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Phytochemical assessment and evaluation of antibacterial potential of selected ethno medicinal plant against skin pathogens from the war-affected region of North-West Pakistan

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Abstract
Medicinal plants have been used for centuries, and various cultures still depend on the plants for their primary health care needs. In the present study selected plant *Oxalis corniculata* is collected from Bajaur region, Pakistan. The plants extracts were obtained and further fractionated into ethyl acetate, chloroform, n-hexane along with their aqueous extracts. By well diffusion method these plant extracts were screened against four skin selected bacterial pathogenic strains e.g. *Staph. aureus, Streptococcus pyogenes, Staphylococcus epidermidis, Pseudomonas aeruginosa.* Minimum inhibitory concentrations (MICs) were also determined of the selected plant extracts against selected bacteria. Results of MICs and well diffusion showed that *Oxalis corniculata* was more potent plant for activity against the selected skin pathogens. Phytochemical analysis showed that *Oxalis corniculata* consists of alkaloid, phenol, tannins and flavonoid in highest concentration. Phytochemical analysis also showed that hexane, ethyl acetate and chloroform were more effective in antibacterial potential while aqueous was less effective. Owing to this importance further research on these plants can be promising as agent for development of novel antibiotics.

Keywords: Plant extracts, bacteria, antibacterial and phytochemical activities, MIC

1. Introduction
Plants have been utilized as folk medicine throughout the world for centuries and indigenous communities have developed their own specific knowledge on plant resources, uses, management and conservation. Today, around 25% of all prescribed medicines in the developed world contain ingredients derived from medicinal plants [1, 2]. It has been estimated that the medicines extracted from plant sources are used by more than 80% of the world's population in developing countries to meet their primary healthcare needs [3]. The traditional use of plants and plant resources is rapidly increasing due to their minimal side-effects and (affordable) accessibility, and because they sometimes represent the only source of healthcare available to poorer communities and the major part of traditional therapy involves the use of plant extract and their active constituents [4]. Active ingredients formed during secondary metabolism are generally seen, have biological properties and are used globally for various purposes such as treatment of infectious diseases and in food industries. Medicinal plants are a great economic value all over the world and about 7,000 types of medicinal plants have been documented all over the world of which more than 900 types belong to valuable medicinal plants [5]. The use of plant extracts as complementary and substitute medicine has been increased dramatically in the last years [6]. Following the beginning of modern medicine, herbal medicine suffered a hold up, but during last two or three decades progress in phyto-chemistry and in recognition of plant compounds valuable against certain diseases have diverted the interest in herbal medicines [4]. In the current years, the increase resistance of bacterial pathogens against numerous antibiotics has become a difficult problem due to random use of modern antibiotics [7, 8]. As a result, the requirement for new and effective antimicrobial agents with broad-spectrum activities from natural sources is raising day by day [9].

Skin diseases occur all over the world an amount of 34% among all occupational diseases encountered [10]. Skin diseases affect people of all ages from neonates to the elderly and constitute one of the five reasons for medical consultation. Skin diseases indicate major health problems in both developed and undeveloped countries. For example, in the United States, skin infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) result in around 12,6000 hospitalizations and 18,650 death each year, a rate which exceed that of acquired immunodeficiency syndrome (AIDS) [11].

Burns wounded are also susceptible to severe and often fatal *Pseudomonas aeruginosa* infections [12].

Socio-economic atmosphere such as household overloading play a massive part in the spread of skin infections. Furthermore, hot and humid climatic circumstances aggravate skin infections. Although death rates for skin diseases are comparatively low, they impact considerably on the quality of life and sometime become persistent and are complicated to treat [13]. Conventional medicinal resources, especially plants have been found to play a great part in managing skin disorder [14]. It has been used in the treatment of skin diseases in many countries around the world where it contribute extensively in the primary health care of the population [15, 16].

In the recent years, the development of resistance of pathogens against several antibiotics has become a difficult issue caused by the indiscriminate use of modern antibiotics [17, 18].

Folk medicine has been used for thousands of years with significant contributions made by its practitioners to human health, particularly as primary health care providers at the society level [19].

The Himalayan region contains large number of medicinal plants due to various ecological and phyto-geographical factors. Nature gave us an affluent botanical resource and a large number of various types of plants grow in different parts in Pakistan [20]. This region alone supports about 18,440 species of plants of which about 45% are having medicinal potential [21, 22].

Fungal infections due to the hot environment and overloading household are common in Bajaur area, as well as burn accidents due to the use of wood as the major fuel for cooking. It is known that the lay men in this area depend on medicinal plants for their basic health care. Still no analysis has been done in Bajaur region to precept the medicinal plants used to treat different skin disorder. North-West of Pakistan is bestowed with medicinal plant resources due to diverse geographical and habitat conditions. The traditional use of plants for curing various diseases forms an important part of the region's cultural heritage. The study was carried out to document medicinal plants used in the Bajaur Agency, an area affected by the "War on Terror".

Mostly a lot of studies have been carried out in different regions of Pakistan; however, the Bajaur Agency has yet to be explored due to limited access. The area represents one of the country's richest centers of biodiversity and it is a strong source of indigenous knowledge. Most of the population of the area is rural with a low literacy rate; hence they are more dependent upon natural resources, and especially on plants for their healthcare needs and livelihoods. War has crippled modern health facilities in the study area, which in turn has resulted in the spreading of gastrointestinal and skin related diseases among others. Tribal communities are compelled to alter their occupation which leads to ethno cultural deprivation. Therefore an attempt has been made to investigate the traditional health care system of the people of the Bajaur region, for treatment of various types of skin diseases, using the ethno medicinal flora.

2. Selected Medicinal plant of Bajaur region

2.1 *Oxalis corniculata* (Family: Oxalidaceae). *Oxalis corniculata* is commonly known as the Creeping wood Sorrel [23]. The plant has a good arrangement in the nature and consist all the important ingredient, essential for normal and good health of humans [24].

The leaves consist of water, niacin, vitamin C, protein, beta-carotene, calcium, carbohydrate, phytosterols, flavonoids, mucilage and phenolic compounds [25]. It is used in conventional remedy for the treatment of fever, burns, and gastrointestinal disorders and has been used as an anti-inflammatory agent [26]. The plant consist of antimicrobial agents which have been investigated on a plant pathogen *Xanthomonas campestris* and on human pathogens such as *Staphylococcus aureus* and *Escherichia coli* [27, 28]. It is also known to cure dysentery, diarrhea and skin diseases [26]. *Oxalis corniculata* decrease myocardial infarction by reducing the amount isoproterenol (produce oxidative stress) compound which have significant role in increasing the amount of creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) a cardiac injury marker enzymes [29]. *Oxalis corniculata* have healing activity by increase contraction rate and decrease epithelization period by using excision and scratch of wound [30]. The fresh leaves of *oxalis corniculata* are used to stop bleeding [31].

3. Materials and methods

3.1 Study area and Sampling

The selected plant samples were collected from various areas of District Khar (Bajaur Agency). The plant material was identified by plant taxonomist and a voucher specimen no KUST 425 was deposited in the herbarium of KUST. The indigenous medicinal plants of the area were analyzed for phytochemical analysis and antibacterial potential at the Department of Microbiology in collaboration with Department of Chemistry, KUST.

3.2 Collection of skin bacterial samples

Samples were collected from Hayat abad Medical Complex (HMC) and North West General Hospital (NWGH) were brought to Microbiology Department, KUST. These bacteria were confirmed on the basis of their microscopy, culture and biochemical tests.

Table 1: List of selected bacteria

S. No	Bacteria isolates	Characteristics
1	*Staphylococcus aureus*	Gram +ve, cluster form, coagulase +ve, cause food poisoning, arthritis, septicemia, skin infection.
2	*Staphylococcus epidermidis*	Gram +ve cocci, cluster form, catalase +ve, normal flora of skin, cause hospital acquired infection, endocarditis, joint infection.
3	*Streptococcus pyogenes*	Gram +ve, spherical form, catalase +ve, cause fasciitis, impetigo, toxic shock syndrome.
4	*Pseudomonas aeruginosa*	Gram –ve, rod form, oxidase +ve, cause skin infection, sepsis, pneumonia, urinary tract infection (UTI).

3.3 Culture Media
The bacteria samples were cultured on Mac Conkey and blood agar. Mac Conkey agar is a differential media for Gram negative bacteria, Blood agar act is a enriched media which facilitate the growth of Gram positive bacteria and differentiate bacteria on the basis of hemolysis of blood [32].

3.4 Staining and Microscopy
The cultured bacteria were stained with Gram,s stain, observed under microscope for identification of their cell morphology.

3.5 Biochemical Tests

Table 2: To confirm the bacteria biochemical test were performed. These were

S. No	Bacteria	Biochemical Tests
1	*Staphylococcus aureus*	Catalase +ve, Coagulase +ve, DNase test +ve,
2	*Staphylococcus epidermidis*	Catalase +ve, Coagulase -ve, Dnas test –ve, Oxidase –ve.
3	*Streptococcus pyogenes*	Catalase –ve, Coagulase –ve, DNase –ve,
4	*Pseudomonas aeruginosa*	Oxidase +ve, Catalase +ve. Coagulase –ve.

3.6 Collection of Medicinal plant
The medicinal plant *Oxalis corniculata* (creeping wood sorrel samples were collected from Bajaur Agency of Khyber Pakhtoonkhwa Pakistan. The botanical characteristics of these plants were confirmed by the taxonomist at KUST. The plants were preserved for further processing.

Table 3: The understudy Medicinal plant characteristics table

S. No	Botanical name	Family	Local name (Pashto)	Parts used	Traditional Medicinal uses
5	*Oxalis corniculata*	Oxalidaceae	Taroky	Stem	Fever, burn, anti-inflammatory, gastrointestinal disorder.

3.7 Plants Grinding and Fractionation
These plant samples were shade dried for 25 days and preserved for further processing. The dried plant samples (1kg) were grinded into powdered form for obtaining extracts. The extracts were then macerated in methyl alcohol with occasional shaking at room temperature for 12 days and were filtered subsequently. The filtrates were concentrated using rotary evaporator at 42°C to obtain crude extracts of the target plants and later to be fractioned with n-hexane, chloroform, ethyl acetate and water, respectively. Different concentration 50mg/ml, 25mg/ml and 12.5mg/ml of each fraction were prepared in Di-Methyle Sulfoxide (DMSO) for antibacterial assay.

3.8 Bacteria culture
The selected bacterial strains were first cultured on different growth media. Nutrient agar is used because it enhances the growth of bacteria. Mueller Hinton Agar (MHA) media is used for susceptibility testing of bacteria against plants extract and antibiotics. Before inoculation of bacteria, culture was refreshed in nutrient broth for 24 hrs at 37 °C.

3.9 Bacterial susceptibility testing
Antibacterial activities of fractions were determined by using well diffusion assay and minimum inhibitory concentration (MIC) were employed. Four skin bacterial strain *Pseudomonas aeruginosa* (NCTC 10662), *Staph. aureus* (NCTC 6571), *Staphylococcus epidermidis* (NCTC 11047) and *Streptococcus pyogenes* (NCTC 8198) in the antibacterial assay. Mueller Hinton agar (Oxide, UK) media was prepared in conical flask according to manufacturer. The media along with petri dishes, pipette and metallic borer were sterilized in autoclave for 15 minutes at 121 °C and 15 psi pressure. The media was poured into Petri dishes under aseptic condition and allowed to solidify. After solidification of media in plates fresh culture (adjusted to 0.5 McFarland turbidity) were inoculated on each plate through spreader further 7 wells were made through cork borer (6mm diameter), then 100µl extract were poured in the wells through micropipette. Di-methyle sulfoxide (DMSO) was used as a negative control in the well and for positive control standard antibiotic imipenem was used; the plates were labeled, placed in incubator for 24 hrs at 37 °C for aerobic incubation. Zone of inhibition was measured by a scale in millimeter [33].

3.10 Minimum Inhibitory Concentrations (MICs)
In order to find out the qualitative antibacterial activity of plant extract against bacterial pathogen the minimum inhibitory concentration (MIC) was used. The 15 ml sterilized, caped tube were taken having 10 ml nutrient broth in each. Different concentration dilutions of the crude extracts were prepared in nutrient broth. The upper limit was 50 mg/ml and subsequent as 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and lower was set as 3.12 mg/ml. Then 100 ul of overnight bacterial broth cultures were matched to 0.5 McFarland standards and inoculated in all the tubes. Negative and positive control was also run. The negative control was only nutrient broth while positive control was nutrient broth having bacterial culture. Then the tubes were incubated at 37 °C for 24 hours, after that results were recorded. The minimum concentration of extracts at which no bacterial growth appeared was considered as MIC for specific bacteria [33].

3.11 Phytochemical analysis
Qualitative analysis of various extract of different plants was performed for the identification of various class of active chemical constituents like glycosides, steroids, tannins, terpenoids, alkaloids, flavonoid, amino acid, carbohydrates, proteins, phenol, starch and saponin etc using standard procedure to identify the constituents [34, 35].

Detection of alkaloids

a) **Mayer's Test:** The 50 mg crude extracts was taken in a glass tube having 1 ml hydrochloric acid mixed with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate indicates the presence of alkaloids.
b) **Wagner's Test:** The 50 mg crude extracts was taken in a glass tube having 1 ml hydrochloric acid mix with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Detection of carbohydrates: The 60 mg crude extract were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a) **Molisch's Test:** Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of carbohydrates.
b) **Benedict's test:** Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Detection of glycosides: The 50 mg crude extract were hydrolyzed with diluted HCl, and then subjected to test for glycosides.

Legal's Test: Extract was treated with sodium nitroprusside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

Detection of saponins
a) **Froth Test:** The 50 mg extract was diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.
b) **Foam Test:** The 50 mg of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of phenols
Ferric Chloride Test: The 30 mg extract was treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of tannins
Gelatin Test: To the 40 mg extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Detection of flavonoids
Lead acetate Test: 20 mg extract was mixed with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Detection of proteins
Xanthoproteic Test: The 30 mg extract was mixed with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Test for steroid
The 5 ml of crude extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlying with 1 ml of concentrated sulphuric acid. A brown ring at the interference indicated deoxy sugar characteristics of cardenolides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may be formed [36-38].

Statistical Analysis. All the experiments were done three times, mean zone of inhibition were calculated.

4. Results

4.1 Antibacterial activity of Plants by Well Diffusion Method

The Medicinal plant *Oxalis corniculata* was used *in vitro* for antibacterial action under optimal condition against skin pathogens: e.g. *Staph. aureus, Streptococcus pyogenes, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. The lowest activity was shown by *Oxalis corniculata*.

4.2 *Oxalis corniculata* (Stem)

The aqueous and hexane extracts showed highest activity (7-10mm) against *Strep. pyogenes, Staph. aureus* and *P. aeruginosa* while lowest activity against *Staph. epidermidis* and Maximum activity were observed in case of *Strep. pyogenes, Staph. aureus* and *Staph. epidermidis* by ethyle acetate. While lowest activity was observed in case of *P. aeruginosa*. The chloroform extract have shown highest activity against *Strep. pyogenes* and *P. aeruginosa*, however lowest activity against *Staph. aureus* and *Staph. epidermidis* were seen as shown in table 3.5. Different concentration of the plant extract which showed different zone of inhibition were shown in figure 3.5. a, b, c, d.

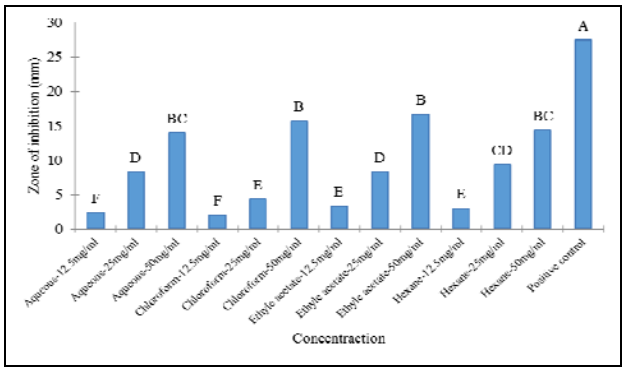

Fig 3.5. a: Effect of different concentration of *Oxalis corniculata* on *S. aureus*

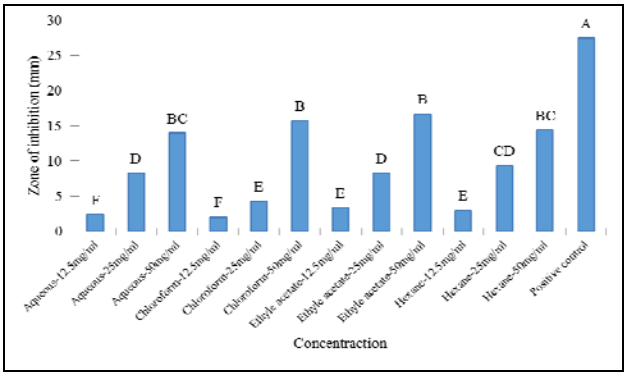

Fig 3.5. b: Effect of different concentration of *Oxalis corniculata* on *P. aeruginosa*

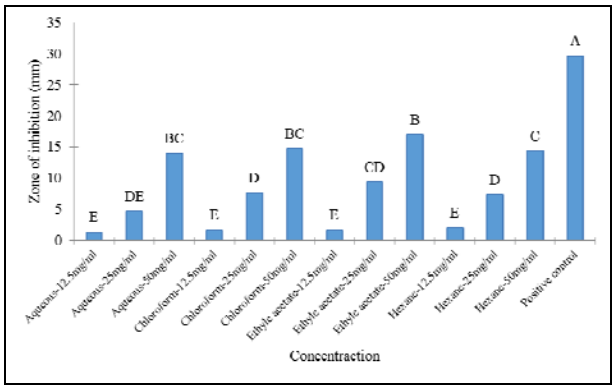

Fig 3.5.c: Effect of different concentration of *Oxalis corniculata* on *S. epidermidis*

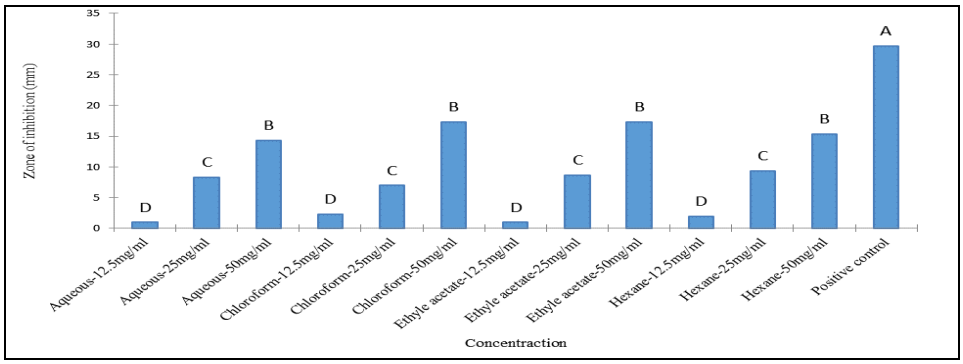

Fig 3.5.d: Effect of different concentration of *Oxalis corniculata* on *S. pyogenes*

Table 3.5: Mean value of antibacterial activity of *Oxalis corniculata* against skin pathogens

Concentration	*S. aureus*	*S. pyogenes*	*S. epidermidis*	*P. aeruginosa*
	Zone of inhibition (mm)			
Positive control	27.45± 1.7	29.667±2	29.667±1.8	27.444±2
Chloroform-50mg/ml	15.667±0.9	17.333±1.7	17.000±2	17.333±1.8
Hexane-50mg/ml	14.333±1.7	17.333±1.2	14.667±1.9	15.000±1.8
Ethyl acetate-50mg/ml	16.667±1.6	15.333±1.8	14.333±2	16.000±2
Aqueous-50mg/ml	14.000±1.8	14.333 ±1.6	14.000±1.4	16.667±1.6
Ethyl acetate-25mg/ml	8.333±1.6	8.667±1.2	9.333±1.6	4.000±1.2
Hexane-25mg/ml	9.333±0.6	8.333±0.5	7.667±1.2	10000±1.2
Aqueous-25mg/ml	8.333±0.8	9.333±0.6	7.333±0.9	8.000±1.2
Chloroform-25mg/ml	4.333±	7.000±	4.667±	7.000±
Ethyl acetate-12.5mg/ml	3.333±0.2	1.000±0.5	2.000±0.3	1.667±0.1
Hexane-12.5mg/ml	3.000±0.2	2.000±0.4	1.667±0.4	1.000±0.2
Chloroform-12.5mg/ml	2.000±0.7	2.333±0.5	1.667±0.5	1.3330±0.3
Aqueous-12.5mg/ml	2.333±0.5	1.000±0.2	1.333±0.9	1.667±0.2
DMSO-Negative control	0.000	0.000	0.000	0.000

4.3 Minimum Inhibitory Concentrations (MICs)

Minimum inhibitory concentration was used to find out the bacteriostatic value of the plant extracts. The MIC values against skin pathogens were in range of 3.12mg/ml to 25mg/ml. Table 3.6.

4.4 *Oxalis corniculata* (Stem)

MIC values of crude extract of Oxalis corniculata were higher against *Staph. aureus, Strep pyogenes* 6.25 mg/ml and 12.5 mg/ml were against *Staph. epidermidis* and *P. aeruginosa*. While no zone of inhibition was observed in 3.12mg/ml concentration of the plant against the Bacterial pathogens.

Table 3.6: Minimum inhibitory concentration of plant crude extract against skin pathogens

Bacteria isolates	*Oxalis corniculata*(mg/ml)
Staph. aureus	6.25
Strep. pyogenes	6.25
Stahp. epidermidis	12.5
P. aeruginosa	12.5

4.5 Phytochemical activity

Qualitative analysis for phytochemical of selected plants were performed by standard procedures.

4.6 *Oxalis corniculata* (Stem)

Crude extract of *Oxalis corniculata* were consist of alkaloids, carbohydrates, saponins, phenols, tannins and Starch was in high concentration, glycosides were in moderate concentration while protein was not found as described in Table 3.7.

Table 3.7: Phytochemical analysis of plants

Plants	Phytochemicals								
	Alkaloid	Carbohydrate	Glycoside	Saponin	Phenol	Tannins	Flavonoid	Protein	Starch
Oxalis corniculata	+++	+++	++	+++	+++	+++	+++	-	+

* - sign shows not detected; + shows compound present in small amount; ++ shows compound present in moderate amount; +++ shows compound present in higher amount.

5. Discussion

The need of antibiotics is increasing day by day in the world for control of antibiotic resistant pathogenic bacteria. To overcome this dilemma the pharmaceutical companies are now looking for alternatives. Plants have been an affluent source of medicines because it is assumed that plant based drugs cause fewer or no side effect and influence a wide range of antibiotic resistant microorganisms.

The medicinal plant *Oxalis corniculata* extracts have been used in recent study for observing their antibacterial potential. The phytochemical were extracted in ethyle acetate, chloroform, n-hexane and aqueous. These extracts were used against skin causing pathogens *Staph. epidermidis, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Staph. aureus* as occasional pathogens.

5.1 *Oxalis corniculata*: In this case the hexane extract indicated a highest activity zone for *Staph. aureus* and *Streptococcus pyogenes,* while less resistance was observed for *P. aeruginosa* and moderate resistance was seen in *Staphylococcus epidermidis* isolates. These results are comparable to related observations for similar bacterial strains [39].

Streptococcus pyogenes, *Staphylococcus epidermidis* and *P. aeruginosa* were resistant to chloroform extract while *Staph. aureus* was sensitive to chloroform extract as also determined in another analysis [40].

Similarly *S. aureus, Streptococcus pyogenes* and *Staphylococcus epidermidis* were resistance to ethanolic extract while *P. aeruginosa* was resistant to the extract. These finding were an agreements with our results [41-43].

The aquous extract depicted better results in the growth inhibation to *Staph. aureus* and *P. aeruginosa* as compared to *Streptococcus pyogenes* and *Staphylococcus epidermidis.* These observations co relate to a study [44]. The MIC value of the plant extract against *Staph. epidermidis* and *P. aeruginosa* was highest, while against *Staph. aureus, Strep pyogenes* were less effective as seen in previous study [45].

Phytochemical analysis determined that the plant extract consist of alkaloids, carbohydrates, saponin, phenols, tennins, flavenoids starch while the presence of glycosides were in lesser amount, but protein could not be detected. These observation are in concordance to the similar research work findings [46].

The present study was carried out to uncover the antibacterial activity and phytochemical constitutents of the selected medicinal plants. Phytochemical investigation can verify the purity and authenticity of different parts of the plants by using standard method. Phytochemical investigation of chloroform, ethyl acetate, n-hexane and water extracts exposed the presence of carbohydrates, proteins, starch, saponnins, terpenoids, tannins and steroid. All these constituents help in determining the pharmacological standard in determining the quality and purity of different parts of the plants.

In the last two decades, drugs resistances as well as side effects of certain antibiotics have lead to find out new antibacterial agents. There are sufficient proofs that showed antibiotics properties in plants extracts. The present study was done to evaluate the antibacterial activity of wildly growing indigenous plants. Aqueous, chloroform, ethyl acetate and hexane extracts of different parts of *Oxalis corniculata* was tested against skin pathogenic bacteria *staph. aureus, Staph. epidermidis, Streptococcus pyogenes,* and *Pseudomonas aeruginosa.* The results indicate that their active compounds can be promising agent for development of novel antibiotics that is green antibiotics.

6. Conclusion

Present work demonstrated that selected plant extracts were efficient against skin isolates. It can be concluded that plant extracts exhibit versatile biochemical molecules which can inhibit or kill bacteria by different site or mode of action. Findings of the current study provide initial evidence that *Oxalis corniculata* against common human skin pathogenic bacteria were effective in their activity. Further in-depth analysis on these, as well as other potential medicinal plants will be helpful for future elucidation of bioactive molecules to treat infectious diseases caused by skin bacteria.

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