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Antioxidant, Antimicrobial and Cytotoxic activity of Albizia lebbeck and Smilax ornataIn Vitro

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Abstract

Drug resistance and a broad range of side effects are the major drawbacks of antibiotics to treat microbial diseases. Medicinal plants are traditionally used to cure various infectious as well as non-infectious diseases. Medicinal plants also show their

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antioxidant and cytotoxicity potential due to the existence of a wide spectrum of phytochemicals. The presentstudy exploresthe antimicrobial, antioxidant and cytotoxic activity of Albizia lebbeck and Smilax ornata. Six extracts of both plants are prepared in different solventsand the extracts are tested for antimicrobial, antioxidant and cytotoxicity through various methods. Both plants strongly show electron and proton donating capacity, but mild antibacterial potential against Staphylococcus aureus and E. colimicroorganisms.We found that phenolic components in the selected medicinal plants are the origin of antibacterial, antioxidant, and cytotoxicity potential of the extracts and these plants could be beneficial towards human and animal health.

Keywords: *Herbal medicine, Antibacterial, Antioxidant, Cytotoxicity, Albizia lebbeck, Smilax ornata*

Introduction

Herbs have prime significance in the modern pharmacology to develop newer pharmaceuticals due to their safety andpotential therapeutic effects.Inhabitants of the developing countries mainly depend upon these herbs as a primary source of treatment for several diseases due to their usage in the traditional medicine system, andtheir therapeutic properties are well recognized (Vyas, Malli, Talaviya, & Ghadiya, 2016). These herbal plants are widely used as decorative, food and beauty products, flavoring agents, insect repellent, and medicinal purposes. World health organization (WHO) also stated that more than ¾ populations depend on herbal remedies due to feweradverse effects and cost-effectiveness (Chaudhuri, Pathak, & Sevanan, 2011).The plant parts or substances are oppressed by various natural and physical processes, such as decontamination, meditation, fractionation, and extraction. The herbal products and related preparation methods are referred to as phytomedicine. Similar formulations may be developed for immediate use or as a source of other polyherbal formulations.

The resulting plant products are accounted for active ingredients and may also include additional static ingredients(Okigbo & Mmeka, 2006). Phytomedicine has a wide range of clinical advantages, and therefore, itissuccessfulintreating chronicillnessesandoccupiesacentralposition in the global healthcare.In comparison to artificial medications, phytomedicine has fewer side effects and is very important in treating communicable diseases (Iwu, Duncan, & Okunji, 1999). phytomedicine has a wide variety of applications in reducing the mortality rate, morbidity, and disability caused by fatal diseases such as HIV/AIDS, tuberculosis, malaria, diabetes, sickle-cell anemiaandmental illnesses(Elujoba, Odeleye, & Ogunyemi, 2005).Phytomedicine is becoming increasingly important in developing countries, owing to its safety, high-quality assurance, and efficacy of herbal drugs (Calixto, 2000; Okigbo & Mmeka, 2006).Phytomedicine comprise a high ability to treat a wide range of contagious diseases and have a strong presence in developing countries. These plant-based medicines play a notable role in modern therapeutics(Pan et al., 2013). The medicines derived from natural resources are gaining attention both scientifically and practically. However, the mechanism of plant'saction andtheir products aremuch more complex than the mechanism of clarification by a single bioactive component. Plants have justified their value by minimizing side effects and ensuring safety(Saxena, Saxena, Nema, Singh, & Gupta, 2013). There is a requirement to bring common harmony between the traditional and modern health care systems. Therefore, integrationof phytomedicine into the current health system is a necessity(Pandey, Debnath, Gupta, & Chikara, 2011). Evaluation of the in vitro antimicrobial, antioxidant and cytotoxicity potential of *Albizia lebbeck* and Smilax *ornata*is the main object of the current study.

Albizia lebbeck(L.) Benthis commonly known as Shirish (put the local name in Pakistan). It is a broadly distributed plant in tropical and subtropical Africa as well as Asia, having significant commercial value for industrial medical purposes. The leaves are high in protein and make good feed. The plant contains flavonides, macrocyclic alkaloids, saponin, and phenolic glycosides (Makki, 2021). It is a leguminous plant that belongs to the Mimosae subfamily of the Fabaceae (Formerly Leguminosae) family. It's a large deciduous tree with greyish bark and young shoots that are glabrous. The leaflets are in 5-9 pairs, 2.5-5.0 cm long, broadly oblong and pale green, with irregular sides, extremely obtuse glabrous above and reticulately veined beneath, and uniformly 2-pinnate leaves. Flowers in globose umbellate heads with a diameter of 2-3.8cm are stalked and scented white. Peduncles 3.8-7.5cm long that emerge from the upper leaf axils alone or in groups of 2-4. Calyx 4 mm long, teeth tiny; corolla 1 cm long; tube glabrous; lobes 2.5 mm long; calyx 1 cm long; tube glabrous; and lobes 2.5 mm long. The stamens are about twice as long as the corolla (Mishra, Gothecha, Sharma, & toxicology, 2010). The pod is 10-30 cm long and 2.5-5.0 cm broad, with a flat straw color and 4-12 pale brown seeds inside (Avoseh, Mtunzi, Ogunwande, Ascrizzi, & Guido, 2021).

Smilax ornata Lem. is a prickly-stemmed perennial trailing vine that is endemic to Mexico and Central America. Sarsaparilla is the common name for this plant (put the local name in Pakistan). It is a member of the Smilacaceae family. It's the backbone for a soft drink. It's also a key ingredient in old-fashioned root beer, which was more readily available until health studies revealed its dangers. *Smilax ornata* was a popular European syphilis cure when it was introduced from the New World, and Native Americans thought it had medical virtues(Vandebroek et al., 2021).

Methods and Materials

Selection and collection of plant material

Selected medicinal plants for the current study were obtained from the local market of Faisalabad, Punjab, Pakistan. Seeds of *Albizia lebbeck* and roots of *Smilax ornata* were taxonomically identified and confirmed from the Botany Department of Government College University, Faisalabad, Pakistan.

Preparation of plant extract

The selected plant parts were collected and washed with distilled water to remove the dirt particles. The plant parts were dried beneath the shadow at room temperature. Dried samples were processed into powder viaa mechanical grinder and stored in a closed container for further procedures. 20 g of plant powders were soaked in 200 mL of different solvents, ethanol, n-Hexane and water for 4 h with constant stirring (Mehwish et al., 2019). At 40°C, the mixture was centrifuged for 10 minutes at 10,000 rpm. The supernatant was gathered, and the procedure was repeated. To obtain the unpurified extracts, the filtrates were evaporated to

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dryness using a rotary vacuum evaporator at 40 °C.Then, lyophilized to remove the solvent effect and obtain the powdered extracts(Hellmuth, 2017).

Antioxidant Study

Using a 2, 2-diphenyl-1-picrylhydrazyl radical scavenging experiment, the antioxidant activity of extracts from several plants were determined (Marcheafave et al., 2019a). Total phenolic contents were estimated using the Folin-Ciocaletu reagent technique (Khan, 2017). An aluminium chloride complex-forming assay was used to evaluate the total flavonoid content of the extracts (Ferysiuk & Wójciak, 2019).

Spectrophotometric method for DPPH free radical scavenging property

The colorimetric test was attained to determine the ability of extracts to scavenge free radicals in a short time(Huang et al., 2020). DPPH is a nitrogen-centered free radical that is stable at room temperature. It was used to see if the plant extracts are helpful toward scavenging free radicals. It is purple in color when it is in its free radical state. During the process, it receives proton from plant bio-actives, mainly phenolics, and the purple color changes to yellow(Li & chemistry, 2017). The change in absorbanceindicates the plant extract's capacity to neutralise free radicals (antioxidant) power (Ajaib, Shafi, Iqbal, Bhatti, & Siddiqui, 2021).

The antioxidant capacity of extracts was evaluated using the 2, 2 – diphenyl -1- picrylhydrazyl radical scavenging test (Marcheafave et al., 2019b) with a slight modification. 10 mL of freshly prepared 0.004% DPPH in methanol was added to 30 mL of varied quantities of the examined plant extracts, and the mixed solution was kept in the dark for half an hour. At 517 nm, the absorbance was measured. A low absorbance reaction combination suggests considerable radical scavenging activity. Samples antioxidant activity was compared to that of ascorbic acid, which was used as a control. A solution without plant extract was used as the control. Each test was repeated for three times. The following formula was used to compute the percentage inhibition of DPPH radical samples(Tohma et al., 2017).

$$\text{Inhibition of DPPH (\% age)} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

TFC analysis of the selected plant extracts

The total flavonoid content of the plant extract was resolute using a colorimetric approach based on the production of a flavonoid-aluminium complex (at 510 nm), with the results represented as mol equivalents of rutin per mg of the dried extract (Sakanaka, Tachibana, & Okada, 2005).Plant extracts' total flavonoid content was assessed using the technique expressed by (Wu et al., 2006). 0.5 mL of the extract was mixed with 2 mL of distilled water and 0.15 mL of a 5% $NaNO_2$ solution for a 6-minute incubation period. Following that, 0.15 mL of 10% $AlCl_3$ solution was added to the mixture and incubated for another 6 minutes before adding 4 percent NaOH solution. By adding methanol to the reaction mixture and thoroughly mixing it, volume of the reaction mixture was increased to 5mL. After fifteen minutes' incubation, the reaction mixture's absorbance was measured at 510nm. The TFC (total flavonoid content) of the selected extracts were calculated using the catechin linear regression curve and presented as catechin equivalents.

Evaluation the TPC (total phenolic content)

Haq et al., described the Folin Ciocalteu method to determinethe total phenolic content of the tested samples (Haq, Sani, Hossain, Taha, & Monneruzzaman, 2011). 1.0 mL of the given sample was joined with 0.5 mL of Folin-Ciocalteu (10%) and0.4 mL of sodium carbonate (20%) and incubated for 10hours. The absorbance of the resultant blue color was calculated at 765nm. The measurement was carried out in compliance with the standard Gallic acid. The calibration curve was made using various quantities of gallic acid. 1mL aliquots of0.1,0.2,0.3,0.4,0.5,0.6,0.7,0.8,0.9 and1.0 mg/mL Gallic acid solution in methanol were mixed with 5.0 mL Folin-Ciocalteu reagent (diluted 10 times) and 0.4 mL sodium carbonate (20%). The absorbance at 765nm was determined after one hour and the calibration curve was produced using the absorbance as a function of concentration. The total concentration of phenolic compounds in the selected plant extracts in GAE (Gallic acid) was determined using the following method.

$$TPC = C^{*}V / M \text{ (mg/gm)}$$

wherein, T is the presented total content of phenolic compound in mg GAE/ g of plant extract,

C is the concentrations of Gallic acid derived from calibration curve in mg/ mL, V is the volume of extract in mL, M is the weight of plant extract in gram.

Antimicrobial assay

The antibacterial properties of the extracts (*Albizia lebbeck* seeds and *Smilax ornata* roots) was assessed. Tagg and McGiven (1971) (Tagg & McGiven, 1971) and Ali (1972) used the agar well diffusion method (2014). To resurrect the bacterial species, sterilized Petri plates were loaded with nutrient agar and the bacterial type with 108 cfu/ml were inoculated (Bisht et al., 2021), in comparison to the 0.50 McFarland turbidity index. Then,the concentrated seed's and root's extract were transferred into the petri plate wells, and the plates were incubated at 37°C for 24 hours. In contrast, no extracts were used in the control plates. The zone of inhibition was estimated to determine their potential against the harmful bacterial species. All of the experiments were carried out in threes. The antibacterial property of each extract was assessed by using the agar well method against *Staphylococcus aureus* ATCC 25923 (Lorenzini, Mercantini, & De Bernardis, 1985; Magaldi et al., 2004). The well had a diameter of 7mm and a height of 4mm. The suspension in sterile normal saline was made for each strain and turbidity was corrected to the 0.5 McFarland standard. To achieve a confluent growth, the suspension of organisms was utilized to inoculate blood agar plates. With the use of a cork borer, wells were cut in the agar surface. Then, 100 mL of the samples preparation was poured into each well.At the same time, Staphylococcus aureus and E. coli were examined using Ciprofloxacin, respectively. Before assessing the inhibitory zone diameters, all of the plates were incubated for 24 hours at 37°C. Any zone of inhibition round the wells containing the decoction was considered sensitive.

Cytotoxicity analysis

The following procedures were used to assess the cytotoxic activity of the selected plant extracts.

Hemolytic activity determined by hemolytic assay

The anti-hemolytic activity of given extracts was assessed using a spectrophotometric technique similar to that published before, with a few changes(Yang, Sun, & Fang, 2005). Three milliliters of human and animal (sheep) blood cells were smoothly mixed and centrifuged for five minutes at 850 xg in a sterile fifteen mL

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polystyrene screw-cap tube at room temperature. The supernatant was drained and the sticky pellet was washed three times with 5 mL of the sterilized isotonic phosphate buffered saline (PBS) solution (KH_2PO_4, 0.2 g/L; NaCl, 8 g/L; Na_2HPO_4, 12 g/L; and KCl, 0.20 g/L, adjusted to pH 7.40. After being rinsed and suspended in 20 mL cold, sterile PBS, the cells were counted using a haemacytometer. The blood cell suspension was stored on damp ice for every test and diluted to 7.068 mL with sterile PBS. 20 mL of sample was deposited in 1.5 mL Eppendorf tubes aseptically. For each experiment, the positive, 100% lytic control was 0.1 percent Triton X-100, while the negative, background (0 percent lysis) control was PBS. A 180 µL diluted blood cell solution was aseptically put into each 1.5 mL tube and smoothly mixed three times with a large mouth pipette tip. Finally, the samples were mixed and incubated at 37 °C for 35 minutes with mix up (80 rev/min), then the tubes were immediately placed on ice for five minutes before centrifugation at 1310 x g for 5 minutes. A total of 100 µL supernatant was collected with vigilance, transferred to a disinfected 15 mL Eppendorf tube, and diluted with 900 µL of chilled and sterilized PBS. All tubes were maintained on wet ice after dilution. The absorbance at 576 nm was measured using Quant (Winooski, BioTek, USA, VT) (Powell et al., 2000). After that, each sample was run in triplicate and the results were averaged. The following equation was used to compute the percentage lysis of RBC. Sample absorbance and triton X-100 absorbance are denoted by As and Ac, respectively (standard).

$$\text{Hemolysis (\% age)} = \frac{\text{A450 of test compound treated sample} - \text{A450 of buffer treated sample}}{\text{A450 of 1 \% Triton X} - \text{100 treated sample} - \text{A450 of buffer treated sample}} \times 100$$

Ames test for mutagenicityssss

Bruce N. Ames introduced the Ames test in 1970s to investigate the mutagens. This analysis is based on the principle of reverse mutation or back mutation. In this test, the bacterial strains (TA-100 and TA-98) are added to the reaction mixture (Turkez, Arslan, Ozdemir, & toxicology, 2017).

Then, the Ames test could be used to assess the mutagenicity of investigational materials using Muta-Chromplate (Ontario, EBPI, Canada). The test has been carried out on the bacteria such as S. typhimurium TA100 & S. typhimurium TA98 in Nutrient agar (liquid culture) (Shahid, Anjum, Iqbal, Khan, & Pirzada, 2020).

Ames test method

A reagent mixture of glucose-D, Davis Mingioli salt, bromocresol purple, histidine-L, and biotin-D was poured in a sterile bottle. The numbers in Table 1 are used to conduct the test. Plant extracts, reagent combination, and test strains were mixed with distilled water to make the sample. In this experiment, micro well plates were produced according to Razak and Aidoo (2011) procedure and incubated at 37 °C for 96 hours. The experimental plates were marked first, followed by the reference plate, and purple wells containing blanks indicated that the evaluation is not polluted. The background, standard, and samples were visually counted. Wells with turbidity and a yellow tint were seen as positive, whereas those with a purple tint were regarded as negative. Purple coloring in the wells of sample indicated toxicity of the sample against the test strain. To be mutagenic, the number of positive wells in an extract should be more than twice of the number of positive wells in the background plate.

Statistical analysis

The obtained results were articulated as Mean ± SEM and further interpreted through statistical analysis by applying one-way ANOVA test.

RESULTS AND DISCUSSION

Scavenging activity of DPPH

The DPPH radical scavenging activity (percent of inhibition) of the plant extracts was studied by utilizing various solvent systems mentioned in the procedure. The antioxidant profiles of various solvent extracts differed significantly. The antioxidant study was done for the two plants. The strongest DPPH radical scavenging activity (inhibition%) was shown by n-hexane extracts for both the plants *Albizia lebbeck* and *Smilax ornata* with 17.70551% and 15.98916%, respectively, and the weakest for ethanolic extracts with 12.82746% and 10.47877%, respectively, as shown in Figure 1.

Similarly, the total flavonoid content was found highest GE to catechin / mgin water extracts for the two plants *Albizia lebbeck*and *Smilax ornata* with 37.5 and 35.13, respectively, and the lowest was found in n-hexane extracts with 24.86 and 26.97, respectively, as shown in figure 3. Whereas, the total phenolic content was found highest GE to catechin (mgGAE/g) in n-hexane extracts for the two plants *Albizia lebbeck* and *Smilax ornata* with 75.2 and 78.0, respectively, and the lowest was found in ethanolic extracts with 58.2 and 65.2, respectively, as shown in figure 4.

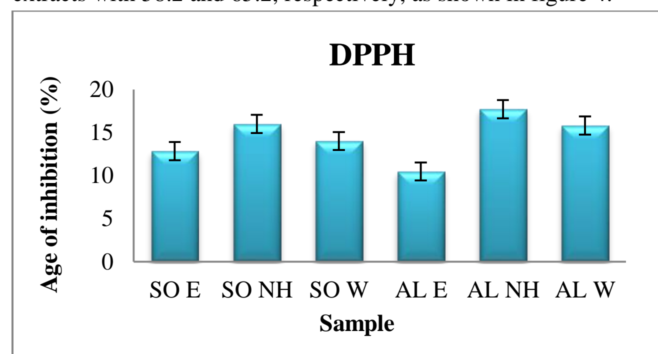

Figure 1. Plant extracts' DPPH free radical scavenging properties.

Total flavonoids content

Plant extracts showed considerable variations in TFC profile. The maximumTFCof *Albizia lebbeck* was observed in water extracts (35.13) and minimum was observed in n-hexane extract (26.97). In case of *Smilax ornata,*maximum TFCwas shown by extracts, 37.5, and minimum TFC was shown by n-hexane extracts 24.86.

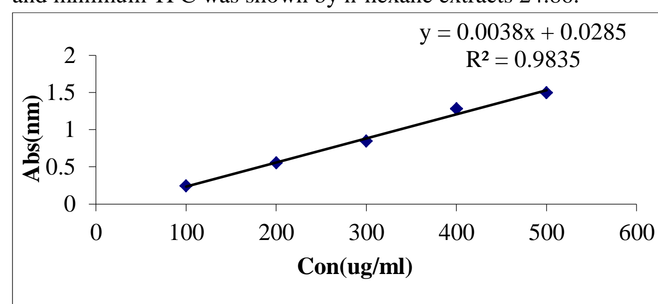

Fig. 2. Standard curve of Catechin.

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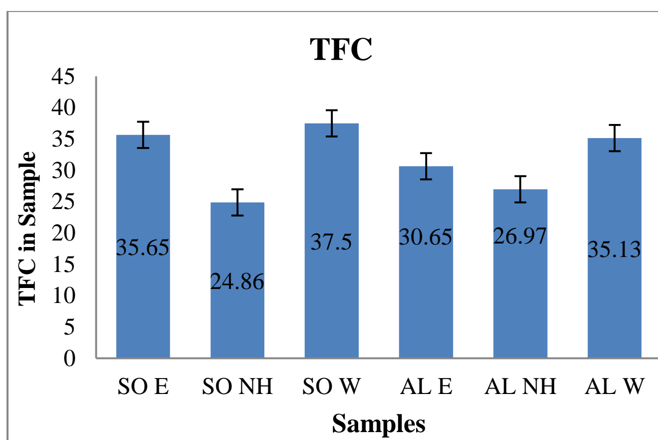

Figure 3. Graphical presentations values of TFC

Total phenolic content determination (TPC)

Different solvent extracts showed significant differences in the TPC profile.The maximum total phenolic contents of *Albizia lebbeck* was shown by n-hexane extracts (75.2mgGAE/g), and the minimum was observed by ethanolic extract (58.2 mgGAE/g). In the case of *Smilax ornata*, maximum total phenolic contents were shown by n-hexane extracts, 78, and minimum GE to catechin was shown by ethanolic extracts 65.2.

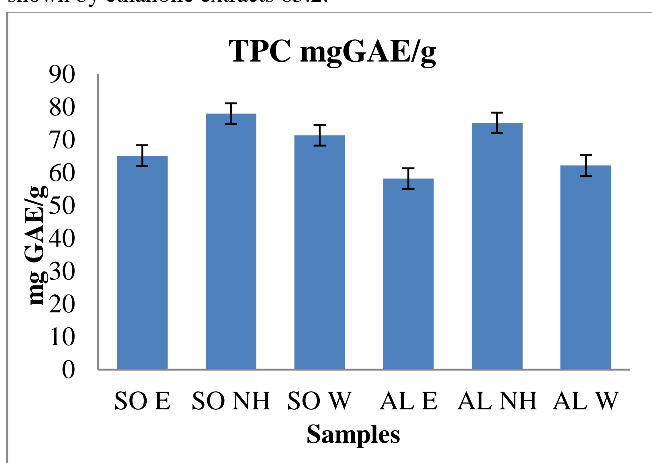

Figure 4. Total phenolic content of various plant solvent extracts.

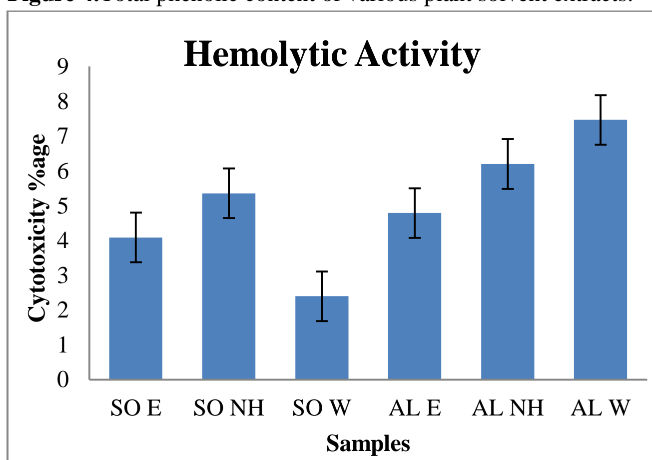

Figure 5. Hemolytic activity of various solvent extracts of plants.

The cytotoxicity profiles of various solvent extracts showed significant differences. We looked at the influence of different solvent systems on the cytotoxicity (percent) of various plants; while using stirring as an extraction technique in the previous chapter. The maximum cytotoxicity (%) of *Albizia lebbeck*was shown by water extracts (7.46%) and minimum was observed by ethanolic extract (4.78%). In the case of *Smilax ornata*, maximum cytotoxicity was shown by n-hexane extracts, 5.35%, and minimum DPPH radical scavenging in water extracts as 2.39%.

Table 1. Setup for Ames assay.

Sr. No.	Treatment	Extracts Samples	Standard Mutagen	Reagents	Distilled Water	Test Strain
01	Standard Mutagen	----	0.1	2.5	17.5	0.005
02	Background	-------	-------	2.5	17.5	0.005
03	Blank	-------	-------	2.5	17.5	------
05	Test Sample	0.005	-------	2.5	17.5	0.005

AMES Results:

Back ground Plate 19 / 96 Wells

Sr. No	Sample	Ta 98 no of positive wells / total number of well	Results	Ta 100 no of positive wells / no of total wells	Results
01	**SO E**	17 / 96	Non mutagenic	21 / 96	Non mutagenic
02	**SO nH**	20 / 96	Non mutagenic	16 / 96	Non mutagenic
03	**SO W**	17 / 96	Non mutagenic	16 / 96	Non mutagenic
04	**AL E**	19 / 96	Non mutagenic	18 / 96	Non mutagenic
05	**AL nH**	23 / 96	Non mutagenic	19 / 96	Non mutagenic
06	**AL W**	19 / 96	Non mutagenic	27 / 96	Non mutagenic

Cytotoxicity (%) was tested through AMES test by using TA 98 and TA 100 of the selected plant extracts, arranged by using altered solvents mentioned in methodology. Considerable variations in the number of positive wells were recorded among different solvent extracts. In the preceding chapter, the impact of various solvent systems on the number of Positive wells of various plants, employing stirring as an extraction process, was discussed. The maximum number of positive wells of *Albizia lebbeckagainst TA 98* was shown by n-hexane extracts (23/96) and minimum was observed by ethanolic extract (19/96). In contrast, the maximum number of positive wells of *Albizia lebbeck* against *TA 100* was shown by water extracts (27/96) and minimum was observed by ethanolic extract (18/96). In case of *Smilax ornata*,against *TA 98,* the maximum number of positive wells was shown by n-hexane extracts, 20/96, and minimum number of positive wells was shown by the ethanol extracts (16/96). AgainstTA100, the maximum number of positive wells was shown by ethanol extracts, 21/96,

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and minimum number of positive wells was shown by water extracts (16/96).

Antibacterial activity

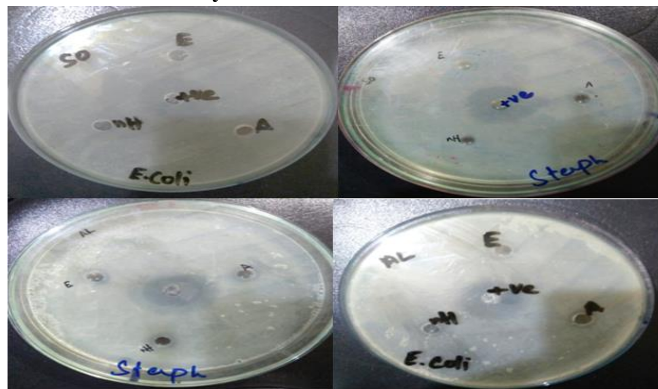

Figure 6. Different solvent extracts of plants with antibacterial property.

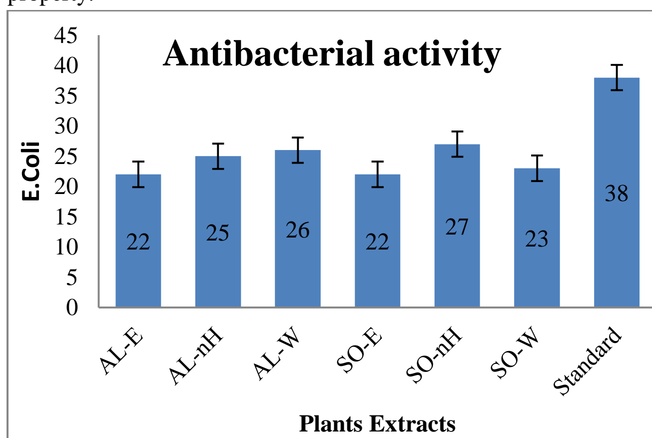

Figure 7. Graphical presentations of antibacterial activity (*E. coli*).

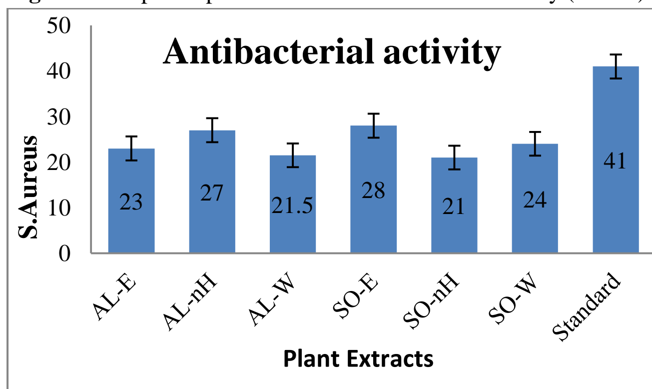

Figure 8. Antibacterial activity (*S. Aureus*).

Discussion

At first, we discuss the findings of phytochemical and antioxidant studies. Due to new incidences of drug resistance, resistance to several broad-spectrum antibiotics has become a global problem (Gootz, 2010). Because of these new cases, as well as rising consumer demand for natural antibacterial agents, there is a need to screen natural antimicrobial compounds that are effective against a variety of drug-resistant diseases. Several new natural antimicrobial substances have been found in recent decades for the treatment of serious illnesses. With this in mind, the current study was conducted to investigate the antibacterial properties of *Albizia lebbeck* seeds, which are medicinally essential. Different *Albizia lebbeck* soluble seed extracts demonstrated different inhibitory patterns against disease-causing bacteria. Hexane extract has been found to be the most effective antibacterial agent against the pathogens examined among the three extracts. In the case of *E. coli*, which can cause infections in immunocompromised people, a maximal zone of inhibition was observed (Ali et al., 2014). Humans are infected with E. coli, a hemorrhagic bacterium that causes severe diarrhea and renal failure. Methicillin-resistant Staphylococcus aureus is a disease-causing bacterium that is frequently resistant to lactam antibiotics (Ali et al., 2014). *Albizia lebbeck* extracts in ethanol and n-hexane effectively suppressed the growth of this multi-drug resistant bacterium. Similar to, extracts of *Smilax ornata* inhibited E. coli in a variety of ways. Among all of the extracts, the *Smilax ornata*n-hexane fraction has been found to be highly effective as an antibacterial agent against all of the pathogens examined(figure 6,7). The mutagenicity of both bacterial strains, S. typhimurium TA98 & TA100, was investigated. The blank plate was examined first, with the rest of the plates being read only until all of the wells in the blank plate had turned purple, indicating that the assay had not been contaminated. There was no change in color on the blank plate, suggesting no contamination. Table 1 shows the mutagenicity test results for TA98 and TA100. No samples were carcinogenic and were found all samples to be nonmutagenic.

Conclusion

In conclusion, this study outlines that plants can minimize side effects and ensuring the safety of patients. There is a requirement to harmonize traditional and modern health care systems to reduce the risk of both because it should be simple to integrate phytomedicine into the current health system. The knowledge of evaluating the in vitro antimicrobial, antioxidant and cytotoxic potential of *Albizia lebbeck* and *Smilax ornata* has been the main aim of the current study. Therefore, medicinal plants can act as microstatic or microcidal agents and show substantial antioxidant and cytotoxic potential due to the presence of a wide range of phytochemicals.

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