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# Phytochemical analysis of aqueous extract of *Azadirachta indica* leaves

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**Abstract**
Plant-based antimicrobial compounds have emerged as a blessing to medical sciences due to the multitude of bioactive phytochemicals or secondary metabolites, ease of availability and a broad spectrum of action with negligible adverse effects. *Azadirachta indica* (*A. indica*) is reported to possess a multitude of phytochemicals. In the present study, the phytochemical constituents of the aqueous extract of *A. indica* leaves were analysed qualitatively. The chemical nature of the extract and the structurally similar compounds present in it were analysed by Fourier transform infrared (FTIR) spectroscopy. Preliminary phytochemical analysis by qualitative biochemical tests revealed the presence of alkaloids, flavonoids, terpenoids, glycosides, phenols, steroids, tannins and saponins. Fourier transform infrared spectroscopic analysis revealed the presence of functional groups such as alcohols, carboxylic acid, amine salt, disubstituted alkenes, sulphates and halocompounds.

**Keywords:** *Azadirachta indica*, aqueous extract, Fourier transform infra-red spectroscopy, phytochemicals

## 1. Introduction
Herbal remedies and plant-based medicines were gaining great importance in recent times. They were considered to be a rich source of structurally and functionally diverse phytochemicals or secondary metabolites that protects them from diseases or predators and possess a multitude of antimicrobial and resistance- modifying properties. Despite the widespread availability of synthetic pharmaceuticals and their relative success in treating a wide range of ailments, several people continue to advocate the use of phytomedicines due to their less critical side effects.

*Azadirachta indica* (*A. indica*) commonly known as neem, Margosa, Nimtree, Nimba or Ariyaveppu (Malayalam) is an evergreen tropical tree of India known for its legendary medicinal value. It belongs to the family Meliaceae (Mahogany), subfamily Meloideae and tribe, Melieae. The tree is native to the Indian and is found across the tropical and semitropical regions. Depending on the agro-climatic conditions, it can reach an average height of 20-30 m and a diameter of 4-5 ft (Girish and Shankara, 2008) [1].

The earliest documentation of the medicinal properties of neem was proven by British archaeologists during the excavation of the 4500-year-old Harappan civilisation. They discovered clay pots with therapeutic herbs such as *A. indica* and skulls on which cranial surgeries were being performed. Neem was the first medicinal plant that gained accession to the annals of ancient Siddha system. It was used widely to prevent contagious diseases such as small pox and was thought to defend against evil spirits (Kumar and Navaratnam, 2023) [2]. It has been regarded as an inevitable component of traditional Chinese medicine and Indian ayurvedic medicine since time immemorial. Traditionally, the different parts of the plant were being used for its proven antibacterial (Altayb *et al*., 2022) [3], antiviral (Hemdan *et al*., 2023) [4], wound healing (Jayalakshmi *et al*., 2021) [5], antioxidant, anti-inflammatory (Naik *et al*., 2014) [6], anti-cancerous (Priyadarsini *et al*., 2010) [7], immunomodulatory (Sarkar *et al*., 2021) [8] and antidiabetic activities (Ghodeswar *et al*., 2023) [9]. All the aforementioned biomedical applications could be attributed to a multitude of bioactive phytochemicals with diverse chemical structures and multiple therapeutic capabilities that paved way for the development of a wide range of medicinally and industrially beneficial formulations. Bioactive chemicals such as nimbolide, azadirachtin, and gedunin found in neem were documented to have enormous ability to infuence a wide range of biological processes, both *in vitro* and *in vivo*.

Hence, the present study was conducted to investigate the phytochemical constituents of the aqueous extract of neem leaves, its chemical nature and structurally similar compounds present in it.

## 2. Materials and Methods

### 2.1 Plant material

The leaves of *A. indica* (Ariyaveppu, Indian Liliac) were collected from Nadathara, Thrissur, Kerala (10.51667°N; 76.21667°E) during January 2023 (Fig. 1). The leaves were washed with double distilled water to remove any unwanted stakes, dried under shade by spreading on a paper, coarsely pulverised using an electric pulveriser and stored under a dark dry area until used (Fig. 2).

**Fig 1:** Leaves of A. indica.

**Fig 2:** Shade dried leaves of *A. indica.*

### 2.2 Authentication of plant material

The plant was assembled for herbarium and the voucher specimen (HERB/VEPM/CVASMTY/1/2023) was deposited in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy (Fig. 3). The herbarium prepared from the leaves of *A. indica* were authenticated at the Raw Material Herbarium and Museum Department (RHMD), Council of Scientific and Industrial Research (CSIR) – National Institute of Science Communication and Policy Research (NIScPR), New Delhi.

**Fig 3:** Herbarium of *Azadirachta indica.*

### 2.3 Preparation of aqueous extract of *A. indica* – Digestion method

The aqueous extraction of the *A. indica* leaves was carried out as described by Abubakar and Haque (2020) [10].

In brief, the shade dried and pulverised leaves (10 g) were transferred onto a clean round bottom flask. Water was used as the solvent and 100 ml of autoclaved double distilled water was added to the plant material. The mixture was then placed over a water bath maintained at 60 °C for about 30 minutes with stirring. Heat was applied throughout the extraction process to decrease the viscosity of the extraction solvent and to enhance the removal of secondary metabolites. At the end of extraction, all the solid particulates were separated from the extract by filtration using Whatman No. 1 filter paper. The filtrate obtained was subjected to screening fo r phytochemicals (Fig. 4)

**Fig 4:** Aqueous extract of leaves of *A. indica* prepared by digestion method.

### 2.4 Qualitative tests for analysis of phytochemical constituents

The aqueous extract from the leaves of *A. indica* was subjected to qualitative analysis for the presence of various phytochemical components namely steroids, alkaloids, flavonoids, diterpenes, triterpenes and saponins (Harborne, 1998; Khanal, 2021) [11, 12].

The presence of steroids and triterpenes was determined by Salkowski's test. Mayer's test, Wagner's test, Hager's test and Dragendroff's tests were used to verify the presence of alkaloids. The sodium hydroxide test was used to confirm the presence of glycosides. The ferric chloride test was used to determine the presence of tannins and flavonoids, while the froth test was performed to determine the presence of saponins.

#### 2.4.1 Detection of steroids

Salkowski's test was used to detect the presence of steroids in the given sample. For this, fifty milligrams of the extract were dissolved in three millilitres of chloroform. Few drops of concentrated sulphuric acid were added through the sides of the test tube and the solution was allowed to stand. The development of red colour indicates the presence of steroids.

#### 2.4.2 Detection of alkaloids

About 500 mg of the extract was mixed with five millilitres of ammonia and extracted with an equal volume of chloroform. To this extract, five millilitres of dilute HCl was added. The acid layer obtained was used for the following chemical tests for alkaloids.

#### 2.4.3 Mayer's Test

To one millilitre of acid layer, a few drops of Mayer's reagent were added. The development of a creamy white precipitate indicated the presence of alkaloids.

#### 2.4.4 Wagner's Test

A few drops of Wagner's reagent were added to one millilitre of the extract. The development of reddish- brown precipitate indicated the presence of alkaloids.

#### 2.4.5 Hager's Test

To one millilitre acid extract, a few drops of Hager's reagent were mixed. The development of a yellow precipitate by the sample taken indicated the presence of alkaloids.

#### 2.4.6 Dragendroff's Test

A few drops of Dragendroff's reagent were mixed with one millilitre of acid extract. The development of a reddish-brown precipitate indicated the presence of alkaloids.

#### 2.4.7 Detection of Glycosides

The sodium hydroxide test was used for the detection of glycosides. To 50 mg of the extract one millilitre of distilled water and six drops of 10 percent sodium hydroxide solution was added and mixed well. The development of yellow colour indicated the presence of glycosides.

#### 2.4.8 Detection of tannins

The ferric chloride test was used for the detection of tannins. For this, treated two milligrams of the extract was mixed with three millilitres of one percent ferric chloride solution. The development of a brownish green or a blue-black colouration indicated the presence of tannins.

#### 2.4.9 Detection of flavonoids

The presence of flavonoids in the given extract was assessed by both the ferric chloride test. For ferric chloride test, two millilitres of alcoholic solution of the extract (500 mg extract in 10 mL methanol) was mixed with a few drops of neutral ferric chloride solution. The development of green colour indicated the presence of flavonoids.

#### 2.4.10 Detection of diterpenes

For this, five milligrams extract was mixed with three millilitres of five percent copper acetate solution. The development of green colour suggested the presence of diterpenes.

#### 2.4.11 Detection of triterpenes

The Salkowski test was used to assess the presence of triterpenes. Three millilitres of chloroform were mixed with three milligrams of extract and this was further shaken with three millilitre concentrated sulphuric acid. The development of yellow colour in the lower layer on standing indicated the presence of triterpenes.

#### 2.4.12 Detection of saponins - Froth test

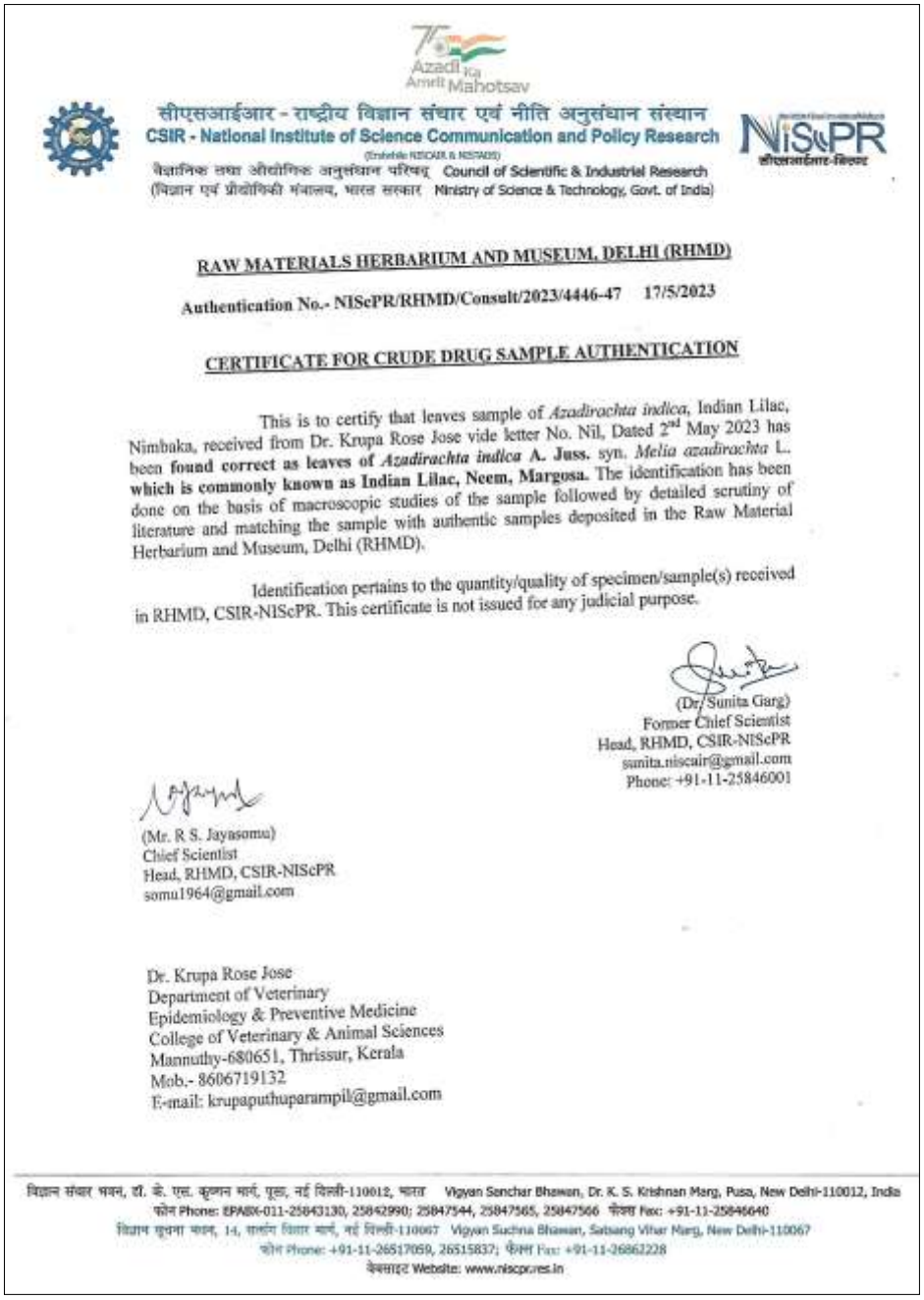
Approximately 200 mg of the extract was shaken with five millilitres of water. The development of foam that persisted for 10 min indicated the presence of saponins.

### 2.5 Fourier transform infrared (FTIR) spectroscopic analysis

To evaluate the chemical bonds and functional atoms present in the extract, Attenuated Total Reflectance- Fourier Transform Infrared (ATR-FTIR) spectroscopic analysis was performed. A Perkin Elmer Spectrum Two™ FTIR spectrometer with attenuated total reflectance was used in the current study. Spectrum was taken from 4000 $cm^{-1}$ to 400 $cm^{-1}$ range using an infra-red spectrophotometer against the blank. An overhead ATR attachment has been mounted at the sample station. The ATR diamond crystal was carefully cleaned with pure isopropanol and a small quantity of sample was placed carefully on the diamond crystal surface to cover the ATR window to focus the laser beam. Each spectrum was recorded and analysed.

## 3. Results

The herbarium prepared from the leaves of *A. indica* was authenticated from the Raw Material Herbarium and Museum Department (RHMD), Council of Scientific and Industrial Research (CSIR) – National Institute of Science Communication and Policy Research (NIScPR), New Delhi vide authentication number NIScPR/RHMD/Consult/2023/4446-47 dated 17.05.2023 (Fig. 5).



Azadi Ka Amrit Mahotsav

सीएसआईआर - राष्ट्रीय विज्ञान संचार एवं नीति अनुसंधान संस्थान
CSIR - National Institute of Science Communication and Policy Research
(Erstwhile NISCAIR & NISTADS)
वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद् Council of Scientific & Industrial Research
(विज्ञान एवं प्रौद्योगिकी मंत्रालय, भारत सरकार Ministry of Science & Technology, Govt. of India)

NISCPR

**RAW MATERIALS HERBARIUM AND MUSEUM, DELHI (RHMD)**

**Authentication No.- NIScPR/RHMD/Consult/2023/4446-47 17/5/2023**

**CERTIFICATE FOR CRUDE DRUG SAMPLE AUTHENTICATION**

This is to certify that leaves sample of *Azadirachta indica*, Indian Lilac, Nimbaka, received from Dr. Krupa Rose Jose vide letter No. Nil, Dated 2nd May 2023 has been **found correct as leaves of *Azadirachta indica* A. Juss.** syn. *Melia azadirachta* L. **which is commonly known as Indian Lilac, Neem, Margosa.** The identification has been done on the basis of macroscopic studies of the sample followed by detailed scrutiny of literature and matching the sample with authentic samples deposited in the Raw Material Herbarium and Museum, Delhi (RHMD).

Identification pertains to the quantity/quality of specimen/sample(s) received in RHMD, CSIR-NIScPR. This certificate is not issued for any judicial purpose.

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**Fig 5:** Herbarium authentication certificate of *Azadirachta indica* from RHMD, CSIR – NIScPR, New Delhi *vide* authentication number NIScPR/RHMD/Consult/2023/4446-47 dated 17.05.2023.

The qualitative phytochemical screening revealed the presence of steroids, alkaloids, glycosides, phenolic compounds, tannins, flavonoids, diterpenes and triterpenes as depicted in table 1.

**Table 1:** Qualitative screening of *A. indica* for phytochemicals

| Sl. No. | Phytochemicals screened | Screening test | Result |
|---|---|---|---|
| 1. | Steroids | Salkowski's test | + |
| 2. | Alkaloids | Dragendroff's test | + |
| | | Mayer's test | + |
| | | Wagner's test | + |
| | | Hager's test | + |
| 3. | Glycosides | Sodium hydroxide test | + |
| 4. | Tannins | Ferric chloride test | + |
| 5. | Flavonoids | Ferric chloride test | + |
| 6. | Diterpenes | Copper acetate test | + |
| 7. | Triterpenes | Salkowski's test | + |
| 8. | Saponins | Foam test | + |

+ indicates presence, - indicates absence

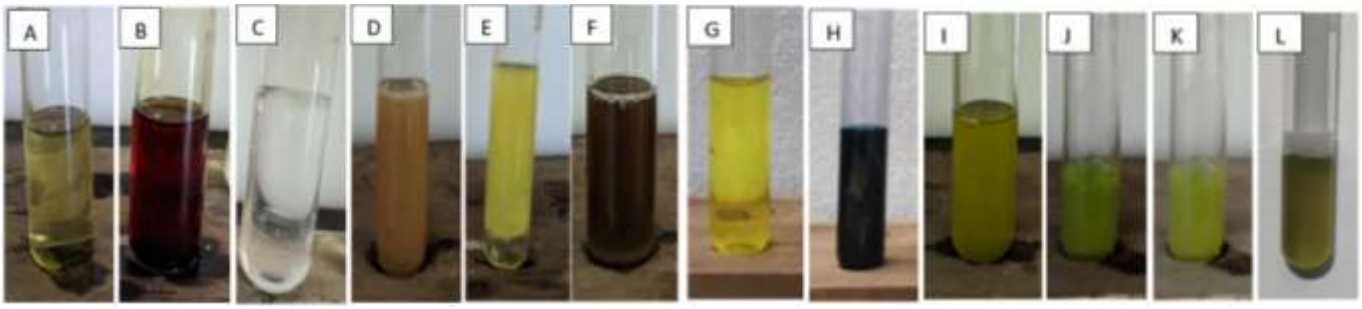


**Fig 6:** Qualitative phytochemical screening of aqueous extract of *Azadirachta indica* (A.) Extract alone (B.) Salkowski's test for steroids (C.) Mayer's test for alkaloids (D.) Wagner's test for alkaloids (E.) Hager's test for alkaloids (F.) Dragendroff's test for alkaloids (G) Sodium hydroxide test for glycosides (H) Ferric chloride test for tannins (I) Ferric chloride test for flavonoids (J) Copper acetate test for diterpenes (K.) Salkowski's test for triterpenes (L.) Foam test for saponins

In the present study, the FTIR spectrum showed major peaks at 3340.32, 2947.97, 2836.20, 1650.55, 1449.68, 1409.58, 1112.38, 1016.18 and 579.63 $cm^{-1}$ (Fig. 7).

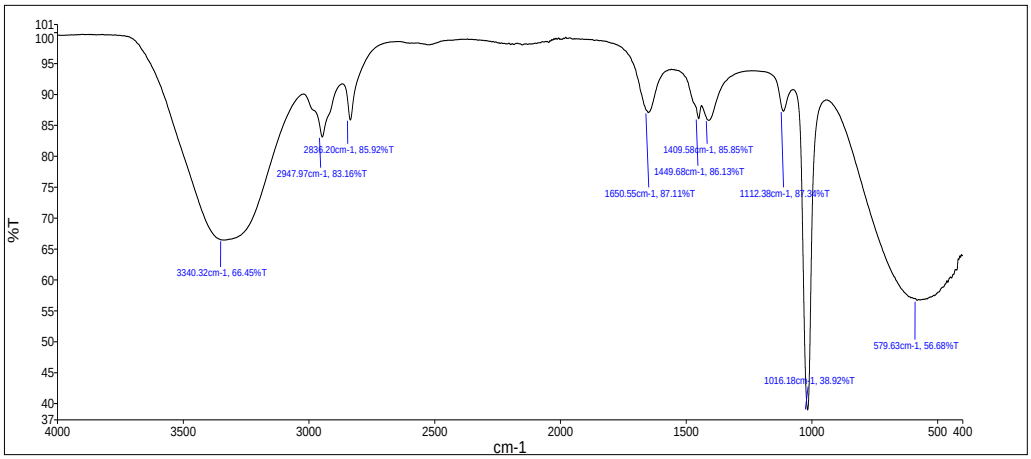


**Fig 7**: FTIR spectra of aqueous extract of leaves of *A. indica* with frequency along x axis and transmittance along y axis

Based on the absorbance spectra as depicted in fig. 7, the structurally related compounds identified in the extract by ATR-FTIR analysis is described in table 2.

**Table 2:** FTIR spectroscopic analysis of aqueous extract of *A. indica* leaves

| Absorption ($cm^{-1}$) | Appearance | Group | Compound class |
|---|---|---|---|
| 3340.32 | Strong | O-H stretching | Alcohol |
| 2947.97 | Strong | O-H stretching | Carboxylic acid |
| | Strong | N-H stretching | Amine salt |
| 2836.20 | Strong | O-H stretching | Carboxylic acid |
| | Strong | N-H stretching | Amine salt |
| 1650.55 | Medium | C=C stretching | Disubstituted alkene |
| 1409.58 | Strong | S=O stretching | Sulphate |
| 1112.38 | Strong | C- F stretching | Fluoro compound |
| 579.63 | Strong | C-Br stretching | Halocompound |
| | Strong | C-I stretching | Halocompound |

## 4. Discussion

Phytochemicals are a complex mixture of secondary plant metabolites that provide them defensive functions. It was found to have extensive use in the field of folk medicine, preservation and food flavouring for centuries (Ashraf *et al*., 2023) [13]. Recently, phytochemicals have gained international popularity due to the fear of adverse effects produced by the synthetic medicines as well as the emergence of antimicrobial resistance. Besides, the ease of availability, ease of administration and cheap rate makes phytochemicals an attractive alternative to synthetic medicines. The extraction and determination of quality and quantity of bioactive constituents was of paramount importance in achieving quality research outcome. Hence, in the current study the aqueous extract of *A. indica* was extracted by digestion method (Abubakar and Haque, 2020) [10] and analysed for the presence of its phytochemical constituents.

### 4.1 Phytochemical constituents in aqueous extract of *A. indica* leaves

The diverse and multiple activities of *A. indica* can be attributed to the presence of active principles such as aldehydes, phenolics, flavonoids, terpenes and other antimicrobial compounds. Their activity varies based on the structure and orientation of these functional groups.

In the present study, the qualitative phytochemical screening revealed the presence of steroids, alkaloids, glycosides, tannins, flavonoids, diterpenes and triterpenes which was in accordance with the findings of Ujah *et al*. (2021) [14]. However, on analysis of its methanolic extract, glycosides were the most abundant followed by alkaloids, flavonoids, tannins and sugars in a moderate concentration and saponins the least (Dash *et al*., 2017) [15].

The percentage of certain phytochemicals varied widely across different parts of the tree. According to a comparative analysis of the phytochemical compositions of the leaf, stem-bark and roots of *A. indica* (Dash *et al.*, 2017) [15] alkaloids, flavonoids, saponins and terpenoids were found in all tested parts but polyphenols, tannins and steroids were found only in leaves and stem-bark. Furthermore, the maximum concentration of alkaloids was detected in stem-bark (12.8 percent) and the lowest in leaves (10.67 percent). The

percentage of flavonoids was highest in the leaves (13.8 percent) and lowest in the stem-bark (12.8 percent). Similarly, seeds contained 2.53 percent saponins, while stem-bark contained the least (2.50 percent). Terpenoids were identified in equal concentrations (13.13 percent) in both leaves and stem-bark, but at the lowest quantity (12.77 percent) in seeds.

Fourier Transform Infrared (FTIR) spectroscopy facilitated the qualitative analysis of the chemical bonds and the functional groups present in the test sample with the help of infra-red scanning (Mourdikoudis *et al*., 2018) [17]. In the present study, the FTIR spectrum showed major peaks at 3340.32, 2947.97, 2836.20, 1650.55, 1409.58, 1112.38 and 579.63 $cm^{-1}$ (Fig. 7). The peak in the range of 3,550–3,200 $cm^{-1}$ were assigned as –OH stretching in alcohols. The peaks in the range of 3,300–2,500 $cm^{-1}$ may perhaps be credited to –OH stretching in carboxylic acid, and amine salts with strong stretching of the N-H group also appeared in this range. The peak in the range of 1662 $cm^{-1}$ to 1626 $cm^{-1}$ might be ascribed to double bond stretching suggestive of disubstituted alkenes. The FTIR peaks that appeared in the range 1,415 $cm^{-1}$ to 1380 $cm^{-1}$ are relevant to S=O stretching while peaks in the range 1400 $cm^{-1}$ to 1000 $cm^{-1}$ demonstrate fluorocompounds. The peaks in the range of 579.63 $cm^{-1}$ were designated to –CBr and –Cl in halocompounds. These observations were comparable with the previous findings of Ali *et al*., (2023) [19] that verified the occurrence of halides, aliphatic amines, aromatic, carboxylic group, amides, alkynes, alkanes and alkenes. Nair and coworkers [18] analysed the powder of *A. indica* leaves using FTIR spectroscopy and confirmed the presence of O-H stretching, C-O stretching, N-H stretching, C-H stretching and C=O stretching and confirmed the presence of alcohols, phenols, amines, amides, carboxylic group, ester, ether and amino acids groups within them.

However, the variation in the chemical bonds and functional groups between different studies might be due to the variations in the solvent used and the extraction systems involved. The decoction system of phytochemical extraction might result in partial damage or destruction of many active constituents owing to the comparatively longer boiling process Ali *et al*., (2023) [19]. Thus, it could be concluded that apart from the solvent used for extraction, the process of extraction also determines the active constituents available in an extract.

## 5. Summary

The results of the present study have confirmed the presence of numerous medicinally important compounds of *A. indica* extract that might serve as a good source of drugs for pharmaceutical industries. The identification and characterisation of phytochemicals in the extract could pave way for the development of a wide range of medicinally and industrially beneficial formulations with diverse biomedical applications.

## 6. Acknowledgments

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## 7. Disclosure

The author(s) reports no conflict(s) of interest in this work.

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