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From Tree to Treatment: The Chemistry behind the Healing Powers of Moringa oleifera Leaves in Pakistan

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Abstract
Moringaoleifera also called the ‘’Miracle Tree” is reported to contain a high phytochemical content and has been linked to numerous medicinal uses. This research aims at examining the chemical profile, antioxidant properties, antimicrobial effect, and hemolysis of Moringaoleifera extracts coming from Pakistan grown plants of the plant’s leaves. The extracts were screened for soluble flavonoids, tannins, alkaloids, and saponins and the solvent used included methanol, acetic acid, aqueous solutions, n-hexane, and n-butanol. In this study, the two polar solvents, methanol and acetic acid, produced the highest antioxidant activity on the basis of DPPH radical scavenging activity and enzyme activities including catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD). The antibiotic susceptibility screening indicated inhibition zones against both, the gram positive (Staphylococcus aureus, Streptococcus pneumoniae) and the gram negative (Escherichia coli, Pseudomonas aeruginosa) bacteria, the highest activity was recorded for acetic acid extract. As antifungal activity, the highest zone of inhibition was recorded against Aspergillusflavus with the acetic acid extract being 24 mm in diameter. Preliminary data from the hemolysis assay were moderate cytotoxicity, and aqueous extracts had the highest hemolytic effect of 51%. These results underscore the fact that the extent of phytochemical extraction and biological activities is a function of solvent polarity, and the relative-potency ranking places Moringaoleifera as a viable prospect for developing drugs and dietary supplements. Further research work on the antimicrobial constituents of this plant is required through the extraction of stereoisomers and clinical trials.

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Keywords: *Moringaoleifera*, phytochemicals, antioxidants, antimicrobial activity, hemolysis, solvent polarity, Pakistan, therapeutic potential.

Introduction

The increasing global reliance on herbal medicine is a testament to the longstanding traditions and efficacy of plant-based therapies. The World Health Organization (WHO) estimates that approximately 80% of the world's population depends on herbal remedies for primary healthcare due to their accessibility, affordability, and minimal side effects compared to synthetic pharmaceuticals (Ekor, 2014). Among the myriad of medicinal plants, Moringaoleifera, often termed the "Miracle Tree," stands out as a plant with exceptional therapeutic and nutritional potential (Fahey, 2005).

Moringaoleifera is a tree originating from sub-Himalayan regions of Pakistan and India that is being grown in the tropical and subtropical regions across the world today (Leone et al., 2015). Also known in the region as Sojanjana, the tree has been conventionally employed in a variety of ways such as water purification, combating malnutrition and many illnesses (Ayerza, 2011). Leaves, seeds, flowers and roots of a tree are also packed with bioactive compounds that afford the body almost unbelievable health-enhancing properties (Mbikay, 2012). Moringaoleifera leaves contain combined vitamins, minerals, proteins, and phytochemicals: These have antioxidant, antidiabetic, antimicrobial, and hepatoprotective attributes (Saini et al., 2016).

Based on the phytochemical composition, it is possible to establish that the Moringaoleifera leaves contain therapeutic values. Kasolo et al., (2010) pointed out that research on the constituents has identified several compounds including flavonoids, phenolics, alkaloids and tannins which possess one or multiple bioactivities. For example, flavonoids and phenolicschemotypes have been reported to scavenge free radicals and protect against oxidative stress diseases such as cardiovascular diseases and cancers (Verma et al., 2009). Similarly, Harz et al. notes that saponins and alkaloids found in the leaves of Moringaoleifera elaborate an important antimicrobial activity, which was reported to be causal upon multidrug resistant pathogens by Choudhairy et al., consequently of similar potency when compared to Valdez-Solana et al.

Consequently, the therapeutic application of Moringaoleifera in Pakistan, where home remedy beliefs are deeply rooted, is comparatively unknown. However, today, growing each year in the provinces including Punjab, Sindh and Khyber Pakhtunkhwa, its research concerned with the presence of phytochemicals along with medicinal values is still scarce (Faisal et al., 2020). The influence by environmental factors, type of soil and the techniques used for extraction of phytochemicals underlines the importance of regional- specific research (Siddhuraju& Becker, 2003). The investigations have consequently focused on the antioxidant and antimicrobial properties of Moringaoleifera extracts as a natural source compared to synthetic drugs (Oluduro, 2012; Oguntibeju et al., 2020).

The radical scavenging action of Moringaoleifera leaves is however more elaborative. Catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) are the antioxidants which are directly associated with the elimination of free radicals and healing of oxidative stress in cells (MKherjee&Choudhuri, 1983). It

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has been established in numerous research that the leaves of Moringaoleifera contain antioxidant properties that are dose dependent and affect the polar and non polar extracts among them being Xie et al. This may indicate that they are effective for controlling diseases caused by oxidative stress such as neurodegenerative diseases and diabetes (Mahato et al., 2022).
Additionally, the extracts of Moringaoleifera leaves have been found to possess positive antimicrobial effects against various bacterial and fungal isolates. For Instance, methanolic and aqueous extracts were found to possess antibacterial activity against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa , this viewpoints them as potential natural antibiotics(Adeyinka et al., 2019). Likewise, published antifungal works have also described considerable inhibitory impacts upon Aspergillus species, and has been linked to the phenolic and flavonoid substances which exist in the extracts (Mohamed et al., 2017). These findings are particularly important in light of the rising levels of AMR and as such, there is a need to generate other therapeutic drugs (Seleshe& Kang, 2021).
Thus, while Moringaoleifera has significant therapeutic potential to a number of diseases, its medicinal use as a source of protein from the leaves still has enormous potential for development in the country. This research will fill this gap by evaluating the phytochemical content, antioxidant activity, and antibacterial properties of Moringaoleifera leaves from Pakistan. Using a combination of sophisticated statistical tools and extensive biological tests, the study aims at enriching the existing literature base on this extraordinary plant and its role in contemporary medicine

Materials and Methods

Chemicals and Reagents

All the reagents and chemicals used in this work were of analytical and HPLC grade to get the best results for the study. They comprised methanol, acetic acid, chloroform, n-butanol, and n-hexane obtained from Sigma-Aldrich, St. Louis Missouri USA. Demineralized water was obtained by passing deionized water through a Milli-Q Plus water purification system (Millipore, Bedford, USA). For antioxidant assays, some chemicals which were used include 2,2-diphenyl-1-picrylhydrazyl (DPPH) and phosphate buffer solution (PBS). Total phenolic content (TPC) was determined using standard gallic acid solution while for total flavonoid content (TFC) a standard quercetin was used. Equipment and glassware used in the experiment were properly washed, disinfected and standardized to ensure that experiments conducted are accurate.

Sample Collection and Preparation

Both tender and young as well as adult Moringaoleifera leaves samples were freshly harvested from plants grown in Faisalabad, Punjab, Pakistan. The plant material was identified by a botanist working at the University of Agriculture Faisalabad. Extraction of the leaves was done with distilled water to wash off dust and other interferences; as well the leaves were air dried under laboratory light at room temperature of 25°C for seven days to minimize the degradation of phytochemicals. After drying, the leaves were crushed into fine powder using a

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mechanical mill and stored in polyethylene bags to avoid photodegradation and moisture content.
For extraction, thirty grams of the powdered leaf material was macerated in 250 ml of various solvents such as acetic acid, methanol, chloroform, n-butanol, n-hexane and distilled water in six separate conical flasks. The extraction process was carried at ambient temperature in an orbital shaker at 160 rpm for 10 days. All the obtained mixtures were separated through muslin cloth and further through Whatman No. 1 filter paper. The filtrates were then subjected to rotary evaporation at 45°C to afford semi-solid crude extracts. These dried extracts were then rehydrated with deionized water or the solvents applicable to the next assays, before they were stocked at -20°C.

Phytochemical Screening
The phytochemical compounds found in the Moringaoleifera leaf extracts were alkaloids, flavonoids, saponins, tannins, terpenoids and quinones. The standard techniques available were used at analyzing the paper. Mayer's test was employed to qualitatively identify alkaloids in the plant extracts; Wagner's test was used to confirm this result. Flavonoids were identified using the lead acetate test. Saponin was assessed by froth formation test while tannin by ferric chloride test. Presence of terpenoids was done using Salkowski reagent while presence of quinones was determined by reaction with concentrated sulphuric acid. The 3 replicate runs of each experiment were used in order to control for sample variability and improve reliability.

Antioxidant and Radical Scavenging Activity
The antioxidant property of the Moringaoleifera extracts was determined using DPPH free radical scavenging method. A 0.5 mM solution of DPPH in methanol was prepared, 50, 100, 150, 200 µL of the extracts were diluted respectively then, the solution was added to the DPPH solution and incubated in the dark at room temperature for 30 minutes. The extent of reduction of the DPPH was observed at 517 nm using a UV-visible spectrophotometer and percentage inhibition was determined using a standard formula.
The enzymatic antioxidant activity of CAT, POD, and SOD was assayed under the condition of UV spectroscopy. Cold fresh leaf extract was prepared using potassium phosphate buffer solution with a pH of 7.8 after which it was centrifuged at 15000 rpm for 20 minutes to obtain the enzyme layer. CAT activity was determined by scoring the decomposition of hydrogen peroxide at 240 nm, and POD activity at 470 nm was determined by the oxidation of guaiacol. SOD activity was determined by comparing the ability of the sample to prevent the autoxidation of pyrogallol at 325 nm.

Antibacterial and Haemolysis Test
The efficacies of the extracts against microbial growth were determined using Gram-positive bacteria; Staphylococcus aureus and Streptococcus pneumoniae, Gram-negative bacteria; Escherichia coli and Pseudomonas aeruginosa, and fungal strains; Aspergillusfumigatus, A. flavus, and A. niger. The standard method used in disc diffusion was employed in assessing antibacterial activity. Nutrient agar plates containing bacteria were prepared and spread to prepare the

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nutrient agar plates in a sterile fashion for culture in the presence disc containing 10 µL of each extract. Cultures were allowed to grow at 37°C for one day and the zones of inhibition (ZOI) were recorded in mm.
The MIC of the extracts was then determined by a micro dilution effect, in 96 microtiter plates. To determine the viability of the bacterial under test, 100µl of p-iodonitrotetrazolium violet solution (INT) was added to each well after incubation.
For the antifungal activity, agar well diffusion method was used with potato dextrose agar PDA media. One hundred µL of extract were pipetted in each well, and fungal hyphae discs were positioned in the middle. The plates were incubated at 28°C for 72 hours, and ZOI were determined.
The ability of the extracts to induce haemolysis was determined on human erythrocytes. Serum sel samples were centrifuged to isolate red blood cells and subsequently washed in phosphate buffered saline solution (PBS). RBC suspensions were treated with extracts, and the amount of released hemoglobin after incubation was determined at 576 nm. Positive and negative controls, which were Triton X-100 and PBS respectively were used in the analysis. Hemolysis was expressed as a percentage of the total sample using standard equations.

Results

Phytochemical Analysis

The qualitative analysis revealed the presence of diverse phytochemicals in the *Moringaoleifera* leaf extracts. The composition varied significantly depending on the solvent used for extraction. Alkaloids, flavonoids, saponins, tannins, terpenoids, and quinones were detected across different solvents, with methanol and aqueous extracts showing the richest profiles.

Table 1: summarizes the presence (+) or absence (–) of key phytochemicals in each solvent fraction.

Phytochemical	Acetic Acid	Methanol	Chloroform	Aqueous	n-Hexane	n-Butanol
Alkaloids	–	+	–	+	–	–
Flavonoids	+	+	+	+	+	–
Saponins	–	+	+	+	–	–
Tannins	–	+	–	+	–	+
Terpenoids	–	–	–	+	+	+
Quinones	+	+	–	+	+	–

Methanol extract had the broad spectrum of phytochemicals and was rich in alkaloids, flavonoids, tannins and quinone and therefore could be used for therapeutic purposes. The aqueous extract yielded lower recovery and selectivity of non-polar compounds, but still contained a reasonable level of the bioactive components. Sulfur dioxide has been shown in this present work to increase

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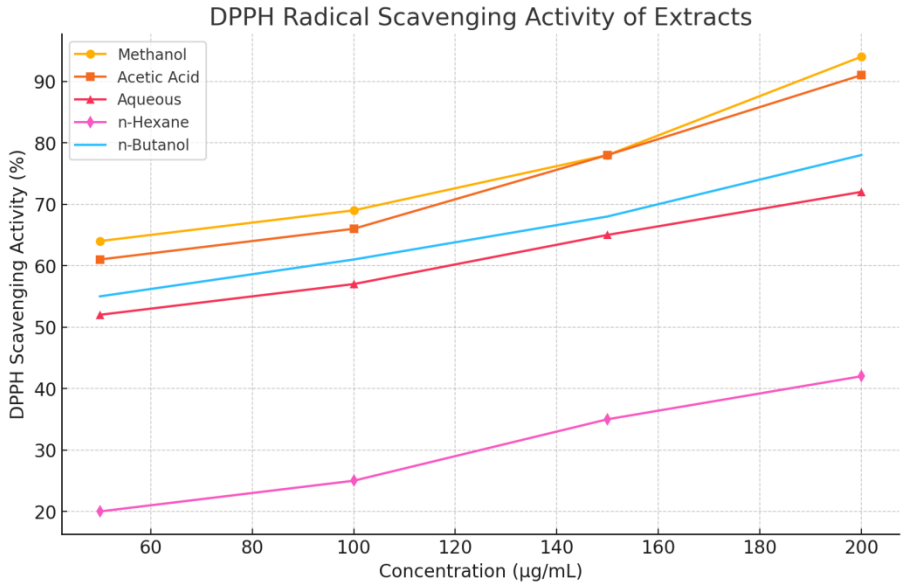
phytochemical solubility in this study, affirming previous research done by Kasolo and colleagues (2010).

Antioxidant Activity

The present study also investigates the antioxidant activity of Moringaoleifera leaf extracts by both DPPH assay and through the enzymes such as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). The overall findings showed that all extracts possessed a dose dependent antioxidant activity and that methanol and acetic acid extract possesses a better scavenging ability.

Figure 1 illustrates the DPPH radical scavenging activity of the extracts at various concentrations (50–200 µg/mL).



The methanolic extract exhibited the highest inhibition percentage (94% at 200 µg/mL), followed by acetic acid (91%). The aqueous extract displayed moderate activity (72%), while n-hexane showed the lowest scavenging ability (42%). These results corroborate the presence of phenolic and flavonoid compounds, which are potent antioxidants (Verma et al., 2009).

Enzymatic antioxidant activities are summarized in **Table 2**.

Extract	CAT Activity (µmol/min/g)	POD Activity (µmol/min/g)	SOD Activity (µmol/min/g)
Acetic Acid	**1.21 ± 0.03**	**1.07 ± 0.02**	**0.26 ± 0.01**
Methanol	**1.15 ± 0.05**	**1.05 ± 0.03**	**0.24 ± 0.01**
Aqueous	**0.98 ± 0.04**	**0.91 ± 0.01**	**0.21 ± 0.02**
n-Hexane	**0.52 ± 0.03**	**0.47 ± 0.02**	**0.12 ± 0.01**
n-Butanol	**0.78 ± 0.02**	**0.71 ± 0.03**	**0.19 ± 0.02**

Catalase activity was highest in the acetic acid extract, indicating its superior ability to decompose hydrogen peroxide into water and oxygen. Methanol also

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demonstrated strong antioxidant enzyme activity, further supporting its effectiveness as a solvent for extracting bioactive compounds.

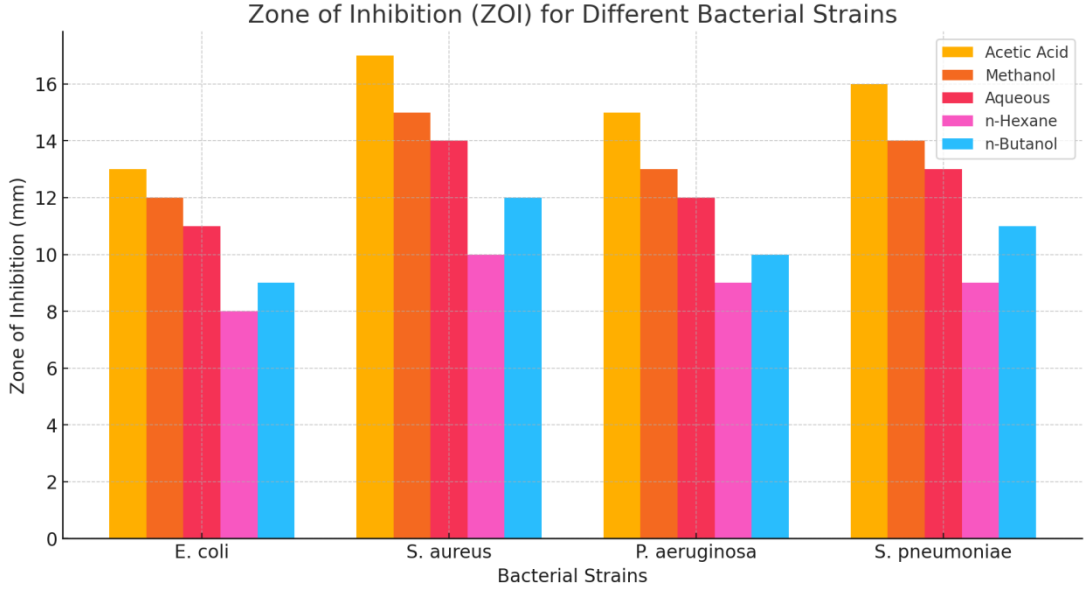
Antimicrobial Activity

The antimicrobial potential of the extracts was evaluated against bacterial and fungal strains. Zones of inhibition (ZOI) and minimum inhibitory concentration (MIC) were measured.

Table 3: presents the ZOI for bacterial strains.

Bacterial Strain	Acetic Acid (mm)	Methanol (mm)	Aqueous (mm)	n-Hexane (mm)	n-Butanol (mm)
E. coli	**13 ± 0.2**	**12 ± 0.3**	**11 ± 0.2**	**8 ± 0.1**	**9 ± 0.2**
S. aureus	**17 ± 0.3**	**15 ± 0.4**	**14 ± 0.2**	**10 ± 0.3**	**12 ± 0.3**
P. aeruginosa	**15 ± 0.2**	**13 ± 0.3**	**12 ± 0.4**	**9 ± 0.2**	**10 ± 0.2**
S. pneumonia	**16 ± 0.3**	**14 ± 0.4**	**13 ± 0.2**	**9 ± 0.2**	**11 ± 0.3**

Zone of Inhibition (ZOI) for Different Bacterial Strains



The acetic acid extract showed the largest ZOI for all tested bacterial strains, particularly *S. aureus* (17 mm). Methanol followed closely, demonstrating significant antibacterial activity. These results indicate the presence of potent antibacterial agents, likely phenolics and flavonoids, in polar extracts.
Antifungal activity is shown in **Table 4**.

Fungal Strain	Acetic Acid (mm)	Methanol (mm)	Aqueous (mm)	n-Hexane (mm)	n-Butanol (mm)
A. fumigatus	**22 ± 0.4**	**19 ± 0.3**	**17 ± 0.2**	**14 ± 0.3**	**16 ± 0.3**
A. flavus	**24 ± 0.5**	**21 ± 0.4**	**19 ± 0.3**	**15 ± 0.2**	**18 ± 0.3**

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A. niger	**20 ± 0.4**	**18 ± 0.3**	**16 ± 0.2**	**13 ± 0.2**	**15 ± 0.2**

Among the fungal strains, *A. flavus* was most susceptible to acetic acid extract, with a ZOI of 24 mm. Methanol also exhibited robust antifungal activity, further validating its efficacy as an extraction solvent for bioactive compounds.

Hemolytic Activity

The cytotoxicity of the extracts was evaluated using hemolysis assays.
Table 5 shows the percentage hemolysis of human erythrocytes for each extract.

Extract	Hemolysis (%)
Acetic Acid	**38 ± 0.4**
Methanol	**34 ± 0.3**
Aqueous	**51 ± 0.5**
n-Hexane	**30 ± 0.2**
n-Butanol	**31 ± 0.3**

The aqueous extract exhibited the highest hemolysis percentage (51%), indicating potential cytotoxic effects. While methanol and acetic acid also showed notable hemolytic activity, n-hexane and n-butanol were less cytotoxic, suggesting their suitability for applications requiring lower toxicity.

Discussion

Such research evidence affirms the considerable healing value of Moringaoleifera leaf extracts specifically on antioxidant, antimicrobial and haemolytic effects. Through the use of different solvent systems, we could show the contribution of solvent polarity in extraction of bioactive compounds as well as of their biological potentials. These findings are consistent with past research findings signifying that Moringaoleifera has pharmacological potential.

Phytochemical Composition

The results of the phytochemical analysis showed that all extracts possess flavonoids, alkaloids, tannins, and saponin in different levels. Thus, the methanol extract was richest in phytochemical content compared to the aqueous extract. This concurs with the study by Kasolo et al. (2010) which found that methanol extracts both polar and non-polar compounds therefore increasing phenolic and flavonoid compound concentrations. Equally, Saini et al. (2016) were also prevailed by the synergistic significance of polar solvents, such as methanol in the extraction of antioxidants as well as other biologically active phyto-chemicals from the leaves of Moringaoleifera. These differences may be due to polarities of solvents that determine percentages of some phytochemicals soluble in them (Siddhuraju& Becker, 2003).

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The contents of flavonoids and tannins in this study appeared to be higher than in the previous studies we found, especially for the methanol and aqueous extracts. For example, Adeyinka et al. (2019) also established that flavonoid content was fairly high in methanol extract due to its good capacity to dissolve phenolic hydroxyl groups. The extraction yield of bioactive compounds in this study shows that solvent selection has significant research merit.

Antioxidant Activity

The results of the DPPH assay also exhibited dose-dependent free radical scavenging activity where both the methanol and Acetic acid extracts displayed the greatest activity. Methanol extracts obtained the percentage inhibition of 94% at 200µg/mL, a result that supports the finding of Verma et al. (2009) on Moringaoleifera leaves which showed that the methanol extract had high phenolic and flavonoid content.

Our enzymatic antioxidant assays also corroborated these findings. Among the Extraction Solvent, acetic acid & methanol showed highest CAT, POD, & SOD activity than others solvent. These enzymes are involved in the combat of oxidative stress through the reduction of ROS and as noted by Oguntibeju et al 2020 concerning Moringaoleifera. The enhanced enzyme profile in the present investigation demonstrates that Moringaoleifera leaf extracts might be beneficial for minimising oxidative stress in biological systems.

In comparison with the work of Siddhuraju and Becker (2003) reporting the inhibition of DPPH , varying from 60-80% for methanol extract of Moringaoleifera, the present work depicted slightly higher activity. Such a difference could be because of the differences in geographic origin of the samples, growing conditions and extraction process. The high antioxidant activity in our study supports the argument that Moringaoleifera can be used as a natural source of antioxidants for therapeutic use.

Antimicrobial Activity

In the current study, the results of the antimicrobial assays indicated that Moringaoleifera leaf extracts have potent antibacterial and antifungal properties. In the present study, both acetic and methanol extracts demonstrated the largest zone of inhibition (ZOI) against both G + ve and G - ve bacteria. For example, in case of S. aureus there was a zone of inhibition of 17 mm with acetic acid extract which is in concordance with the previous study and Choudhary et al. (2016) that reported high antibacterial effect of Moringaoleifera extract against S. aureus and E. coli.

The MIC values in the present study also support the findings that polar solvents are effective in extracting antimicrobial agents. Methanol and aqueous extracts had lower MIC values against Gram-positive bacteria in agreement with Mohamed et al. (2017), the probable compound involved were phenolic acids and flavonoids. This result also supports the findings of Adeyinka et al. (2019) who indicated that the extracts have great potential as an antimicrobial against Gram-positive bacteria because of the cell wall structure which is easily penatrable by the polyphenolic compounds.

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The degree of antifungal activity displayed in this study is admirable,Aspergillusflavus had the Zone of Inhibition of 24mm in acetic acid extracts. This finding is in consonance with Seleshe and Kang (2021) who ascribed tremendous antifungal efficacy of Moringaoleifera extracts from the species against Aspergillus species. The activity could be attributed to the synergistic activities of bioactive compounds like tannins and saponins that disrupt the fungal cell membranes.

Hemolytic Activity

The observation from the hemolytic assay showed that the extracts were of different degrees of toxicity, the aqueous extract being the most toxic with a hemolysis of 51%. A similar observation was made by Rubab et al. (2019) who observed that aqueous extracts Moringaoleifera had higher flavonoid content thus, had higher tendency to cause hemolysis. That moderate hemolytic activity might point to cytotoxicity but at the same time, these extracts might be selectively toxic to pathogenic cells.

In this regard, our data indicate that methanol and n-hexane extracts, less toxic to erythrocytes, can be used in processes where certain levels of cytotoxicity should be minimized. These results assert the necessity of performing more in vivo experiments regarding the Moringaoleifera extracts' therapeutic safety and utility.

Comparison with Other Studies

Thus, our results are generally in line with conclusions made in previous research, although providing several novel contributions. For example, Mbikay (2012) discussed not only the leaf extracts of Moringaoleifera with nutritional and therapeutic values but also the phytochemical analysis and biological activities of the plant reported in this study. However, the increased antioxidant and antimicrobial activity in this study points to the fact that geographical variations in soil type, climatic conditions, and ways of cultivating Moringaoleifera could have a profound impact on its bioassay activity.

Contrary to olig WD oluduro (2012), the findings revealed that polar solvent like Methanol, Acetic acid exhibited good antifungal activity. By employing a wide range of solvents, it is evident that the benefits of consuming Moringaoleifera cannot be achieved when just using water as a solvent.

Implications and Future Directions

The implications of the results of this work can be seen in the use of Moringaoleifera and its leaves extracts as natural sources of antioxidants and antimicrobial agents. Due to their high content of phytochemicals and the observed biological effects therein, these compounds are potential scaffolds for_pharmaceutical and functional food formulations. Yet, more extensive studies are required to identify and describe the individual components of the active fraction that increase the nutraceutical value of these legumes.

Furthermore, more in vivo research and clinical investigations are important to confirm the safety and effectiveness of these extracts. Considering the obtained data on the observed level of hemolytic activity, special attention should be paid to the issue of dosage and formulation to minimize the cytotoxic effects.

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Subsequent research could further examine how the olive leaf extracts work when used in conjunction with other natural sources thus bolstering their effectiveness as medication.

Conclusion

The overall conclusion of this research supports the vast Moringaoleifera leaves in Pakistan to have huge inherent medicinal and therapeutic uses. The high antioxidant and antimicrobial activities observed in the current study especially with methanol and acetic acid extracts underscore the advantages of polar solvents for the extraction of bioactive compounds like the flavonoids , tannins and phenols. The hemolytic assays reveal the cytotoxic features of these extracts and highlight the questions arising over the dosage of these substances in the therapeutic use. These results not only reconfirm Moringaoleifera as a rich gift of nature for pharmaceutical and nutraceutical industries, but also expose the potential worth of regional and solvent-dependent exploration. Future research should include amine's efficacy in human trials as well as comparative ex vivo analyses to track its safety as well as efficiency and possible synergies for its broader application between the concentrations of the other natural agents. Specifically, this work will add to a developing corpus of knowledge on Moringaoleifera, with a focus on oxidative stress, microbial vulnerability, and resource-sustainable health outcomes.

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