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Evaluation of Antioxidant and Antibacterial Activities of Leaf and Seeds Extracts of Neem (*Azadirachta indica*) Using Different Extraction

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Abstract

Neem (*Azadirachta indica*), has long been recognized for its medicinal properties. This study evaluates the antibacterial, antioxidant using the Ferric-Biperidine Reducing Capacity of Total Antioxidants (FBRC), Ferric-Reduced Antioxidant Method (FRAP), and scavenging activity by the 1, 1-diphenyl 2-picrylhyorazyl (DPPH) method. The total phenolic content of neem leaf and seed extracts was determined using Folin-Ciocalteu Aliquot method. Among the extracts analyzed, the aqueous soaked leaf extract exhibited the highest antioxidant activity, followed by the ethanolic-soaked leaf extracts, while the ethanolic Soxhlet seed extracts demonstrated the lowest activity. the total phenolic content was the highest in ethanolic Soxhlet seed extracts (6.319 mg/mg AAE) and aqueous soaked leaf extracts (4.838 mg/mg), with ethanolic soaked leaf extracts showing the lowest concentration (4.165 mg/mg). Antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* was observed exclusively in ethanol-soaked leaf extracts, with significant inhibition of S. aureus (18 mm). These findings highlight the potent antioxidant and antimicrobial properties of neem extracts, supporting their potential application in medicinal treatments.

Article Info

Keywords:

Azadirachta indica, Soxhlet ethanolic seeds extract , Maceration aqueous leaf extract, Maceration ethanolic leaf extract, Antioxidant and antibacterial activity

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1. INTRODUCTION

The flora in our vicinity not only aids in purifying our surroundings but also serves as an abundant reservoir of antioxidants and medicinal phytochemicals. Historically, India has relied heavily on plant-based remedies for disease prevention and treatment. Not only are allopathic medications costly, but they also have numerous adverse effects. It is undeniable that numerous beneficial phytochemicals with medicinal properties are derived from plants [1]. Medicinal plants have been shown to combat cancer through the appearance of free radicals, especially oxygen radicals. God provides the human body with natural defense systems, including enzymes that help eliminate free radicals and toxins. Among these, glutathione peroxidase (GPx) and glutathione-S-transferase (GST) depend on reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT), [2]. Antioxidants are an ideal means of protecting against free radicals. The use of plants as treatments for various diseases dates back to ancient times, and this tradition is prevalent on all continents [3]. It is well-established that antioxidants neutralize free radicals through oxidation. Free radicals are molecules that lack partners, leading to instability and high reactivity. As their numbers increase, the balance between antioxidants and free radicals is disrupted, potentially resulting in cellular oxidative stress. Prolonged imbalance may lead to the development of severe conditions, such as cardiovascular diseases, inflammation, diabetes, and cancer [4]. Phenolic and polyphenolic compounds are the primary natural antioxidants found in plants and food sources [3]. As is known in

human and animal medicine, antibiotics are used extensively, which has led to microbial resistance to these antibiotics [5]. The bacteria that are found in the lower intestinal tract of warm-blooded animals as well as humans are gram-negative bacteria, namely *Escherichia coli*, a rod-shaped organism from the Enterobacteriaceae family. These bacteria are found in environments containing sewage or feces [6]. This Gram-Negative bacteria's diameter ranges from 0.5 to 1.5 μm [7]. Gram-positive *Staphylococcus aureus* with approximately 0.5-1.5 μm in diameter is commonly found on the skin, nose, or mouth of human hosts without causing illness. Diseases caused by *Staphylococcus aureus* such as abscesses, pneumonia, meningitis, endocarditis, and septicemia, are caused by the penetration of these bacteria through wounds and damaged skin [8]. One of the well-known plants among the Yemeni population is the neem plant from the Meliaceae family, commonly found in Southeast Asian countries such as Laos, Myanmar, Cambodia, and Thailand [9]. Different parts of the neem tree (seeds, leaves, flowers, and bark) exhibit a wide-range of pharmacological activities and are used as raw materials for pesticides, medicines and other products. [10]. There is a wealth of research data available on the pharmacological and antiviral properties of neem, making it a valuable resource for the synthesis of medications to treat a variety of human diseases, it also has biological activity represented by antimicrobial (antibacterial and antifungal) antiviral, antiparasitic, antiseptic, antipyretic and anti-inflammatory [11]. Neem is recognized for its ability to generate potent antioxidant activity [4]. This study aimed to evaluate and compare the antioxidant activities of neem leaf extract prepared using ethanol, water, and neem seed extract (*Azadirachta indica*) in ethanol. These extracts have demonstrated effectiveness against certain pathogens, particularly those causing intestinal infections; such as *Staphylococcus aureus* and *Escherichia coli*.

Figure 1. Neem Leaves.

Figure 2. Neem seeds.

2. MATERIALS AND METHODS

2.1. Plant Materials

The leaves and seeds of Azadirachta indica (Figures 1 and 2) with herbarium code (743) were collected from the Al-Jrahi area near Alhudida, Yemen, in April 2022. Neem leaves and seeds were washed with fresh water and then dried.

2.2. Extraction of Plant Material for Chemical Studies

2.2.1. Ethanol extract from dry Neem plant

An electric blender was used to grind dried leaves into fine particles.Twenty grams of powdered leaves were immersed in 250 ml of 95% ethanol in separate conical flasks. The mixtures were then placed in a shaking water bath and left to stand for approximately 48 h to facilitate extraction at room temperature, after which the resulting solutions were filtered using muslin cloth. The filtrate was subsequently transferred to a beaker and placed in a water bath (60 ℃) to evaporate the solvent. The final leaf extracts were stored in a tightly sealed glass vials below 5 ℃.

2.2.2. Preparation of aqueous leaf extracts

Dried leaves were pulverized into fine particles using an electric blender. Subsequently, 20 g of the ground leaves was steeped in 250 ml of distilled water in conical flasks, then these mixtures were placed in a shaking water bath, heated at 60 ℃ for 15 min, and left for approximately

48 h to facilitate extraction, and the resulting solutions were filtered through a muslin cloth. The filtrate was then transferred to a beaker and placed in a water bath to evaporate the solvent at 60 ℃ to, yield the final leaf extract. The extracts were stored in tightly sealed glass vials below 5 ℃.

2.2.3. Preparation of Neem Seed Extracts

Extraction process with Soxhlet extraction using ethanol as the solvent. Fifty grams of the sample and 100 ml of the solvent were weighed, and the extraction process was carried out at 60 ℃, which is the temperature of the Soxhlet, for five hours. Subsequently, the mxture was cooled for 30 min. The filtered solution was then poured into a beaker and placed in a water bath (60 ℃) to remove the solvent, obtain the final extracts of the seeds, and then stored in a tightly sealed glass bottle at a temperature below 5 ℃, according to [12] with slight modifications.

2.3. Antioxidant Capacity Determination

2.3.1. Ferric-Bipyridine Reducing Capacity of Total Antioxidants (FBRC)

Mixing 1.0 mL of a 0.01 M $FeCl_3$ solution with 1.0 mL of bipyridine (0.1%) was followed by adding 2.0 mL of 0.3 M acetate buffer (pH 4). Subsequently, 50 µL of a 0.025 mg/mL extract solution was added to this mixture, which was then diluted to 10 mL using deionized water. All samples were then compared against a blank solution composed of 1.0 mL $FeCl_3$, 2.0 mL acetate buffer (pH 4), 1 mL bipyridine, and 6 mL deionized water, with measurements taken at 535 nm [13].

2.3.2. Ferric Reducing Antioxidant Power (FRAP)

The FRAP reagent was prepared by combining 300 mmol/L sodium acetate trihydrate in glacial acetic acid buffer (pH=3.6), 10 mmol/L (2,4,6-tripyridyl-s-triazine in 40 mmol/L HCl), and 20 mmol/L $FeCl_3$ at a ratio of 10:1:1 (v:v:v). Ascorbic acid was used as the standard. Following the reaction of 50 µL of 0.025 mg/mL extract solution with 2 mL of the FRAP reagent, the volume was adjusted to 10 mL. Absorbance was measured at 593 nm [3] after incubation at room temperature for 10 min and the experiment was repeated three times. The assay was performed at 37 ℃ (pH = 3.6).

2.3.3. Total Phenol Contents (TPCs)

The Folin-Ciocalteu Aliquot method was used. Total phenolics in the leaf and seed extracts were determined by preparing a mixture of 0.05 ml of leaf and seed extract (0.25 g/ml) with 2.0 ml of 20 g/L sodium carbonate solution, followed by the addition of 1 ml of Folin-Ciocalteu reagent (1:10) and distilled water, incubation for 30 min, and measurement of the absorbance at 750 nm [3].

2.3.4. Determination of scavenging activity by the 1, 1-diphenyl 2-picrylhyorazyl (DPPH) method

The radical scavenging activity of the extracts obtained using various extraction methods and standards, quercetin and rutin, was assessed by their ability to react with stable DPPH-free radicals [14]. This method relies on the reduction of the stable DPPH when it receives hydrogen from an antioxidant compound. The color change (from deep violet to light yellow) was quantified at 517 nm using a UV-visible light spectrophotometer. Two solutions were prepared: extracts were dissolved in absolute ethanol, and ascorbic acid was dissolved in deionized water. A concentration $3x10^{-4}$ M of DPPH was prepared by dissolving 0.0058 g in 50 mL of absolute ethanol. Each extract solution (50 μL) was transferred to an Eppendorf tube. The concentration was tested in five replicates by pipetting 1 mL of DPPH solution into the sample solution and filling it up to 2 mL with ethanol. The mixture was then shaken and incubated at room temperature for 30 min. For each experiment, a control solution was prepared by mixing 1 mL of ethanol and 1 mL of DPPH ($3x10^{-4}$ M). Percentage inhibition was calculated using the following equation:

$\%inhibition = [(A_0 - A_1)/A_0] \times 100$

2.4. Bacterial isolates and Inoculation

2.4.1. Isolation of bacteria

E. coli and *S. aureus* were obtained from the National Center for Public Health Laboratories, Sana'a, Yemen. The Cultures were grown on nutrient agar (NA) at 37 ℃ for 24 hours [15].

2.4.2. Activity of Neem plant extracts by the well diffusion method

The antimicrobial efficacy of the neem extracts was tested using the agar well diffusion method after incubating *E. coli* and *S. aureus* cultures in brain heart infusion (BHI) broth at 37 ℃ on a rotary shaker (150 rpm), and bacterial growth was monitored by changes in turbidity after 24 h. *E. coli* and *S. aureus* cultures (200 μl) were spread onto agar plates. Wells 6 mm in diameter were created on the agar surfaces using a cork borer. Neem leaf extract, maceration aqueous neem leaf extract, and Soxhlet ethanolic seed extract of neem plants were dissolved in DMSO and added to the respective wells, and the plates were then incubated at 37 ℃ for 24 h. The zone of inhibition was recorded after incubation. The experiment were conducted simultaneously on three culture plates [16].

3. RESULTS AND DISCUSSION

3.1. Antioxidant Activity by FBRC Method

This is a method for measuring antioxidant levels. The Neem plant exhibited varying antioxidant activity (mg/g AAE) for the different extracts. The quantification of antioxidant capacity is illustrated in Figure 3. In this study, it was stated that the maceration aqueous leaf extract had higher antioxidant activity (7.263±0.0040 mg extract/mg AAE), whereas the Soxhlet ethanolic seed extract had lower antioxidant activity (2.461±0.0060) mg extract/mg AAE, and the maceration ethanolic leaf extract at (5.263±0.0500 mg extract /mg AAE as shown in Table 1.

3.2. Antioxidant Activity by FRAP Methods

Reduction of ferric ions to ferrous ions by antioxidant compounds. Ferrous Reduction Capacity of Plasma (FRAP) values indicated higher antioxidant capacity. This is a method for measuring antioxidant levels. The Neem plant exhibited varying antioxidant activity (mg/g AAE) for the different extracts. The quantification of antioxidant capacity is illustrated in Figure 4. This study showed that maceration aqueous leaf extract had higher antioxidant activity (6.912 ±0.001 mg extract/mg AAE). In contrast, the Soxhlet ethanolic seed extract had a lower antioxidant activity at (5.723 ±0.020) mg extract/mg AAE and the maceration ethanolic leaf extract at 6.449 ±0.001) mg extract/mg AAE, as shown in Table 1. The maceration ethanolic and aqueous leaf extracts of *A. indica* exhibited significant antioxidant activity.

3.3. DPPH Scavenging Activity of Neem Extracts

This study presents a comparison of maceration ethanolic leaf extract (as illustrated in Figure 5), maceration aqueous leaf extract, and Soxhlet ethanolic seed extract, at concentrations of 80, 120, and 160 $\mu g/L$. It was found that the maceration aqueous leaf extract had a high free radical scavenging activity of IC_{50} 53.46 $\mu g/g$, the Soxhlet ethanolic seeds extract had a lower free radical scavenging activity with IC_{50} 98.6 µg/g, and the maceration ethanolic leaf extract was found to have an IC_{50} of 75.944 $\mu g/g$.

They showed potent scavenging activity against DPPH free radical, FBRC assay, and FRAP assay. The results showed that the highest antioxidant activities were obtained from the maceration aqueous leaf extract, followed by the maceration ethanolic leaf extract, and the lowest in the Soxhlet ethanolic seed extract of both suburban and rural areas. These differences in antioxidant activities are attributed to differences in the polarity of the solvents, and the method of extraction has been attributed to the presence of bioactive compounds.

3.4. olyphenolic Burden in Neem Leaves and Seeds Successive Extracts

Phenolic compounds represent a prominent class of secondary metabolites in plants [17–19]. The TPC was determined from the extracts of neem leaves and seeds, the total phenolic content (TPC) was determined as shown in Figure 6. The results in Table 1 shows varying levels of total phenolic content for different extracts, with the ethanolic leaf extract showing the lowest concentration (4.165 ±0.034 mg extract/mg AAE), whereas the Soxhlet ethanolic seed extract exhibited the highest concentration of (6.319 ±0.054 mg extract /mg AAE). The macerated aqueous leaf extract also showed a significant phenolic content of (4.838 ±0.015) mg/mg. The Soxhlet ethanolic seed extract and maceration aqueous leaf extract exhibited higher total phenol content; however, there was no significant difference.

The scavenging activity of the soxhlet ethanolic seed extract in this study was determined to have an IC50 value of 98.6 $\mu g/g$, demonstrating superior performance compared with the findings of a previous study. The seed extracts were analyzed using the DPPH method, and the IC_{50} value for the ethanol extract was 102 $\mu g/ml$. Additionally, the free radical scavenging activity of the maceration ethanolic leaf extract in this study was 75.944 $\mu g/g$, which also exhibited improved activity compared to the results reported in [20]. The enhanced activity of the leaf extract could be due to the preservation of heat-sensitive antioxidants during maceration and the presence of a high concentration of phenolic and flavonoid compounds in the leaves. In the present study, total phenolics were significantly found higher in Soxhlet ethanolic seeds extract as compared with maceration aqueous leaf maceration ethanolic leaf extract in both rural and suburban areas, which agrees with [21], that Phenolic compounds, flavonoids and tannins are present in the leaves of the Neem tree [22]. Qualitatively containing phenolic compounds. This variation in phenolic content greatly depends on the extraction processes, various abiotic and biotic stresses, maturity stages, processing of samples, spatial factors, etc., [23, 24]. The leaf ethanol extracts of *Azadirachta indica* showed pronounced antibacterial activity against S. *aureus* but the other extracts did not (Figure 7).

All extracts showed no inhibition zone against E. *coli*, indicating that Gram-positive bacteria are mostly susceptible, whereas Gram-negative bacteria are mostly resistant because of their cell walls [25]. The leaf ethanol extract of A. *indica* exhibited greater activity than the other extracts did. Thus, phytochemical screening of neem leaves with solvent extracts of different polarities revealed the presence of many phytochemical components.

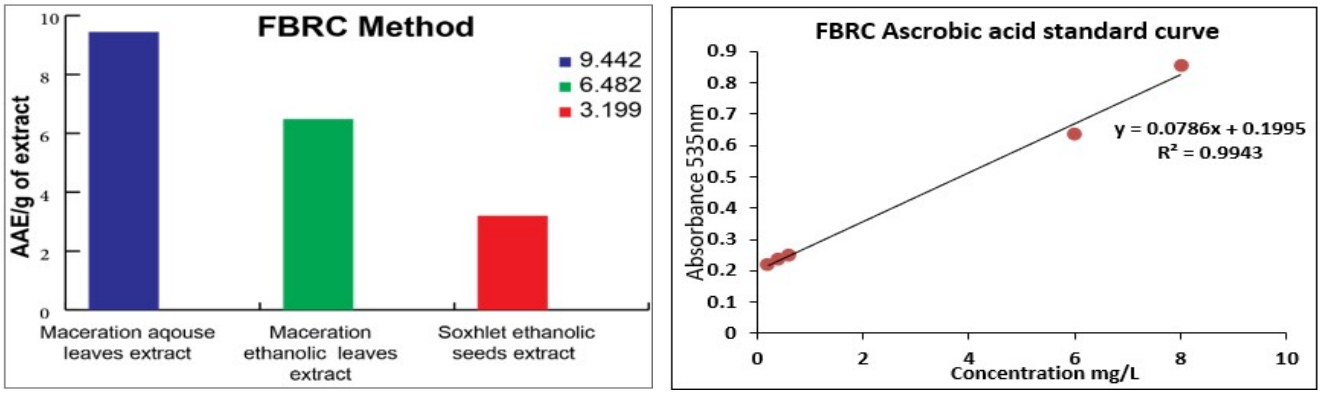

Figure 3. Antioxidant activity FBRC Method of different Neem extracts

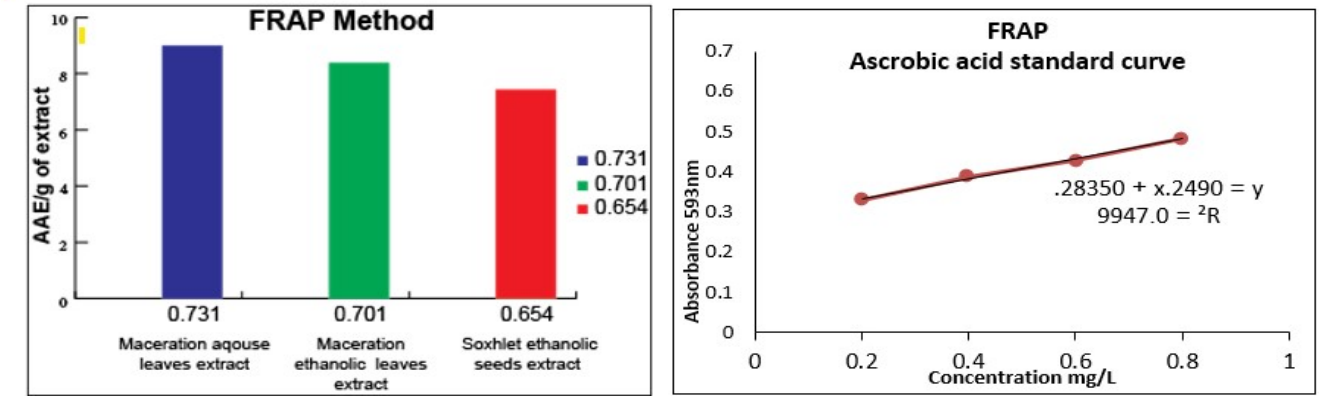

Figure 4. Antioxidant activity FRAP Method of different Neem extracts

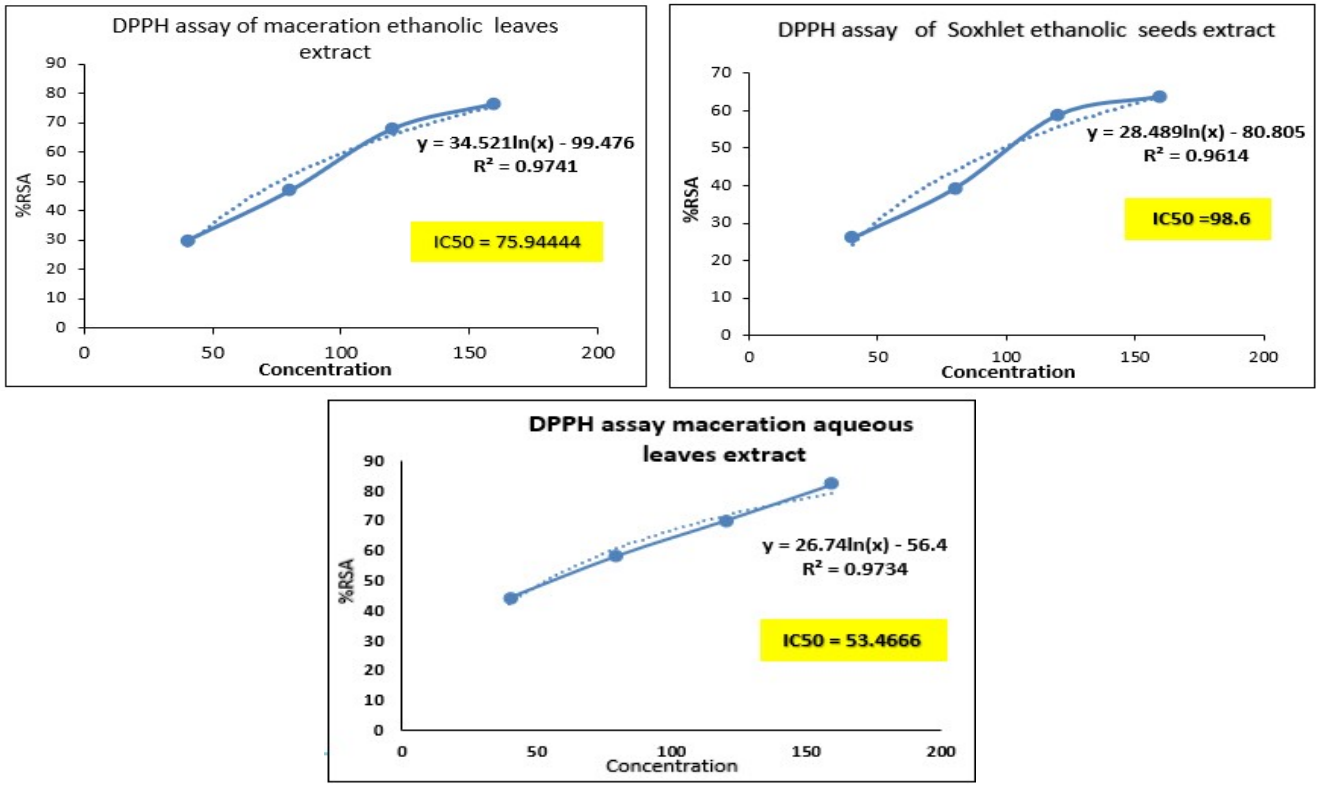

Figure 5. DPPH Scavenging Activity of Different Neem Extracts.

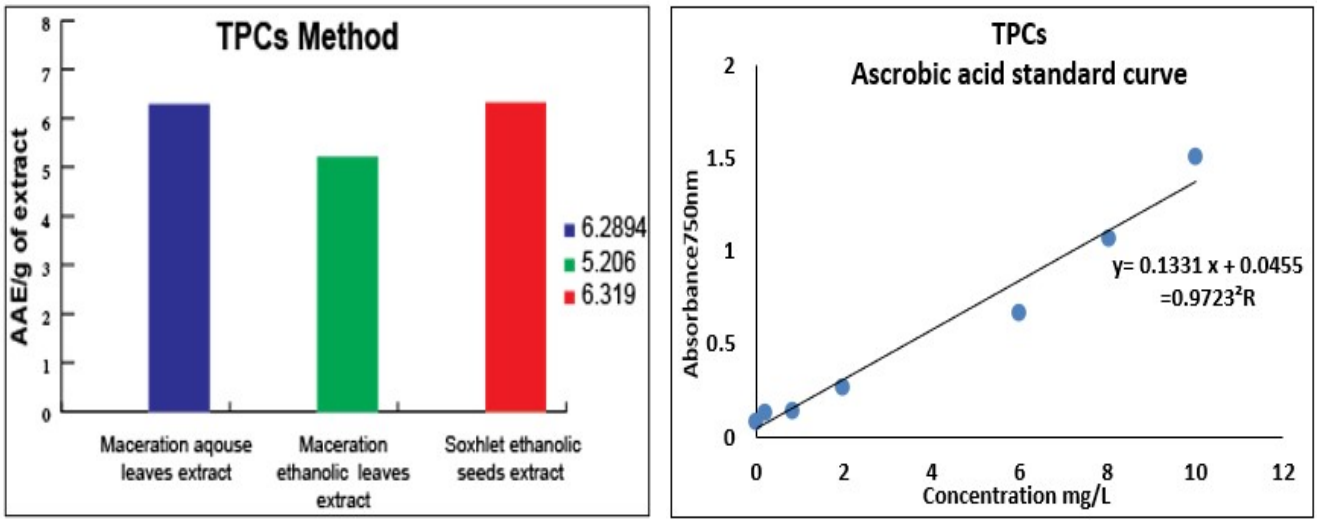

Figure 6. The total phenolic content of plant extract as ascorbic acid equivalent of different Neem extracts

Table 1. Antioxidant activity of different neem extracts

Type of extract	Total Phenols Contents (TPCs)	DPPH Scavenging Activity	FBRC	FRAP
	mg/mg AAE	μg/g	mg/g AAE	mg/g AAE
maceration ethanolic leaf extract	4.165 ±0.034	75.944±0.001	5.263±0.0500	6.912 ±0.001
Soxhlet ethanolic seeds	6.319±0.054	98.6±0.020	2.461±0.0060	5.723 ±0.020
maceration aqueous leaf extract	4.838±0.015	53.46 ±0.001	7.263±0.0040	6.449 ±0.001 Z

The results are means ± SD (n = 3)
AAE/ascorbic acid equivalent

Figure 7. The total phenolic content of plant extract as ascorbic acid equivalent of different Neem extracts

Neem leaves were more effective against S.*aureus* than the aqueous extracts. These results are the same as those obtained in a previous study by [26]. In all cases, the ethanol extract showed the best efficacy against S. *aureus*. According to a report by [27], whose results were similar to ours, the active components were slightly soluble in water and freely soluble in organic solvents such as alcohols, including azardirachtin, 1-maliantriol, salanin, nimbin, nimbdin, and others. Our results also confirm the information reported in [28], where ethanol extracts were more effective than aqueous extracts.

4. CONCLUSION

It can be concluded that the leaves and seeds of Azadirachta indica extract possess antioxidant and antibacterial properties represented by polyphenols at high concentrations. All experimental results show that all leaf extracts have the ability to remove free radicals associated with oxidative stress and exploit them in treatment

against a range of diseases and thus benefit from them in food and medicine by combining them.

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