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Phytochemical Screening and Isolation of Bioactive Compounds from the Ethyl Acetate Extract of *Indigofera nummulariifolia*

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ABSTRACT

Indigofera is the third-largest genus in the family Fabaceae and consists of approximately 750 species, *Indigofera numulariifolia,* a species within this genus is an annual herb or shrub that is widely distributed throughout tropical and subtropical regions of the world including Nigeria. In ethno-medicine, it is used to treat liver ailments and some viral infections. In this study, the pulverized plant sample was subjected to cold maceration to afford the crude extracts of n-hexane, dichloromethane, ethyl acetate, and methanol. Phytochemical screening of crude extracts was carried out according to standard procedures while the antibacterial activity was assessed using the agar well diffusion method against *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Isolation of bioactive compounds from the ethyl acetate extract was carried out using open column chromatography and the pure isolates were characterized using IR and GCMS in comparison with the literature. Phytochemical screening of crude extracts were carried out according to standard procedures while the antimicrobial activity was assessed using agar well diffusion method against *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Purification of the crude extract was done on open column chromatography and characterized using IR and GCMS. The results of phytochemical screening revealed the presence of Saponins, flavonoids, tannins, and steroids. The extracts also showed antimicrobial activity with inhibition zones in the range of 6-20 mm, where the highest activity was recorded against *E. coli* with a zone of inhibition of about 20 mm. Meanwhile, results from the GC-MS analysis of one of the isolated compounds, revealed the presence of six fatty acid derivatives obtained as a single isolate from the n-hexane ethyl acetate mixture in a ratio of 95:5. These findings enrich the understanding of *Indigofera numulariifolia's* chemical composition and highlight its potential bioactive constituents.

Keywords: Fatty acid derivatives, *Indigofera numulariifolia*, Isolation, Phytochemical screening,

INTRODUCTION

Indigofera, an extensive genus encompassing over 750 flowering plant species within the *Fabaceae* family, displays a widespread presence in tropical and subtropical regions globally (Schrire, 2013). Within this diverse genus, the majority of species manifest as shrubs, though some take the form of small trees or annual and perennial herbs. It is characterized by pink flowers, 10-15 in racemes, flowering and fruiting around September to October and rarely found growing in sandy soils along water streams (Santhosh *et al,* 2019). Certain species of *Indigofera*, such as *Indigofera aspalathoides*, *Indigofera heterantha*, *Indigofera tinctoria*, and *Indigofera suffruticosa*, have been traditionally utilized for various purposes. For instance, *Indigofera nummulariifolia* often used by people of the rural communities liver diseases and other viral infections although the use is however without scientific investigation (Maroyi, 2023). *Indigofera aspalathoides*, specifically, are recognized for their

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efficacy in addressing skin disorders (Mohammad *et al*., 2011). *Indigofera heterantha* serves medicinal purposes, particularly in the treatment of gastrointestinal disorders and abdominal pain, while *Indigofera tinctoria* is employed for alleviating constipation, liver disease, heart palpitations, and gout (Amrithpal, 2006; Warrier, 2007).

Chemical analyses of *Indigofera* species have revealed the presence of commonly occurring compounds such as flavonoids, fatty acids, and steroids (Taj *et al.,* 2016). Notably, flavonoidal compounds isolated from Indigoferahebepetala include Kaempferol 3,7diarabinoside, Kaempferol 7-alloside, Triflin-2-O-β-L-ramnopyranosyl, and 7-O-β-L-arabinofuranoside (Gerometta *et al.*, 2020) In the case of *Indigofera suffruticosa*, fatty acid compounds such as Tetradec-11-en-1-ol lactate, oleic acid, 9-octadecenoic acid[z]-, 2-hydroxy-1-[hydroxyl dimethyl], heptanoic acid, docosyl ester, and octadecanoic acid have been identified (Vijisaral *et al.,* 2014). However, despite the extensive work done in revealing the phytochemical compounds in the *Indigofera* genus, little attention was paid to the isolation of those bioactive compounds with zero records of isolation from *Indigofera numulariifolia*. Therefore, this research aims to contribute to the understanding of the chemical composition of *Indigofera nummulariifolia* and explore its potential for applications in medicine, agriculture, or other fields.

MATERIALS AND METHODS

General Procedure

All organic solvents used were of general purpose grade (LOBA CHEMIE PVT. LTD. India). All equipments and capacity are quoted accordingly and operated to standard procedures. Glass ware was dried in an oven at 120 °C. Column chromatography was carried out using a glass column of diameter 0.25mm thickness and silica gel (60 – 120) mesh size. Thin Layer Chromatography (TLC) was carried out on Machery-Nagel polygramSil/G/UV254 pre-coated plates. Gas Chromatography Mass spectrometry (GC-MS) were recorded using an Agilent 19091S-433UI instrument at multi user laboratory, ABU Zaria. Infrared spectroscopy (IR) was recorded using INFRA FTIR 3000A at NARICT Zaria. Melting points analysis were recorded using an Electrothermal 9100 melting point apparatus. All other chemicals were purchased from Sigma Aldrich and used without further purification.

Phytochemical Screened and Bioactive Compounds

Phytochemical constituents are the basic source of raw materials for the pharmaceutical industries (Nicolas *et al.,* 2021). The most important of these bioactive constituents of plants are alkaloids, saponins, terpenoids, tannins, flavonoids and phenolic compounds.

Biological and pharmacological properties of phytochemical compounds depend on several parameters such as species type, ecological factors and environmental conditions. Thus, each plant species will present a unique profile of phytochemicals based on factors such as phenological age of the plant, percentage humidity at time of harvest and method of extraction constitute possible sources of variation for phytocompounds toxicity and bioactivity of extracts (Musyimi, 2008).

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Collection and Identification of Plant Materials

Indigofera nummulariifolia was harvested in October 2022, from Malam Inna Area Gombe, GombeState, Nigeria and was identified at the Department of Botany, Gombe State University.The shade-dried sample was pulverized to powder with a motorized miller and successfully macerated with solvents of varying polarity (n-hexane, dichloromethane, ethyl acetate & methanol)as described by (Greatman, *et al*., 2019).

Extraction and Fractionation

About 1 liter of n-hexane was added to the sample (500g) in the amber bottle and was shaken vigorously the mixture was left in the laboratory for 3 days with continuous shaking every day. It was then filtered using a Whatman filter paper. After previous extraction with n-hexane and DCM, the plant residues were then extracted with ethyl acetate and filtered again. The filtrate from the ethyl acetate extract was evaporated using a rotary evaporator at ambient temperature to afford an ethyl acetate extract 12 g. fraction of the ethyl accetate extract was used for phytochemical screening and antimicrobial analysis while the rest was purified using column chromatography after it was dried under water bath and vacuumas described by (Brusotti *et al*., 2013)

Preliminary Phytochemical Analysis

The crude extracts obtained was tested for the presence glycosides, tannins, flavonoids, saponins, anthraquinones, alkaloids, and steroids using the standard procedures (Prashant *et al.,* 2011).

Column Chromatography

The ethyl accetate fraction (12 g) underwent column chromatography packed using a silica gel of (60 – 120 mesh size). The column was initially eluted with 100% n-hexane, followed by a sequential elution using hexane: ethyl acetate ratios of 95:5, 90:10, and 85:15, reaching 100% ethyl acetate a procedure similar to a procedure reported by Kwaji*et al* 2019. Fractions were collected and those with similar retention factor (rf) values were pulled together based on their thin-layer chromatography (TLC) profiles. This consolidation resulted in the isolation of a white amorphous compound **INE1** with an rf value of 0.59 at solvent ratio hexane-ethyl acetate 95: 5.

Gas Chromatography Mass Spectrometry GC-MS Analysis of the isolate

The isolated **INE1** fraction was then subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis. In this analytical procedure, approximately 1 µl of the extract in Acetone, was introduced into the GC-MS instrument through the injection port, employing a 10µL micro syringe. (Omar *et al.,* 2022). The obtained mass spectrum was then calibrated, and the components' identity was established by comparing retention time and mass spectra fragmentation patterns with those stored in the computer library and referenced in published literature (Mohammed *et al.,* 2016).

Antimicrobial Analysis

The crude ethyl acetate extract was screened for in vitro antimicrobial activities against some selected bacterial strains namely; two gram-negative bacterial strains (*Escherichia coli and Salmonella typhi*) and two gram-positive (*Pseudomonas aeruginosa and Staphylococcus aureus*) using a procedure reported by Kwaji*et al* 2018. The result shows significant activity with a zone of inhibition in the range of 6mm-20mm. The stock solution was prepared by dissolving 5 mg of the extract in 5 mL of DMSO solvent. A serial dilution of different concentrations of 200 µg/mL, 400 µg/mL, 600 µg/mL, and 800 µg/mL were prepared from the stock. The solvent DMSO was used as negative control and standard antibacterial drug

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(Augmentin) 125 µg/disc was used as a positive control for comparison of activities with the crude extract. The bacteria were then subcultured in the Muller Hilton agar medium. What Man filter paper discs of size 6 mm diameter were sterilized in an autoclave and then soaked in the chosen concertation of the compounds and placed in the Petri dishes containing the Muller Hilton agar media seeded with the respective bacteria strain. The culture was then incubated in an oven at 37°C. The diameters of the zones of inhibition were measured after 18 hours of incubation. The antimicrobial activities were calculated as an average of three replicates. The zones of inhibitions were measured using a ruler in millimetres (mm), and the following criteria were adopted. Strong activity (> 14 mm), moderate activity (9–14 mm), weak activity (5–8 mm), NA; no activity (inhibition zone < 5 mm), solvent: DMSO (NA)

RESULTS AND DISCUSSION

The research conducted on the ethyl acetate extract of *IndigoferaNumularifolia* yielded insightful results that shed light on its phytochemical composition and antimicrobial potential. The phytochemical screening (Table 1) revealed the presence of saponins, flavonoids, tannins, and steroids, while alkaloids, anthraquinones, and glycosides were absent. These findings are consistent with the known phytochemical profile of *Indigofera*species, which are recognized for their diverse array of bioactive constituents (Ngoci *et al.,*2011). Saponins, flavonoids, and tannins, in particular, have been associated with various biological activities including antioxidant, anti-inflammatory, and anticancer properties, suggesting the potential pharmacological significance of the extract (Gerometta *et al.,* 2020).

Table 1: Results for Phytochemical screening.

Phytochemical constituents	Tests	Inference
Alkaloids	Wegner'sTest	-
Saponins	FrothTest	+
Flavonoids	Alkalinereagenttest	+
Tannins	Ferric Chloridetest	+
Anthraquinones	Free Anthracenestest	-
Steroids	Salkowski'stest	+
Glycosides	Borntrager'stest	-

NB. – Absent, + Present

Structural Elucidationusing IR and GC-MS

Column chromatography further refined the extract, yielding a colorless viscous compound with a retention factor of 0.59 at a hexane-ethyl acetate solvent ratio of 95:5. Infrared Spectroscopy IR spectrum (Figure 1) provided structural insights into the isolated compounds, identifying functional groups characteristic of ester derivatives. The presence of C=O ester around 1780 $cm^{-1}$and C-O groups cica 1200 cm^{-1}suggests the presence of esters, which are common in fatty acid derivatives. Additionally, the detection of C=C around 1654 cm^{-1} and sp3/sp2 C-H bonds further justified the identification of the compounds.

Further elucidation of the compounds was supported through Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The various fatty acids derivatives found are presented in Table 2. The isolated fatty accids includes hexadecanoic acid methyl ester, Z-9-octadecenoic acid methyl ester, and octadecanoic acid ethyl ester. The presence of theses group, highlights the lipidic nature of the plant. Comparing these results with relevant literature, similar phytochemical profiles have been reported for other *Indigofera* species. For example, studies on *Indigoferatinctoria* have identified saponins, flavonoids, and fatty acids among its major constituents, corroborating the findings of the present study (Ahmadu*et al.,* 2011). Moreover, the bioactivities attributed to the compound, are

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consistent with the reported pharmacological activities of *Indigofera* extracts (Campos *et al.,* 2018).

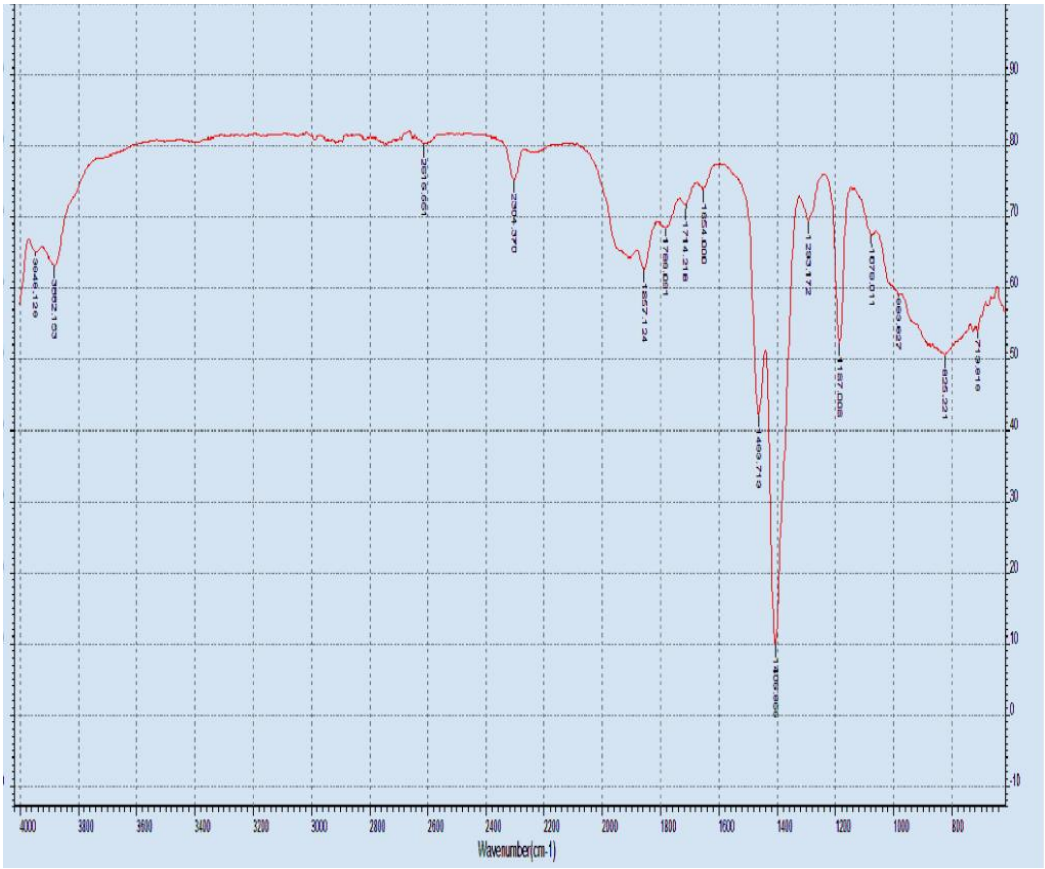

Figure 1: FTIR Spectrum of the compound.

Antimicrobial analysis

The antimicrobial analysis (Table 3) further revealed the extract's notable activity against both gram-negative and gram-positive bacteria, indicating its potential as a source of novel antimicrobial agents. The observed zone of inhibition ranging from 6 mm to 20 mm at various concentrations suggests a concentration-dependent antimicrobial effect, with higher concentrations yielding larger zones of inhibition. Such findings align with the growing interest in natural products as alternative sources of antimicrobial agents, particularly in the face of rising antimicrobial resistance (Bueno *et al.,* 2013).

CONCLUSION

The study on *Indigofera nummulariifolia* has provided valuable insights into the medicinal properties of this plant, justifying its traditional use in ethno-medicine. The extensive phytochemical analysis revealed the presence of various bioactive compounds, including saponins, flavonoids, tannins, and steroids. The antibacterial activity testing against common pathogens demonstrated significant inhibition with the ethyl acetate extract, suggesting the potential of this plant in treating bacterial infections. Further investigation through column chromatography led to the isolation of six fatty acids. The identified compounds open up possibilities for diverse applications in various industries, including food, cosmetics, and pharmaceuticals. The structural determination of these compounds through IR and GCMS analyses adds credibility to their potential therapeutic properties.

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Table 2: The GC-MS identified ester derivatives in the ethyl acetate extract of *Indigofera Nummulariifolia.*

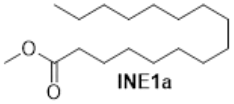
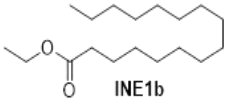
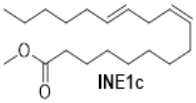
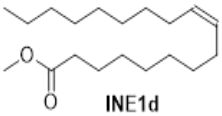
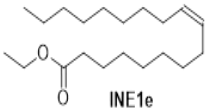
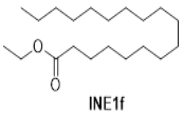
S\N	Chemical Compound	Chemical Structure	RT(Min)	Molecular weight	Extract mass	MS fragment ion
1	Hexadecanoic acid methyl ester	 INE1a	29.3	270.46	270.26	43, 74, 97, 143, 185, 227, 270
2	Hexadecanoic acid, ethyl ester	 INE1b	30	284.48	284.27	55, 88, 115, 157, 199, 241, 284
3	9,12-Octadecadienoic acid	 INE1c	30.9	294.48	294.26	41, 67, 95, 123, 150, 178, 263, 294
4	Z-9-Octadecenoic acid, methyl ester	 INE1d	31	296.50	296.27	55, 83, 111, 138, 180, 222, 264, 296
5	E)-9-Octadecenoic acid ethyl ester	 INE1e	31.4	310.52	310.29	55, 83, 111, 137, 180, 222, 264, 310
6	Octadecanoic acid, ethyl ester	 INE1f	31.6	312.54	312.30	43, 88, 115, 157, 213, 241, 269, 312

Table 3: AntimicrobialSensitivityofEthylacetateextract of *IndigoferaNumularifolia.*

Clinical isolate	Zoneofinhibition(mm)				Control
	800µg/ml	600µg/ml	400µg/ml	200µg/ml	Augmentin 125 µg/disc
E. coli	20	17	13	11	22
S. aureus	17	16	12	12	24
T. typhi	18	16	14	10	21
P. aeruginosa	16	13	12	11	22

Strongactivity(>14mm),moderateactivity(9–14mm),weakactivity(5–8mm),NA;no activity (inhibition zone < 5 mm), solvent: DMSO (NA).

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