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Original Article

Comparative Study on Phytochemical, Antioxidant properties, Heavy Metal and FT-IR Analysis of *Azadirachta indica* L. from IMFA Industrial Area, Rayagada District, Odisha

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ABSTRACT

Background: Various plant parts are utilized in traditional medicine for their therapeutic capabilities. *Azadirachta indica* (Neem) is widely recognized for its medicinal properties, including antibacterial, antifungal, and anti-inflammatory effects. The presence of bioactive compounds in methanolic extracts of *A. indica* makes it a potential candidate for pharmaceutical applications. However, industrial contamination may influence its phytochemical profile, antioxidant potential, and heavy metal content, necessitating a comparative study between decontaminated and contaminated samples. **Objectives:** This study aims to evaluate and compare the phytochemical composition, antioxidant properties, heavy metal accumulation, and FT-IR spectral characteristics of *A. indica* samples collected from the IMFA industrial area, Rayagada district, Odisha, India. **Materials and Methods:** Both qualitative and quantitative phytochemical analyses were conducted on methanolic extracts of *A. indica* leaves. Antioxidant potential was assessed using DPPH, Heavy metal concentrations, including Chromium (Cr), were determined using ICP-OES. FT-IR spectroscopy was employed to identify functional groups present in the plant extracts. **Results:** The bioactive compounds detected among all the samples included alkaloids alongside phenols as well as tannins and saponins together with proteins. The contaminated sample contained greater amounts of total phenolic and flavonoid content at 65.14 mg GA/g and 245.74 mg RU/g than the decontaminated sample which had 58.72 mg GA/g and 189.13 mg RU/g. The antioxidant capacity analysed through DPPH assay spanned from 8.57% to 97.88% for contaminated samples and from 48.64% to 89.94% for decontaminated samples. The presence of heavy metals which evaluated through ICP-OES. Through FT-IR showed distinctive functional groups to confirm the chemical modifications between the evaluated samples. **Conclusion:** The findings emphasize the impact of industrial pollution on the phytochemical and antioxidant properties of *A. indica*. The presence of heavy metals in contaminated samples raises concerns about their safety for medicinal use. Further studies are recommended to explore detoxification strategies and assess the long-term effects of heavy metal exposure on medicinal plants.

Keywords: *A. indica*, Phytochemical screening, Antioxidant properties, ICP-OES method, FT-IR analysis.

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INTRODUCTION

Traditional healing practices have relied on plants and herbs as the primary source of bioactive compounds while also serving as a base for modern herbal medicine.[1] A total of 25% of both conventional pharmaceuticals and primary healthcare medications derive from plant resources.[2] In India, the *Azadirachta indica* is commonly known as "Neem tree" which belongs to family Meliaceae. This plant is highly regarded because its remarkable therapeutic features include anti-inflammatory effects, antimicrobial benefits, and antioxidant activities.[3] The continuous environmental pollution, primarily through heavy metals has led to increased worries about medicinal plants safety and performance.[4] Industrialization and mining operations have accelerated heavy metal accumulation in the environment, leading to combined pollution of water and soil.[5] Soil contaminants found in heavily toxic pollution zones cause plants to absorb the poisonous elements, leading to substantial chemical composition and medicinal properties changes in these plants.[6] Ogbaga and Okoro evaluated the phytochemical features of *A. indica* leaves obtained from polluted and clean sites, and they reported essential variations of alkaloids, flavonoids, and tannins because environmental stress factors impact secondary metabolite

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production.[7] A vast number of research papers have proven that heavy metals like Lead (Pb), Cadmium (Cd), Chromium (Cr), Arsenic (As), Mercury (Hg), Iron (Fe), etc., chemically disrupt plant metabolism, which causes oxidative processes. It leads to Reactive Oxygen Species (ROS).[8] The oxidation process applies plant-based antioxidants, including vitamins, phenolics and flavonoids, tannins and carotenes that protect cells by intercepting free radicals and creating no adverse effects on biological systems.[9,10] A high level of natural dietary antioxidants provides positive results for treating chronic heart diseases and cancer.[10] The increasing demand for natural antioxidants has surged dramatically throughout the past few years in the pharmaceuticals, nutraceuticals, and food additives markets.[11] Due to the immense medicinal potential of *A. indica*, the present research paper was designed to carry out a comparative study of phytochemical screening, *in vitro* antioxidant properties and heavy metal analysis using the ICP-OES method. Further, the research paper focused on identifying the functional group of bioactive compounds using the FT-IR method.

MATERIALS AND METHODS

Collection of plant sample

The leaves of plant sample *Azadirachta indica* were collected from both the IMFA industrial area and away from the industrial area of Therubali village of Rayagada district (19.33 ° N Latitude and 83.43° E Longitude) in December 2023. A renowned taxonomist authenticated the plant material, and the herbarium specimen was submitted to the Botany Department of Berhampur University, Odisha, India.

Preparation of Plant extracts

The collected samples were placed in sterile polythene bags upon reaching our laboratory. The leaves were thoroughly washed with tap water to remove any surface impurities. They were then dried in an oven at 80°C for 8 hr. After drying, the plant materials were ground into a fine powder using a mechanical grinder. Approximately 7 g of the dried powder was extracted with 300 mL of methanol utilizing a Soxhlet apparatus. Each extract was filtered separately, and the solvent was evaporated in a water bath to yield the crude extracts.[12] After drying, these extracts were stored in Eppendorf tubes and refrigerated for future analysis.

The dried extracts were weighed using the formula to determine the yield percentage of the soluble constituents.[13]

Yield % = W1/W2×100

Where W1 represents the net weight of the powder in grams after extraction and W2 indicates the total weight of the wood powder in grams used for extraction. The dried extracts were preserved at 4°C for further studies.

Qualitative Analysis of Bioactive Compounds

Standard phytochemical screening procedures were employed to assess the presence of various bioactive constituents in the methanolic extracts. Compounds such as alkaloids, terpenoids, phenolics, tannins, reducing sugars, saponins, proteins, steroids, anthocyanins, coumarins, leucoanthocyanins, and glycosides were identified following established qualitative protocols.[14-16]

Quantitative Analysis of Bioactive Compounds

Total Phenolic Content (TPC) Estimation

The total phenolic content in the methanolic plant extracts was quantified using a modified Folin-Ciocalteu method.[17] A volume of 20 µL of methanolic extract (1 mg/mL) was mixed with 1.58 mL of pure distilled water in a well test tube, followed by adding 100 µL of 7% Folin-Ciocalteu's reagent. After standing for 8 min, 300 µL of 10% sodium carbonate solution (250 g/l) was added. The mixture was withstanding at room temperature for 2 hr. Absorbance was recorded at 765 nm in triplicate. A calibration curve was created using gallic acid (0-500 mg/l) as standard, and results were expressed in milligrams of gallic acid equivalents per gram (mg GAE/g) of dry plant material. The TPC was calculated as:

$$C = c \times V/m$$

Where:

C = total phenolic content (mg GAE/g),

c = concentration of gallic acid from the standard curve (mg/mL),

V = volume of extract (mL),

m = weight of dry methanolic plant extract (g).

Total Flavonoid Content (TFC) Estimation

Determination of the flavonoid content was done spectrophotometrically using a standard protocol.[18] A 1 mL crude of the methanolic extract (1 mg/mL) was combined with 1 mL of 2% $AlCl_3$ solution in methanol. The resulting mixture was left at room temperature for 1 hr. Absorbance readings at 415 nm were taken in triplicate. A calibration curve was constructed using rutin standard solutions, which facilitated the determination of the flavonoid content. The amount of flavonoid which was expressed as milligrams of rutin equivalents per gram (mg RE/g) of dry plant material and calculated as follows:

$$C = c \times V/m$$

Where: C = total flavonoid content (mg RE/g),

c = concentration of rutin from the calibration curve (mg/mL),

V = volume of extract (mL),

m = weight of dry methanolic plant extract (g).

Measuring Antioxidant Activity DPPH Method

The antioxidant activity associated with the methanolic extracts was determined from the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity.[19] DPPH solution (0.004% w/v) was prepared by using methanol. Stock solutions of the extract (1 mg/mL) and standard ascorbic acid (0.05 g/mL) were prepared. Each sample was placed in test tubes at various concentrations (10-500 µg/mL). To each, 1 mL of freshly prepared DPPH solution was added. The tubes were wrapped tightly with aluminum foil to shield them from light, and methanol solvent was added to make 2 mL in total volume. 30 min later, absorbance was taken at 517 nm after incubation at room temperature in the dark. Control without extract or standard and blank with only methanol was also prepared. The percentage of DPPH inhibition was determined using the following formula:

$$\% \text{ Inhibition} = [(A_{control}-A_{sample}) / A_{control}]\times 100$$

Heavy Metal Analysis Using ICP-OES

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (PerkinElmer Avio 200 dual view instrument) with Syngistix software was employed for quantifying heavy metals (As, Cd, Pb, Co, Cr, Hg, Na, Zn, and Fe). The axial view was used for trace concentrations (µg/L) due to the increased plasma path length, while the radial view was used for higher concentrations (mg/L) to ensure linear response. A sea spray settler with a baffle was used.

Equipment

Multiwave digestion system (Anton Paar Multiwave).

Sample Preparation Procedure

Multiwave Digestion System (Anton Paar Multiwave) Sample Preparation Protocol Weigh between 0.2 and 0.5 g of the sample into a PTFE digestion vessel. Allow the vessels to cool within the digestion unit for 15 to 20 min to avoid pressure accumulation. Once cooled, transfer the digested solution into 50 mL volumetric flasks and dilute with ultrapure water. Prior to ICP-OES analysis, filter the solution using a 0.45 membrane filter (Table 1).

Instrument condition

- Setting Gas Flow: 8 L/min,
- Auxiliary gas flow: 0. 2 L/min,
- Nebulizer gas flow: 0.7 L/min,
- RF power: 1450 Watts,
- pump flow rate: 1. 50 mL/min,
- plasma Air flow: 10 L min-1,
- Stabilization time: 20 s.

Analysis by FTIR

The methanolic extracts of plant samples were analysed with a Perkin Elmer Spectrum Two FTIR spectrometer (PerkinElmer, Waltham, MA, USA) in the wave number range of 4000-400 cm^{-1} with air background. The equipment was operated in attenuated total reflection ATR mode with a diamond crystal.

PerkinElmer Spectrum Two FTIR, ATR Mode

- **Instrument Model:** PerkinElmer Spectrum Two.
- **Serial Number:** 104789.
- **Software:** NIOS2 Main 00.02.0100.
- **Detector:** $LiTaO_3$ (Lithium Tantalate).
- **Source:** MIR (Mid-Infrared).
- **Beamsplitter:** Opt KBr (Potassium Bromide).
- **Spectrum Type:** Absorbance/Transmittance.
- **Phase Correction:** Magnitude.
- **Scanning Parameters**
- **Scan Range:** 4000-450 cm^{-1} (default).
- **Resolution:** 4 cm^{-1}.
- **Number of Scans:** 4.
- **Scan Speed:** 0.2.
- **Apodization Function:** Strong.
- **IR-Laser Wavenumber:** 11,750 cm^{-1}.
- **Beam Type:** Ratio.
- **Scan Direction:** Combined.
- **Gram Type:** Double.

Statistical Analysis

Graphical analysis was done using Origin Pro-2021.

Table 1: All the phases and its parameters were calibrated properly in the instrument.

Phase	Temperature	Ramp time (min)	Hold time(min)	Pressure(bar)
Preheating	100	5-10	-	20-30
Digestion	100-220	10-20	20-40	40-60
Cooling	Room temp.	10	-	-

All experiments were conducted in triplicate, unless otherwise stated. The results were presented as mean±SD (Standard Deviation). The correlation analysis results of Ascorbic acid with TPC and TFC.

RESULTS

The yield percentage of methanolic extract of both contaminated and decontaminated samples of *Azadirachta indica* was found to be 13.5% and 13.64%, respectively.

Qualitative analysis of bioactive compounds

A preliminary phytochemical investigation of bioactive compounds was conducted using methanol extracts from contaminated and decontaminated plant samples. Initial analysis of methanolic extracts of both plant samples of *A. indica* demonstrated variations in the presence and absence of bioactive compounds. The contaminated plant sample revealed bioactive compounds such as alkaloids, phenols and tannins, saponins, proteins, steroids, coumarin, and glycosides, and the remaining bioactive compounds are absent. Similarly, the decontaminated plant sample showed the presence of bioactive compounds like alkaloids, terpenoids, phenols and tannins, saponins, proteins, anthocyanins, coumarins, and glycosides in Table 2.

Quantitative analysis of bioactive compounds

The Total Phenolic Content (TPC) of the plant extract was expressed in terms of Gallic Acid Equivalent (GA) using the standard curve equation in (0.00014x+0.01069) with a correlation coefficient (R^2) of 0.9677 (Figure 1).

Here, the phenolic content of the contaminated plant sample was 65.14±0.006 mg GA/g, and the decontaminated plant sample was 58.72±0.002 mg GA/g, as shown in Table 2. Similarly, the Total Flavonoid Content (TFC) of the plant extract was expressed in terms of Rutin equivalent (RU) using the standard curve equation in (y=0.00024x+0.0863) with a correlation coefficient (R^2) of 0.9689 (Figure 2).

At this point, the flavonoid content of both contaminated and decontaminated plant samples was 245.74±0.003 mg RU/g and 189.13±0.045 mg RU/g, respectively.

Antioxidant Properties by DPPH method

The DPPH scavenging activity of the methanolic plant extract was compared with the standard antioxidant compound, i.e., Ascorbic acid. This assay is based on scavenging DPPH radicals from the antioxidants, which decreases absorbance at 517 nm. The value of antioxidant properties of the known compound, i.e., Ascorbic

Table 2: Qualitative phytochemical screening of both contaminated and decontaminated plant sample of *A. indica*.

Parameter	Contaminated plant sample	Decontaminated plant sample
Alkaloid	+	+
Terpenoid	-	+
Phenol and tannin	+	+
Reducing sugar	-	-
Saponin	+	+
Protein	+	+
Steroids	+	-
Anthocyanin	-	+
Coumarin	+	+
Leucoanthocyanin	-	-
Glycosides	+	+

+=Present; -=Absent.

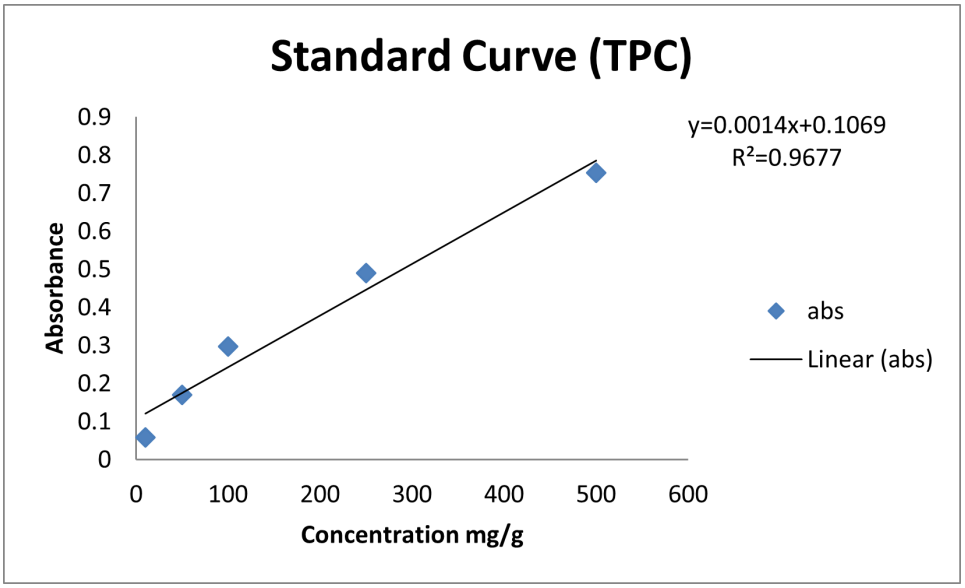

Figure 1: The standard curve of Total Phenolic Content.

acid, was from 34.43% to 96.77%. The antioxidant properties of methanolic extracts of contaminated and decontaminated plant samples ranged from 8.57 % to 97.88 % and 48.64% to 89.94%, respectively, at 10 to 500 µg/mL concentrations (Figure 3).

ICP-OES Analysis

The results of the heavy metals concentration in the methanolic extract of the leaf sample are shown in Table 3. ICP-OES of contaminated and decontaminated plant samples were measured, and the following elemental values were obtained. The values of heavy metals like as (0.007 mg/L), Cd (0.000 mg/L), Pb (0.005 mg/L), Co (0.000 mg/L), Cr (0.038 mg/L), Hg (0.002 mg/L), Na (0.076 mg/L), Zn (0.642 mg/L), Fe (1.896 mg/L) in contaminated plant sample. Similarly, the values of heavy metals in decontaminated plant samples are as (0.000 mg/L), Cd (0.000 mg/L), Pb (0.003 mg/L), Co (0.002 mg/L), Cr (0.030 mg/L), Hg (0.000 mg/L), Na (0.856 mg/L), Zn (0.555 mg/L), Fe (0.427 mg/L).

FT-IR analysis

The FTIR analysis revealed the presence of various functional groups in both contaminated and decontaminated samples of *A. indica*. Functional groups like N-H, C=C, C=C, and O-H were identified by matching the frequency range with the sigma -Aldrich Table reference. The absorption peak at 3326.09 cm^{-1} corresponds to N-H stretching, which confirmed the presence of an amine functional group in a contaminated plant sample (Figure 4). It also shows the absorption band at 2123.65 cm^{-1} is C=C stretching, indicating alkyne groups and 1634.95 cm^{-1} is C=C indicating the presence of alkenes groups in the contaminated plant samples. However, the absorption band at 3327.15 cm^{-1}, 2107.62cm^{-1}, and 1634.98 cm^{-1} of a decontaminated sample of *A.*

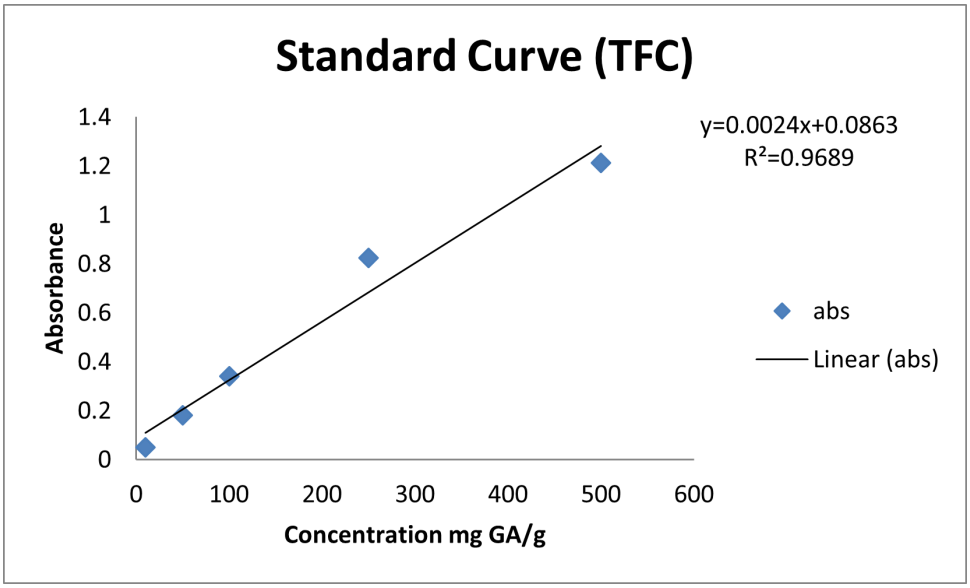

Figure 2: The standard Curve of Total Flavonoid Content.

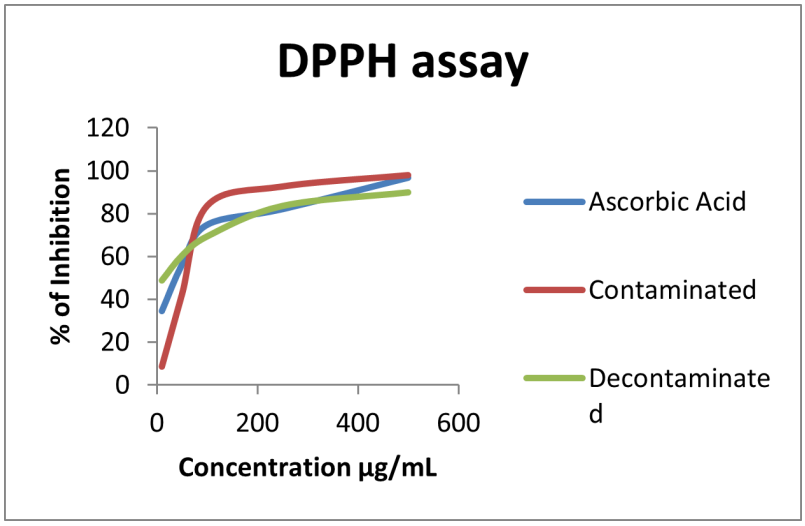

Figure 3: DPPH Comparison of % inhibition of Ascorbic acid with contaminated and decontaminated of *A. indica.*

indica is O-H, C=C, C=C stretching, which revealed the presence of alcohol, alkyne and alkene respectively (Figure 5).

DISCUSSION

Traditional medicine plays a vital role in the primary health care of people in developing countries, including India, and medicinal plants contribute to this practice.[20] The present study evaluated the bioactive compounds, antioxidant properties, heavy metal contents and functional group of contaminated and decontaminated plant samples of leaves of *Azadirachta indica* (Neem), which were collected from different sites of IMFA industrial area of Rayagada District, Odisha, India. The phytochemical composition of plants depends on the geographical region, climatic conditions, and edaphic factors in the soil. Therefore, the results showed significant differences between the two plant samples. These differences indicate that the stress caused by heavy metals affects the biosynthesis of secondary metabolites, resonating with previous studies that supposed that metabolic shifts under stress boost plant defensive attributes.[8,21] The phenolic compounds are naturally occurring bioactive compounds in plants that exhibit significant antioxidant activity.[13] These compounds are present in different parts of the plant and contribute to their health benefits. The flavonoid consists of a large group of polyphenol compounds having a benzoyl- γ- pyrone structure and is omnipresent in plants.[20] These compounds work as effective antioxidants in reducing the deleterious effects of ROS generated due to metal-induced stress.[22] After decontamination, the decrease in total phenolic and flavonoid content indicates that the remediation process can influence secondary metabolism. Some other studies have also reported that soil remediation reduced the accumulation of secondary metabolites in medicinal plants.[21] DPPH free radicals determined the free radical scavenging activity of the methanolic plant extract. The antioxidant activity is one of the most important pharmacological properties of the plant. DPPH is a commonly used free radical because of its chemical properties.[23] When an antioxidant donates an electron or hydrogen atom, the DPPH radical is reduced to its non-radical form, DPPH-H. This causes a colour change from violet (radical form) to pale yellow (reduced form).[24]

Medicinal plants have been reported as a potential source of heavy metal toxicity to both humans and animals.[22] The most common heavy metals implicated in human toxicity include Fe, Zn, Na, Cr, As, Pb, and Hg may cause toxicity. Therefore, the World Health Organisation (WHO) recommends that the raw materials of medicinal plants should be checked for the presence of heavy metals. However, most people live in industrial areas where the medicinal plants grow. The study collected the plant samples in two different geographical regions. The present study revealed that the contaminated plant sample possesses a high amount of Iron (Fe), i.e., 1.896 mg/L and Chromium (Cr), i.e., 0.038 mg/L. similarly, the decontaminated plant sample possesses a high amount of Na (0.856 mg/L), Zn (0.555 mg/L), Fe (0.427 mg/L), and Cr (0.30 mg/L).

Table 3: Concentration of Heavy metals in contaminated and decontaminated sample of *A. indica*.

Heavy metals	Amount of heavy metals in Contaminated sample in mg/L	Amount of heavy metals Decontaminated sample in mg/L
As	0.007	0.000
Cd	0.000	0.000
Pb	0.005	0.003
Co	0.000	0.002
Cr	0.038	0.030
Hg	0.002	0.000
Na	0.076	0.856
Zn	0.642	0.555
Fe	1.896	0.427

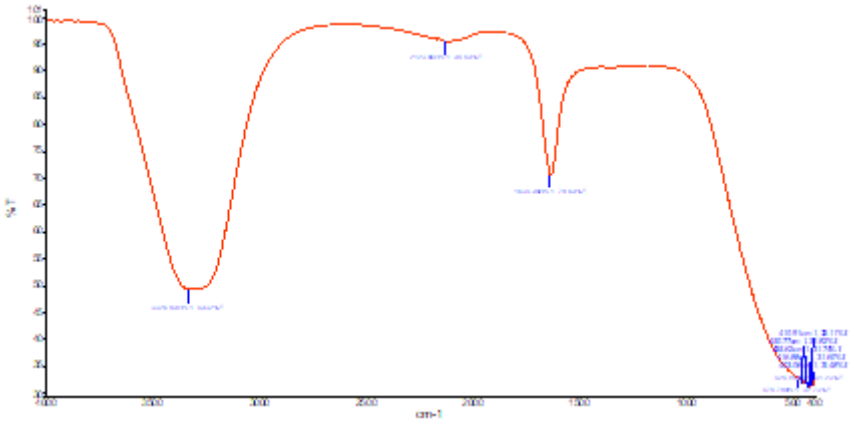

Figure 4: FTIR spectrum of contaminated sample of *A. indica*.

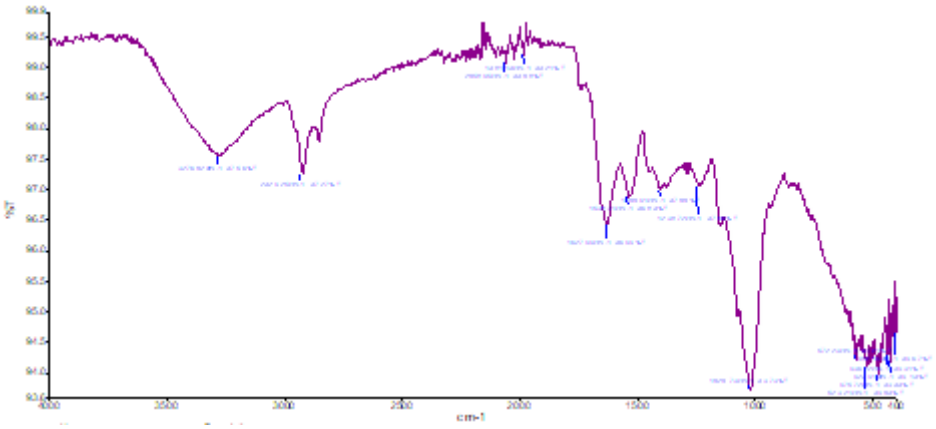

Figure 5: FTIR spectrum Of decontaminated sample of *A. indica.*

FTIR Analysis

The essential functional groups detected by FT-IR analysis in both samples were N-H, C=C, C=C, and O-H, marking amines, alkynes, alkenes, and alcohols, respectively. These functional groups play important functional roles in establishing bioactivity in medicinal plants. An extra number of O-H functional groups on other decontaminated samples probably points to biochemical modifications from changes in environmental conditions. Additional studies have indicated that FT-IR spectroscopy could be relied on to document stress-induced biochemical changes in medicinal plants.[25,11] The spectral pattern variances from polluted and decontaminated samples indicate a high likelihood of the exposure of heavy metals causing a probable change in any biochemical pathways, which here could dictate the therapeutic potential of *A. indica* to some extent.[21]

The detected variations between phytochemical and metal content levels demonstrate environmental pollution affecting medicinal plants. Interaction with heavy metals in the environment triggers secondary metabolite stimulation, leading to probable modifications in herbal medicine pharmacological activities. Soil remediation activities would destroy environmental contamination while affecting plant chemical composition. Further research about metal contamination needs to explore both bioactive compound pharmacology and metal accessibility after soil remediation steps. Enhancing advanced soil remediation methods requires prioritizing metal elimination without affecting other phytochemicals to achieve safe and effective medicinal plant products.

CONCLUSION

This study provides insight into monitoring the impact of industrial contamination on *A. indica*. The result showed that stress due to heavy metals affects the biosynthesis and elemental composition of secondary metabolites, which might influence their medicinal value. Additional research is needed to decode the pharmacological impact of these compositional alterations and optimal remediation practices for medicinal plants, ensuring confirmation of their therapeutic potential.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TPC: Total Phenolic Content; **TFC:** Total Flavonoid Content; **ICPO-ES:** Inductively Coupled Plasma Optical Emission Spectroscopy; **FT-IR:** Fourier Transform Infrared Spectroscopy; **DPPH:** 1,1 Diphenyl 2, Picryl Hydrazyl.

SUMMARY

In this research paper, active phytochemicals, heavy metals, bioactive compounds, and antioxidant properties of the leaf plant extract of *Azadirachta indica* is identified through the ICP-OES, and FT-IR methods which exhibit different pharmacological properties. This information may help many pharmaceutical companies to manufacture wonder drug for the society. The relative concentration of bioactive compounds and mineral elements is reasonable and can be considered for medical purposes.

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