Extraction And Pharmacological Profile Screening Of Neem Seed Oils

Bilawal Manzoor[1], Muhammad Suleman[1], Freeha Hafeez[1*], Mehmood Hassan[1], Muhammad Usman[1], Ali Raza[1], Muhammad Bilal Shafique[1], Asma Zafar[1], Hajrah Ali[1]

[1]Department of Chemistry, Faculty of Engineering and Applied Sciences, Riphah International University Faisalabad, Faisalabad, Pakistan

[2]Department of Chemistry, Government College University Faisalabad

Abstract:

Objective: The main objective of this investigation is to carry out an in-depth examination of both qualitative and quantitative phytochemical compounds, while concurrently evaluating the antioxidant properties of methanol extracts obtained from *Azadirachta indica* seeds.

Methods: Undertake a comprehensive evaluation of the antioxidant potential of botanical extracts through a range of techniques, including analysis of total phenolic and flavonoid content, as well as DPPH, ABTS, and FRAP assays. Highlight the significance of initial phytochemical analysis and *in vitro* antioxidant tests.

Results: The findings demonstrate that the seeds represent a remarkable reservoir of antioxidant potential, indicating their capacity as a potential source for phenolic chemicals and antioxidants.

Conclusions: Based on the outcomes of this investigation, it can be deduced that the species displays effectiveness in counteracting free radicals and possesses the ability to serve as a potent antioxidant.

Introduction:

Plants have been used as medicines for a long time. Many parts of plants were used in the past to cure illnesses. Plants have provided important drugs for human health. Some drugs still come from trees. Medicines started as crude drugs and then became more refined. The discovery of drugs from plants continues today. Many people still use traditional remedies for medicine (Balunas & Kinghorn, 2005). The World Health Organization (WHO), an international agency dedicated to improving global health, has provided an estimate that a significant proportion, up to 80%, of individuals across the globe continue to heavily depend on conventional and time-honored treatments, primarily encompassing the utilization of botanical substances, such as herbs, in order to address their healthcare needs and manage their ailments. The plant species known as Neem can be observed in the regions of Sindh and specific areas in southern Punjab within the nation of Pakistan. *Azadirachta indica*, commonly referred to as "Semi" in Pakistan, has

successfully adapted in the majority of tropical and subtropical countries. Furthermore, it possesses significant medicinal properties and is widely distributed throughout the world. This specific plant stands out due to its versatility in traditional medicine, as its various components such as leaves, bark, flower, seed, and root have been extensively utilized in Chinese , Iranian, and Indian medicinal practices (Sani & Baburo, 2020).

Experimental Work:

Collection of bioactive parts from plant:

An extensive investigation was conducted at the Faisalabad Campus of Riphah International University, focusing on Azadirachtin indica, commonly known as neem trees, within the specialized field of Medicinal and Aromatic Plants. The study involved a meticulous collection of various plant components, including fresh green and yellow fruits, as well as leaves, carried out in the month of June. To perform our chemical analysis, we obtained all necessary substances and solvents exclusively from Naeem Trades, a reputable Petro Chemical Company located in Faisalabad.

Methanol Extract Preparation:

A portion of dry seed powder weighing 10 grams was immersed in a solution consisting of 80% methanol in water (volume/volume) with a total volume of 100 mL. The resulting mixture was subsequently placed in an orbital shaking incubator and incubated at a temperature of 25ºC for duration of 72 hours. Throughout the entire incubation period, the mixture was subjected to agitation at a speed of 100 revolutions per minute. Following the completion of the incubation, the mixture underwent a filtration process employing Whatman No. 1 filter paper. The resulting filtrates were then subjected to centrifugation at a force of 3354 times the acceleration due to gravity for a duration of 10 minutes. The supernatants, which constituted the liquid portion located above the solid pellet, were collected in a conical flask. The remaining solid pellets were subjected to a second extraction process using the identical procedure as previously described. This extraction process was repeated twice, with each subsequent extraction employing 50 mL and 25 mL of methanol, respectively. The supernatants obtained from each extraction were combined.

Evaluation the Antioxidant Capacity of Plant Extract:

Determination of TPC and TFC:

A reaction mixture was created by combining distilled water, methanol extract,

and Folin-Ciocalteu phenol reagent. After a 6-minute incubation period, sodium carbonate and distilled water were introduced. Absorbance readings were then recorded at 760 nm using a spectrophotometer. A series of gallic acid concentrations were employed to construct a standard curve. The outcomes were articulated in milligrams of gallic acid equivalent per gram of dry weight (Wolfe, Wu, & Liu, 2003). For the quantification of total flavonoids, the methanol extract was blended with an aluminum chloride solution and allowed to stand for 60 minutes. The resulting color's absorbance was measured at 420 nm using a spectrophotometer. Various concentrations of quercetin were utilized to establish a standard reference curve. The total flavonoid content in the methanol extracts was expressed in milligrams of quercetin equivalent per gram of the sample. This method enables precise quantification of the flavonoid content in a given sample (Ordonez, Gomez, & Vattuone, 2006).

In-vitro Antioxidant Assays:

The assessment of the antioxidant activities of the methanol extracts from A. indica seeds was conducted using different models. The models included the DPPH, ABTS, and FRAP assays to measure the antioxidant potential of the extracts.

The experimental procedure involved using the DPPH radical assay. The methanol extract was mixed with a solution of 0.135 mM DPPH and incubated for 30 minutes. The absorbance of the solution was measured at 517 nm using a UV-visible spectrophotometer. This methodology is widely used for evaluating antioxidant activity. A decrease in absorbance indicates successful scavenging of DPPH radicals by the substance under investigation (Liyana-Pathirana & Shahidi, 2005).

To evaluate the ABTS cation scavenging activity in methanol extracts, a stock solution was prepared by dissolving 7 mM ABTS and 2.4 mM potassium persulphate in methanol. A working solution, which generates ABTS radicals, was then prepared by combining equal volumes of 7 mM ABTS and 2.45 mM potassium persulphate. The methanol extract was mixed with the ABTS to form a solution with a specific absorbance level. The absorbance level was measured at 0.706 ± 0.001 at a wavelength of 734 nm. The reaction mixture was shaken before measuring the absorbance using a spectrophotometer. This methodology allows for the assessment of the methanol extract's ability to scavenge ABTS cations by analyzing changes in absorbance. These

changes serve as an indicator of the substance's antioxidant potential. Valuable insights can be gained regarding the effectiveness of the methanol extract in combating ABTS cations through the analysis of absorbance readings (Re et al., 1999).

A new working solution was carefully prepared for the FRAP assay. The solution contained acetate buffer, TPTZ, and FeCl3.6H2O. The working solution was derived from a stock solution with specific concentrations. The working solution was heated to 37°C. The methanol extract was combined with the working solution to create a reaction mixture. The mixture was incubated in a dark environment for 30 minutes. The absorbance of the mixture was measured at 593 nm using a UV-vis spectrophotometer. The FRAP assay assesses the extract's ability to reduce ferric ions. The percentage of inhibition was converted into ascorbic acid equivalent per gram of dry weight (Benzie & Strain, 1996). Specific equations were used to convert the inhibition percentages into ascorbic acid equivalent. These equations establish a standardized measure to compare the antioxidant capacities of the methanol extracts.

Determination of IC_{50} Values for Inhibition Concentration Estimation:

The estimation of the concentration of IC_{50} is a critical element in evaluating the potential for inhibition of the methanol extract. Specifically, it measures the concentration at which 50% inhibition of DPPH radical activity is achieved. To determine the IC_{50} value for the extract from the seed, a simple linear equation was utilized. This equation was derived from the correlation between the increasing concentrations of extracts from the kernel and their corresponding potential for inhibiting DPPH. The IC_{50} value, in this particular instance, was expressed in milligrams per milliliter. Additionally, a comparative analysis was carried out by comparing the obtained IC_{50} values for each extract with those of synthetic antioxidants, such as benzenehexatoluene (BHT) and ascorbic acid (AA).

Fourier Transform Infrared Spectrometer:

FTIR analysis was employed to discern the specific functional groups present in the fruit samples.

Statistical Analysis:

The results were shown as the average ± standard deviation from three separate analyses. Duncan's multiple range tests found significant differences between treatment means at $p \leq 0.05$. A two-way ANOVA test was done to evaluate the impact of variables on biochemical parameters. All statistical analyses were conducted using SPSS software, Version 16.

RESULT AND DISCUSSION

Total Flavonoids Content and Total Phenolics Content:

Polyphenols, derived from plants, can be hydrolyzed tannin or condensed tannin. Research has shown that A. indica plants contain polyphenols and antioxidants in their bark, leaves, and fruits, which have various health benefits (Khan et al., 2022; Kharwar et al., 2020). In this study, it was observed that the levels of phenolics and flavonoids were lower in raw and overripe seeds compared to ripe seeds. This may be due to delayed synthesis and degradation processes. Similar findings were reported in studies on Olea ferruginea Royal seeds (Sharma, Kundra, Samant, & Nandi, 2017) and Pimpinella anisum L. (Rebey et al., 2019) plants. These studies highlight the complex dynamics of phenolic and flavonoid synthesis and the need for further investigation. The aim of this study is to determine the phenolics and flavonoids content in neem fruits at different stages of maturity. The chosen solvent for extraction was 80% MeOH. The TPC values for the overripe, ripe, and raw fruits were 11.55 mg GAE/g, 33.98 mg GAE/g, and 12.34 mg GAE/g, respectively. The TFC values were 0.19 mg QE/g dw, 0.29 mg QE/g dw, and 0.17 mg QE/g dw, respectively. These findings contribute to further research in the field.

The reported values indicate statistically significant differences at the $p \leq 0.05$ level.

SN	Total Phenolics and Flavonoids Content	Solvent	Overripe	Ripe	Raw
1	In (mg GAE/g)	80 % MeOH	11.55 ± 0.01	33.98 ± 0.02	12.34 ± 0.01
2	In (mg QE/g dw)	80 % MeOH	0.19 ± 0.01	0.29 ± 0.01	0.17 ± 0.01

In-vitro Antioxidant Activity:

Polyphenols, comprised of organic acids, aromatic rings with hydroxyl groups, and acetylated sugars, are an important class of compounds. They have unique structures that distinguish them from other molecules and exhibit high antioxidant activity, inhibiting harmful free radicals (Borges, Mullen, & Crozier, 2010). In plants, the most abundant polyphenols include flavan-3-ols, flavonols, phenolic acids,

anthocyanins, and hydroquinones (Brahem et al., 2017). Various chemical assays are used to assess the antioxidant efficacy of polyphenols in plants, allowing for a comprehensive evaluation of their potential (Amorati & Valgimigli, 2015). Extracted neem fruit seeds were subjected to in vitro assays to determine their antioxidant activity. The DPPH assay showed an activity of 90.64 ± 0.24 µM AAE/g dw in the overripe state, 100.35 ± 1.23 µM AAE/g dw in the ripe state, and 82.72 ± 0.98 µM AAE/g dw in the raw state. The ABTS assay displayed an activity of 80.5 ± 0.23 µM AAE/g dw in the overripe state, 92.85 ± 2.32 µM AAE/g dw in the ripe state, and 70.45 ± 2.13 µM AAE/g dw in the raw state. The FRAP assay revealed an activity of 54.35 ± 1.65 µM AAE/g dw in the overripe state, 162.67 ± 0.87 µM AAE/g dw in the ripe state, and 50.62 ± 3.34 µM AAE/g dw in the raw state. These in vitro assays showed the antioxidant potential of neem fruit seeds extract in different ripeness states.

SN	*In vitro* assays (µM AAE/g dw)	Seeds Extract of MeOH		
		Overripe	Ripe	Raw
1	DPPH Assay	90.64±0.24b	100.35±1.23a	82.72 ±0.98c
2	ABTS Assay	80.5 ± 0.23b	92.85 ± 2.32a	70.45± 2.13c
3	FRAP Assay	54.35±1.65b	162.67±1.87a	50.62± 3.34c

The values reported are the average ± standard error (SE) of three repeated analyses. Rows with different alphabets show significant differences at the $p \leq 0.05$ level, determined by Duncan's Multiple Range Test.

IC_{50} Values Assessment of Seed Extracts for Inhibition Concentration:

The assessment of IC_{50} values, which indicate the concentration at which seed extracts hinder 50% of unbound radicals, carries significant importance in the realm of antioxidant investigation. The determination of the minimal extract concentration necessary to achieve this level of radical elimination yields vital insights into the efficiency of the seeds being examined as antioxidants. Specifically, the examination revealed that mature A. indica seeds displayed lower IC_{50} values in comparison to other seed varieties. The IC_{50} values were most advantageous in the case of mature seeds, measuring roughly 0.74 mg/mL. Conversely, excessively ripe seeds exhibited the highest IC_{50} values, reaching levels as high as 2.18 mg/mL. These findings underscore a direct relationship between seed maturity and antioxidative potential. It is crucial to acknowledge that a higher IC_{50} value signifies a lower degree of antioxidant

capacity, while a lower IC_{50} value indicates a more vigorous antioxidant capability.

Standards	IC_{50} (mg/mL)
Overripe	2.18 ± 0.14
Ripe	0.74 ± 0.03
Raw	1.35 ± 0.59

The mean ± standard error (SE) values are statistically different at $p \leq 0.05$.

Conclusion:

The results indicated neem seed oils possess significant antioxidant property. The effect can be enhanced by modifications in the extraction methodology. The method should be efficient way for extracting essential oils from neem.

Acknowledgement

Authors are thankful to Riphah International University for providing facilities for the research work.

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