4 times and the solvents was recovered through Rotary evaporator at 40-50°C (Ramachandra *et al.*, 2010).

Phytochemical screening

The extract was screened out for the presence of different bioactive constituents i.e. flavonoids, alkaloids, steroids and tannins etc. (Rajurkar & Gaikwad, 2012).

Test for alkaloids

For evaluating the presence of alkaloids, 2-3 ml of filtrate, Mayer's reagent and 1 ml of HCl (diluted) was added in a test tube and shaken. Formation of yellow precipitation showed the presence of alkaloids.

Test for tannins

FeCl₃ (5%) solution was added to the extract. The appearance of deep blue color confirmed the presence of tannins.

Test for flavonoids

Dilute sodium hydroxide was added to 1 ml of extract. The solution changed the color to yellow followed by colorless solution after the addition of dilute acid. This confirmed the presence of flavonoids.

Test for saponins

The saponin content was measured by dissolving 0.5 gram of extract in water and boiled. The height of the froth was used for the measurement of saponin in the sample.

Antibacterial activity

Urine Samples were collected from Ayub Medical Complex, Abbotabad using sterile urine bags and bottles. Samples were transported to the Microbiology Laboratory at Abasyn University Peshawar, for culturing and identification of organisms.

Sample Collection

Gram staining and biochemical tests were applied for the confirmation of bacterial species.

Culture Media Used

The isolated samples were cultured on the following media:

- Nutrient Agar media: general purpose media for culturing bacteria
- MHA: for checking the sensitivity pattern of bacterial isolates

Nutrient agar media:

Nutrient Agar is a general purpose used for the culturing of all types of bacteria. It is popular because variety of bacteria can grow on it since it has all the basic nutrients required for the growth of non-fastidious bacteria.

28 grams of the powdered media was added to 1 liter of distilled water. The suspension was then heated to boiling to dissolve the medium completely. The dissolved medium

was autoclaved at 121°C for 15 minutes. After autoclaving, the beaker was taken out and allowed to cool at room temperature. The media was then poured into sterile Petri plates under sterile conditions.

Mueller Hinton Agar Media

It is recommended media for evaluating the antibiotic sensitivity test using Kirby-Bauer disc diffusion method for non-fastidious bacteria (both aerobes and facultative anaerobes).

The media was prepared by dissolving 38 g of media in 1000 ml of distilled water. The media was then stirred and heated for dissolving the components. It was autoclaved for 15 minutes at 121 °C. After autoclaving, the media was left to cool. It was then poured in petri plates. The sample was inoculated and plates were incubated at 37°C for 24 hrs.

Sample Processing

Streak plate method was used for the inoculation of samples on the media. All plates were incubated for 24 hours at 37°C.

The bacteria were then identified using standard gram staining and biochemical test procedures.

Identification of MDR Isolates

Disc diffusion method was used for the identification of antibacterial activity. A total of 10 different antibiotics were used i.e. CIP, CHL, CRO, CFM, AML, GEN, CAZ, DO, AMP and MEM.

Antibacterial Activity of Extract

Well diffusion method of (Ishaq *et al.*, 2014) was followed with some modifications for the antibacterial activity of extracts. Pre-autoclaved MHA plates were

inoculated with diluted bacterial culture, using sterile cotton swabs to achieve uniform lawn of growth and sterile borer were used to bore wells in the agar.

Extracts of galls of *Pistacia integerrima* in three different concentrations i.e. 100 µl, 75 µl and 25 µl were used for the evaluation of antibacterial activity.

RESULTS

Phytochemical Analyses

From phytochemical analyses, it was ascertained that alkaloids were present in galls-extracts, obtained with ethanol, methanol and water. Flavonoids and tannins were present in ethanolic and aqueous extract whereas absent in methanolic extract. However, saponins were not found in all the tested extracts.

Table 1: Phytochemical Analysis of Extracts of *P. integerrima* galls with different Solvents

Constituents	Ethanol	Methanol	H ₂ O
Alkaloids	+	+	+
Flavonoids	+	-	+
Saponins	-	-	-
Tannins	+	-	+

Key: + = Detected, - = Not-Detected

Identification of bacteria:

A total of 36 urine samples were collected from the infected individuals. Among them 19 were collected from female patients and 17 were collected from male patients having urinary tract infections (Figure 1).

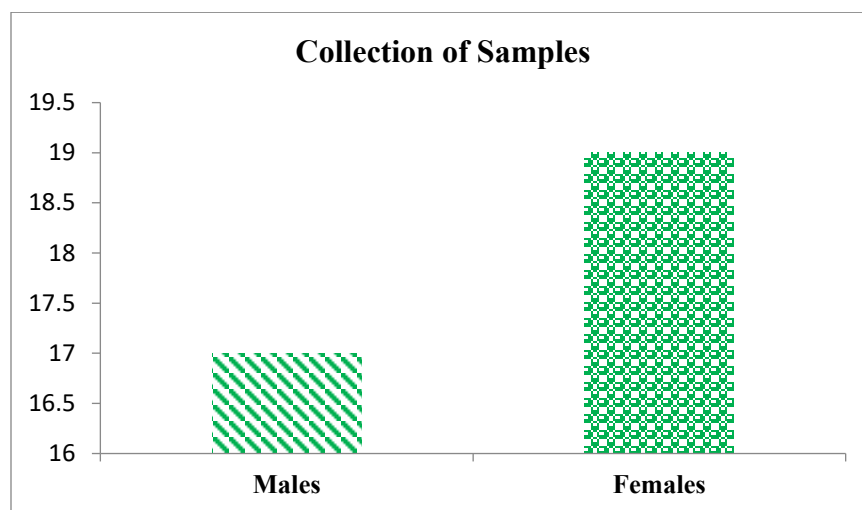


Figure 1 Gender wise Collection of Urine Sample

Identified Organisms	Gram' s	Reaction Citrate	Test Indole	Test Oxidase	Test Coagulase	e Test Urease	Test Catalase	Test	TSI test
<i>E. coli</i>	-	-	+	-	-	-	+		A/A
<i>Proteus spp.</i>	-	+	-	-	+	+	+		K, H ₂ S
<i>S. aureus</i>	+	-	-	+	+	-	+		K, H ₂ S
<i>Pseudomonas spp.</i>	-	+	-	+	-	-	+		NC
<i>Bacillus spp.</i>	+	+	-	+	-	-	+		A/K

A total of 5 bacterial species were identified using standard procedures of gram staining and biochemical tests. The samples were processed according to the standard procedures i.e. gram staining and biochemical tests. The results of the gram staining and biochemical tests are shown in the table 2.

Table 2: Identification of bacterial species

Key: +: Positive, -: Negative, ±: Variable, NC: No change, A: Acid production, K: alkaline reaction; K/AG: Red/yellow with gas production, K/A: Red/yellow, H₂S: Sulfur reduction, It was observed that the *E. coli* (33%) was the most predominant specie isolated from UTI patients followed by *Proteus spp.* (22%). *Pseudomonas spp.* accounted for 19% whereas *Bacillus* for 13.8% (Figure 2).

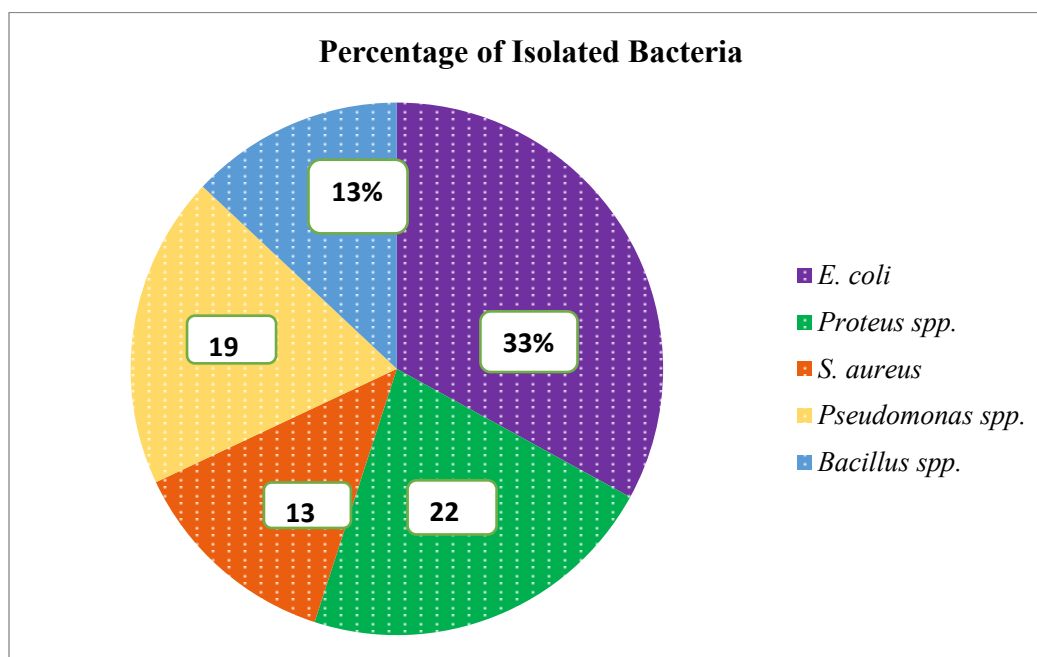


Figure 2 Percentage Wise Distribution of Bacteria Isolated from UTI

Antibacterial activity

For the identification of MDR bacteria species, a total of 10 antibiotics were used i.e. CHL, MEM, AMP, DO, CAZ, GEN, AML, CFM, CRO, CIP. It was noticed that the all the bacterial species isolated showed Multi Drug Resistant pattern. The zones of inhibition of antibiotics against bacterial isolates are shown in table 3.

Table 3: Identification of multi drug Resistant bacterial isolates

Bacterial species	CIP	CHL	CRO	CFM	AML	GEN	CAZ	DO	AMP	MEM
<i>E. coli</i>	R	17	16	R	R	20	14	11	R	16
<i>Proteus spp.</i>	25	18	15	20	R	17	R	13	13	16
<i>S. aureus</i>	20	19	18	19	R	19	R	22	12	22
<i>Pseudomonas spp.</i>	25	17	22	R	18	21	23	R	19	20
<i>Bacillus spp.</i>	18	21	17	R	R	R	14	11	R	17

Antibacterial sensitivity of the bacterial species

The extracts were screened for the antibacterial activity using well diffusion method. A total of 05 bacterial isolates were used for evaluating the antibacterial activity i.e. *E. coli*, *Pseudomonas spp.*, *Proteus spp.*, *S. aureus*, and *Bacillus spp.*

S.NO	Species name	100 µl concentration	75 µl concentration	25 µl concentration
1	<i>Bacillus spp.</i>	24 mm±0.5	19 mm±0.5	14 mm±0.5
2	<i>E. coli</i>	18 mm±0.5	13 mm±0.5	10 mm±0.5
3	<i>Pseudomonas spp.</i>	16 mm±0.5	12 mm±0.5	08 mm±0.5
4	<i>S. aureus</i>	17 mm±0.5	12 mm±0.5	09 mm±0.5
5	<i>Proteus spp.</i>	14 mm±0.5	09 mm±0.5	06 mm±0.5

Antibacterial activity of ethanolic extract:

It has been revealed that the highest zone of inhibition was produced against *Bacillus spp.* (22 mm) followed by *S. aureus* (19 mm) and *Pseudomonas spp.* (17 mm)

when the ethanolic extract was used at 100 µl concentration. The results of 75 µl and 25 µl concentration are shown in the Table 4.

Table 4: Antibacterial activity of Ethanolic extract of galls against different bacterial species

S.NO	Species name	100 µl concentration	75 µl concentration	25 µl concentration
1	<i>Bacillus spp.</i>	10 mm±0.5	08 mm±0.5	04 mm±0.5
2	<i>E. coli</i>	Nil	Nil	Nil
3	<i>Pseudomonas spp.</i>	Nil	Nil	Nil
4	<i>S. aureus</i>	06 mm±0.5	04 mm±0.5	02 mm±0.5
5	<i>Proteus spp.</i>	Nil	Nil	Nil

Antibacterial activity of methanolic extract:

It has been revealed that the highest zone of inhibition was produced against *Bacillus spp.* (24 mm) followed by *E. coli* (18 mm) and *S. aureus* (17 mm) when the methanolic extract was used at 100 µl concentration. The results of 75 µl and 25 µl concentration are shown in the table 5.

Table 5: Antibacterial activity of methanolic extract of gall against different bacterial species

Antibacterial activity of aqueous extract:

At 100 µl concentration of aqueous extract the highest zone of inhibition was noticed against *Bacillus spp.* (10 mm) followed by *S. aureus* (6 mm) whereas no zone was

observed against other bacterial species. Similarly, the results of using 75 μ l and 25 μ l aqueous extract are given in the Table 6.

S. No	Species Name	100 μ l concentration	75 μ l concentration	25 μ l concentration
1	<i>Bacillus spp.</i>	22 mm $\pm$ 0.5	17 mm $\pm$ 0.5	12 mm $\pm$ 0.5
2	<i>E. coli</i>	15 mm $\pm$ 0.5	11 mm $\pm$ 0.5	08 mm $\pm$ 0.5
3	<i>Pseudomonas spp.</i>	17 mm $\pm$ 0.5	13 mm $\pm$ 0.5	10 mm $\pm$ 0.5
4	<i>S. aureus</i>	19 mm $\pm$ 0.5	15 mm $\pm$ 0.5	12 mm $\pm$ 0.5
5	<i>Proteus spp.</i>	12 mm $\pm$ 0.5	08 mm $\pm$ 0.5	05 mm $\pm$ 0.5

Table 6: Antibacterial activity of aqueous extract of gall against different bacterial species

DISCUSSION

There is an increasing resistance to antibiotics by bacteria, therefore there is a need to develop alternative antimicrobial compounds for treating infections. So, new compounds are in search that can be combined with antibiotics to overcome the resistance in bacteria. There are variety of medicinal plants present in the ecosystem. These medicinal plants can be used for the treatment of various infections as they contain the biologically active compounds or phytochemicals that shows antimicrobial properties (Sibanda & Okoh, 2007).

The present study aimed to evaluate the antibacterial activity of the galls of *P. integerrima* against bacteria isolated from urinary tract infections. The extract was

prepared in three different solvents i.e. ethanol, methanol and distilled water. It was observed that the *E. coli* (33%) was the most predominant specie isolated from UTI patients followed by *Proteus spp.* (22%). *Pseudomonas spp.* accounted for 19% whereas *Bacillus* for 13.8%.

The galls extract of the plant is common practice in folk medicine which revealed the presence of alkaloids, terpenoids, flavonoids and tannins. The bark showed the presence of terpenoids, flavonoids while the leaves and roots extracts showed the presence of terpenoids and tannins (Uddin *et al.*, 2011). *P. Integerrima* Galls Stewart was used for the investigation of phytochemicals and antimicrobial activities of ethyl acetate, methanol, chloroform and *n-hexane* (Uddin *et al.*, 2012). The bark of the plant was tested for the presence of phytochemicals and it was noticed that tannins, alkaloids, flavonoids etc. were present (Ismail *et al.*, 2011). In the current study, it was ascertained that alkaloids were present in galls-extracts, obtained with ethanol, methanol and water. Flavonoids and tannins were present in ethanolic and aqueous extract whereas absent in methanolic extract. However, saponins were not found in all the tested extracts.

The antibacterial activity of *P. integerrima* was noticed against six clinical isolated that were identified as multidrug resistant bacteria. The activity was performed on *Esch. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus* pathogenic bacteria. Significant activity was observed by the galls extract. Among the solvents, water and chloroform extract showed moderate activity while maximum activity was noticed by the methanol and ethanol extract (Bharathirajan, 2015). The antibacterial efficacy and phytochemical observation of *P. integerrima* and some other plants against seven different microorganisms such as *Esch. coli*, *S. typhi*, *K. pneumoniae*, *P. vulgaris*, *Pseudomonas*, *B.*

subtilis and *Staph. aureus* by using disc diffusion method was done (Selvi, 2016). In the current research study, MDR bacterial species were identified using several antibiotics i.e. CIP, CHL, CRO, CFM, AML, GEN, CAZ, DO, AMP and MEM. Additionally, the growth of test organisms was markedly inhibited by ethanol extract of *P. integerrima* which was exactly similar to the present research in which the growth was inhibited by ethanol extract.

Ramachandra (2010) used the ethanol and aqueous extracts of galls of *P. integerrima* for anti-bacterial activity. All the strains of Bacteria showed susceptibility towards both the extracts. The ethanol extract showed better activity profile as compared to the aqueous extract. Uddin and Rauf (2012) evaluated the antimicrobial phytochemical properties of ethyl acetate, methanol, chloroform and n-hexane fraction of *P. integerrima* in which the extract in ethyl acetate, chloroform and methanol showed potent activity against Gram positive and Gram negative bacterial strains. In the present research work, when ethanolic extract was used, highest zone of inhibition was produced against *Bacillus spp.* (22 mm) followed by *S. aureus* (19 mm) and *Pseudomonas spp.* (17 mm) when the ethanolic extract was used at 100 µl concentration. Similarly, for methanolic extract, highest zone of inhibition was produced against *Bacillus spp.* (24 mm) followed by *E. coli* (18 mm) and *S. aureus* (17 mm) when the methanolic extract was used at 100 µl concentration. Furthermore, using aqueous extract, the highest zone of inhibition was noticed against *Bacillus spp.* (10 mm) followed by *S. aureus* (6 mm) whereas no zone was observed against other bacterial species.

CONCLUSION

From the present study, it is concluded that *E. coli* (33%) was the most predominant specie isolated from UTI patients followed by *Proteus spp.* (22%). *Pseudomonas spp.* accounted for 19% whereas *Bacillus* for 13.8%. From phytochemical analysis, it was noticed that alkaloids were present in galls-extracts, obtained with ethanol, methanol and water. Flavonoids and tannins were present in ethanolic and aqueous extract whereas absent in methanolic extract. However, saponins were not found in all the tested extracts. *Bacillus spp.* showed highest zone of inhibition against ethanolic, methanolic and aqueous extract i.e. 22 mm, 24 mm and 10 mm respectively. It was noticed that the extract showed best results at 100 µl concentration.

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Fig 1: Galls of *Pistacia integerma*

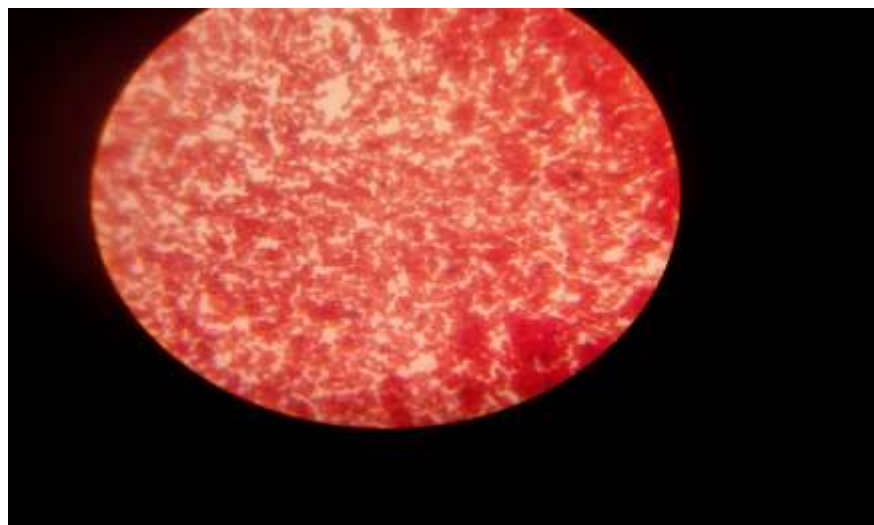


Fig 2. Results of Gram Staining

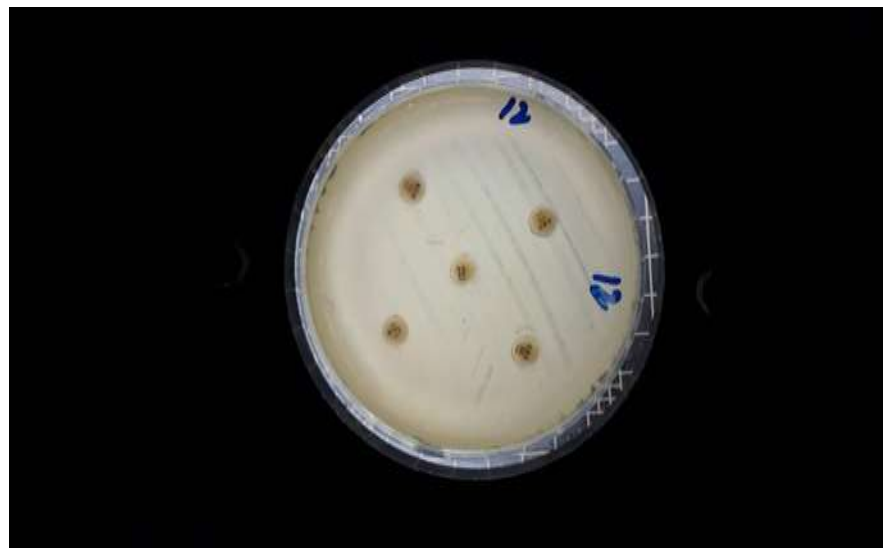


Fig 3. Identification of Multi Drug Resistant Bacteria