https://dx.doi.org/10.4314/dujopas.v10i3b.6
ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
Dutse Journal of Pure and Applied Sciences (DUJOPAS), *Vol. 10 No. 3b September 2024*

Phytochemical screening, Antibacterial activity, and Isolation of bioactive compounds from methanol extract of *Indigofera nummulariifolia* (Livera) Ex. Alston (Fabaceae)

Abdulkadir Abubakar[1], Samaila Abubakar[1]*, Andrew Kwaji[1]
Department of Chemistry,
Faculty of Science,
Gombe State University,
PMB 127, Gombe,
Nigeria

Email: sabubakar@gsu.edu.ng

Abstract
*The herb Indigofera nummulariifolia, belonging to the Fabaceae family, exhibits a global distribution in tropical and subtropical regions, including Nigeria, India, Madagascar, Australia, and parts of Asia. Herein, we reported the results of phytochemical screening, antibacterial activity, and isolation and characterization of bioactive compounds campesterol **INE2** and stigmasterol **INE3** from the methanol extract of the plant. The crude extracts (n-hexane, dichloromethane, ethyl acetate, and methanol) was obtained by cold maceration of the pulverized plant sample. Phytochemical screening of these extracts, detected alkaloids, saponins, flavonoids, tannins, steroids, and glycosides in the methanol extract. Ethyl acetate extract tested positive for saponins, flavonoids, tannins, and steroids, while dichloromethane and n-hexane extracts tested negative for flavonoids, tannins, and steroids. All extracts exhibited antibacterial activity against Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, and Staphylococcus aureus, with inhibition zones ranging from 6 mm to 20 mm. Particularly, the dichloromethane extract demonstrated significant inhibition, recording an 18 mm zone against E. coli at 800 µg/mL, as well as comparable activity against S. typhi and P. aeruginosa. Further purification of the methanol extract via column chromatography resulted in the isolation of campesterol **INE2** and stigmasterol **INE3**. The isolated compounds were characterised using FTIR and GCMS.*

Keywords: Campesterol, *Indigofera Numulariifolia*, Isolation, Phytochemical screening, Stigmasterol,

INTRODUCTION
The field of natural product chemistry has long captivated the scientific community with its diverse array of intriguing compounds derived from the world's most complex and awe-inspiring chemical factories: living organisms. These compounds, synthesized by plants, microbes, marine organisms, and even some animals, have played a pivotal role in human history, serving as the foundation for numerous pharmaceuticals, agrochemicals, and other valuable products.(Luo *et al.,* 2011; Atanasov *et al.,* 2021; Newman, 2022)

The *Fabaceae* family or pea family, represents a vast and diverse group of plants with profound economic and ecological implications. Their remarkable nitrogen-fixing abilities, achieved through symbiosis with nitrogen-fixing bacteria, have drawn considerable interest due to the

Author for Correspondence

array of bioactive compounds they contain, offering potential applications in medicine, agriculture, and industry (Grygier *et al*., 2022). *Indigofera species*, including *Indigofera aspalathoides*, *Indigofera heterantha*, *Indigofera tinctoria*, and *Indigofera suffruticosa*, have been historically utilized for various purposes, particularly in traditional medicine. For example, *Indigofera aspalathoides* leaves have been employed in treating a range of ailments, while *Indigofera heterantha* and *Indigofera tinctoria* serve medicinal purposes for gastrointestinal disorders and constipation, among others (Pullaiah, 2006; Amrithpal, 2006; Warrier, 2007; Muhammad and Choudhary 2011).

Chemical analyses of *Indigofera species* have identified several common compounds such as flavonoids, fatty acids, and steroids. Notable among these are flavonoidal compounds isolated from *Indigofera hebepetala* and fatty acid compounds from *Indigofera suffruticosa* (Vijisaral *et al.,* 2014; Taj *et al.,* 2016). Even though, extensive research has elucidated the phytochemical compounds in the *Indigofera genus*.

In view of the absence of any such report given its significance in herbal medicine practice of the plant base on the literature available, consequently, there is need for the isolation and characterization of the bioactive compounds of *Indigofera nummularifolia* (Lev, 2008).Therefore, this study sought to validate and provide scientific substantiation for the traditional medicinal uses associated with *Indigofera nummulariifolia,* offering valuable insights for researchers in natural products and bioactive compound-related fields. Through isolating and characterizing phytochemical compounds from the plant. In view of that, we have reported the isolation of campesterol **INE2** and stigmasterol **INE3** from the methanol extract of *Indigofera Numulariifolia*.

MATERIALS AND METHODS

Collection and Identification of the Plant Materials

Indigofera nummulariifolia was collected in October 2022 from Malam Inna Area, Gombe, Gombe State, Nigeria, and authenticated at the Department of Botany, Gombe State University.

Laboratory procedures

Extraction and Fractionation

Approximately 1 litre of n-hexane was added to the sample (500 g) in an amber bottle and vigorously shaken. The mixture was left in the laboratory for 3 days with daily agitation. Subsequently, it was filtered using Whatman filter paper. The resulting residue underwent sequential treatment with dichloromethane, ethyl acetate, and methanol, following the aforementioned procedure (Brusotti *et al.,* 2013). All crude extracts were concentrated under reduced pressure at 45°C using a rotary evaporator. The crude fractions from all extracts were subjected to phytochemical and antimicrobial analyses, while the dichloromethane and methanol fractions were later combined for purification through column chromatography (Brusotti *et al.,* 2013).

Antimicrobial Analysis

The crude extracts (n-hexane, dichloromethane, ethyl acetate) were evaluated for in vitro antimicrobial activity against selected bacterial strains obtained from (Microbiology department GSU) and were screened using gram staining technique including two gram-negative strains (*Escherichia coli* and *Salmonella typhi*) and two gram-positive strains (*Pseudomonas aeruginosa* and *Staphylococcus aureus*), following the procedure outlined by Kwaji

et al, 2018. A stock solution was prepared by dissolving 5 mg of the extract in 5 mL of DMSO solvent. Serial dilutions were then made to obtain concentrations of 200 µg/mL, 400 µg/mL, 600 µg/mL, and 800 µg/mL from the stock solution. DMSO served as the negative control, while a standard antibacterial drug (Augmentin) at 10 µg/disc was used as the positive control for comparison with the crude extract. The bacterial strains were sub cultured in Muller Hilton agar medium. Sterile Whatman filter paper discs (6 mm diameter) were autoclaved and soaked in the desired concentrations of the Compounds, then placed on Petri dishes containing Muller Hilton agar media inoculated with the respective bacterial strains. The cultures were incubated at 37 °C in an oven, and after 18 hours, the diameters of the inhibition zones were measured. Antimicrobial activities were averaged over three replicates, and the zones of inhibition were measured in millimeters (mm). The criteria for interpreting activity were as follows: strong (> 14 mm), moderate (9–14 mm), weak (5–8 mm), no activity (inhibition zone < 5 mm), with DMSO serving as the solvent control for comparison (Nicolas *et al*., 2021).

Phytochemical Analysis

The crude extracts were subjected to standard procedures to detect the presence of glycosides, tannins, flavonoids, saponins, anthraquinones, alkaloids, and steroids, as outlined by Prashant *et al*. in 2011.

Column Chromatography

The methanol fraction (13 g) was subjected to column chromatography packed with silica gel (60 – 120 mesh size). Initially, the column was eluted with 100% n-hexane, followed by stepwise elution using hexane:ethyl acetate ratios of 95:5, 90:10, and 85:15, gradually transitioning to 100% ethyl acetate. Subsequently, elution continued with ethyl acetate:methanol ratios of 95:5, 90:10, down to 70:30, resulting in the collection of two hundred and forty-three (243) fractions (each 20 mL). These fractions were combined based on their thin-layer chromatography (TLC) profiles to yield twenty-nine (29) major sub fractions (C1-C29), following a methodology similar to that reported by Kwaji *et al.* 2019.

The fractions were further analyzed, and those with similar retention factor (rf) values were pooled together based on their TLC profiles. Subsequently, two major sub fractions, C27 and C29, were obtained using a ethyl acetate methanol at ratio of 3:7 as the mobile phase solvent system. Each of these subfractions exhibited one major spot with Rf values of 0.46 and 0.47, respectively, resulting in the isolation of pure yellow crystalline solid compounds coded as **INE2** and **INE3**. The compound **INE2** has a melting point value of 154-156 ^{o}C while **IN3** has a melting point of 161-163 ^{o}C.

Gas Chromatography Mass Spectrometry GC-MS Analysis of the isolates

The two isolates **INE2** and **INE3** fractions were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). Around 1 µL of the extract dissolved in acetone was introduced into the GC-MS instrument via the injection port, utilizing a 10 µL micro syringe, as described by Omar *et al.*2022. The resulting mass spectrum was calibrated, and the identification of components was achieved by comparing retention time and mass spectra fragmentation patterns with those stored in the computer library and referenced in published literature, following the methodology outlined by Mohammed *et al*. 2016.

RESULTS AND DISCUSSION

Table 1: Preliminary phytochemical screening of *Indigofera nummulariifolia*

Phytochemical constituents	Test	n-hexane extract	DCMextract	Ethylacetate extract	Methanol extract
Alkaloids	Wegner's	-	-	-	+
Saponins	Froth	-	-	+	+
Flavonoids	Alkaline reagent	+	+	+	+
Tannins	Ferric Chloride	+	+	+	+
Anthraquinones	Free Anthracenes	-	-	-	-
Steroids	Salkowski's	+	+	+	+
Glycosides	Borntrager's	-	-	-	+

Note:+Present,-absent

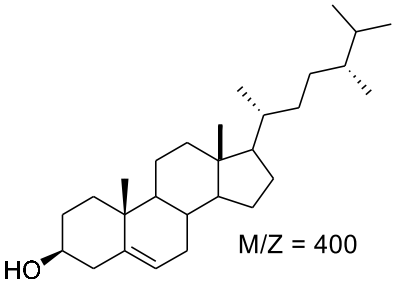
From the results as presented in Table 1, it was observed that both n-hexane and dichloromethane extracts revealed the presence of flavonoids, tannins, and steroids. At the same time, other phytochemicals tested like saponins, anthraquinones, alkaloids, and glycosides, were absent. However, in addition to the phytochemicals in the n-hexane and DMC extracts, ethyl acetate extract tested positive for saponins. These results agreed with a similar work reported by (Ngoci, *et al.,* 2011) where 25 saponins were reported present in ethyl acetate extract but absent in n-hexane due to the presence of its polar OH groups. Methanol extract was the only sample that tested positive for Alkaloids and Glycoside. It is worth noting that, based on the results obtained using the procedure outlined above, all the extracts tested negative for anthraquinones while tannins, flavonoids, and steroids were present in all four extracts. The presence of these phytochemicals in the plant testifies to its numerous therapeutic values as had been reported in many pharmacological of other plants (Cowan, 1999). Flavonoids and steroids have been found to possess good antimicrobial activities (Uddin, *et al.,* 2011).

Table 2: Results for Antimicrobial analysis of *Indigofera nummulariifolia*

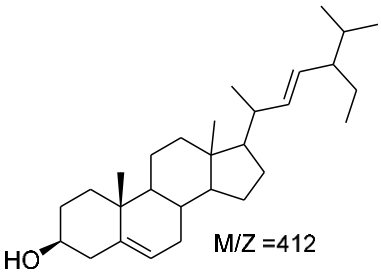
Extract	Conc µml	*E. coli*	*P.Aurogenusa*	*S.typhi*	*Staph*
n-hexane	800	11	12	9	9
	600	8	11	7	8
	400	6	8	7	7
	200	6	6	7	6
etthylaccetate	800	20	16	18	17
	600	17	13	16	16
	400	13	12	14	12
	200	11	11	10	12
DCM	800	18	17	18	12
	600	17	16	16	11
	400	12	14	12	10
	200	10	11	10	8
Augmentin	**10 µg/disc**	**23**	**22**	**26**	**19**
Solvent	**DMSO**	**00**	**00**	**00**	**00**

The antimicrobial assay results, as shown in Table 2, revealed that the n-hexane extract displayed low to moderate activity against both gram-positive and gram-negative bacteria, with inhibition zones ranging from 6mm to 12mm. Conversely, the dichloromethane extract demonstrated a substantial degree of inhibition against all tested organisms, with inhibition zones ranging from 18mm to 20mm at a concentration of 800 µg/mL against *E. coli*, *S. typhi*, and *P. aeruginosa*. These results were notably comparable to those of the standard drug

augmentin, which exhibited inhibition zones of 23 mm, 22 mm, and 26 mm against the respective bacterial strains. Additionally, it was observed that the antimicrobial activity increased proportionally with the concentration of the extract in all cases. These findings are consistent with several reports in the literature, as highlighted by Greatman *et al*. (2019), further validating the efficacy of the crude extracts from *Indigofera numulariifolia* in combating bacterial infections.



Campesterol **INE2**



Stigmasterol **INE3**

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis results of INE2 and INE3 provided valuable insights into the chemical composition of the analyzed fractions, particularly regarding the presence of stigmasterol and campesterol. Campesterol INE2 was detected at 36.9 minutes retention time with the highest peak area of 38, while stigmasterol INE3 was identified with a retention time of 30 minutes and a peak area of 37. The mass spectra of both compounds further confirmed their identity. For instance, the mass of the isolated compound, INE2, Figure 1 showed the parent molecular ion peak at m/z 400 and a base peak at m/z 43(100%) abundance. Further analysis using FTIR supported the idea that the compound isolated was campesterol. This is because in the IR spectrum of INE2, Figure 1, an absorption band due to the presence of H-O group was observed around 3874 cm^{-1} there is another peak s at 3200cm^{-1} indicating the intermolecular hydrogen bonding O-H stretch, 1406cm^{-1} indicates the presence of C-H bending stretch, absorption at 1187.01cm^{-1} for C-O stretching of tertiary alcohol while peak at 1654.0cm^{-1} indicates the C=C stretch and at 988cm^{-1} of RC=CH is due to C-H out of plane bending, (Nariya, 2014).

Similarly, for INE3, results obtained from the GCMS analysis of the compound showed parent molecular ion peak at m/z 412 Figure 3. A careful study of the supporting information provided by the library suggested the compound to be Stigmasterol, a member of the steroid family previously reported in the *Fabaceae* (Habib *et al*., 2007). The base peak is at m/z 55 (100%). The fragments of other peaks relative to the base peak are m/z 379, 351, 300, 255, 213, 159.2, 119, 83 and 55. Other spectral characterization such as FTIR and Mp confirmed our claim. For instance, the OH absorption band was observed circa 3800 cm^{-1}similarly, the other prominent peak due to C=C stretching vibration was seen around 1664 cm^{-1}. All results are in agreement with similar reports for stigmasterol FTIR data (Erwin *et al*., 2020). The presence of characteristic absorption peaks in the FTIR spectra, combined with the mass spectral data obtained from GC-MS analysis, confirms the identity of stigmasterol and campesterol in the analyzed fractions. These results are consistent with previous reports in the literature, corroborating the accuracy and reliability of the analytical methodology employed in this study (Jain *et al*., 2010).

CONCLUSION

The phytochemical screening revealed the presence of alkaloids, saponins, flavonoids, tannins, steroids, and glycosides in the methanol extract. Ethyl acetate extract tested positive for saponins, flavonoids, tannins, and steroids, while dichloromethane and n-hexane extracts tested negative for flavonoids, tannins, and steroids. Particularly, the dichloromethane extract demonstrated significant inhibition, recording an 18 mm zone against *E. coli* at 800 µg/mL, as well as comparable activity against *S. typhi* and *P. aeruginosa*. Further purification of the methanol extract via column chromatography resulted in the isolation of campesterol INE2 and stigmasterol INE3. In conclusion, this study contributes to validating the ethnomedicinal use of *Indigofera nummulariifolia* and provides scientific evidence for its antimicrobial properties. The study further enrich our understanding of the pharmacological potential of this plant species, particularly in combating bacterial infections, and pave the way for future research endeavours in natural product discovery and drug development.

ACKNOWLEDGMENT

Abdul-kadir A. Samaila A. and Kwaji A. thanks Gombe State University for providing enabling environment to conduct the research. AA also thanks Gombe State ministry of education for study fellowship.

REFERENCES

Atanasov, A. G., Zotchev, S. B., Dirsch, V. M., Orhan, I. E., Banach, M., Rollinger, J. M., *et al.,* (2021). Natural products in drug discovery: advances and opportunities. *Nature Reviews Drug Discovery*, **20**(3): 200–216.

Amrithpal, S. (2006). Medicinal plant of the wourld oxford &IBH" publishing Co. Pvt. Ltd. New Delhi. 168-169.

Brusotti, G., Cesari, I., Dentamaro, A., Cacciallanza, G., Massolini, G. (2013) isolation and characterization of bioactive compounds from plant source. *Journal of pharmaceutical and biomedical analysis* **8**: 5-7.

Erwin, Pusparohmana, W. R., Safitry, R. D., Marliana, E., Usman, and Kusuma, I. W. (2020). Isolation and characterization of stigmasterol and β-sitosterol from wood bark extract of baccaurea macrocarpa miq. Mull. arg. *Rasayan Journal of Chemistry*, **13**(4): 2552–2558.

Greatman, C.O., Jibrin A. N. (2019). Phytochemical and Antimicrobial Investigation of *Indigofera nummulariifolia. International Research Journal of Engineering and Technology* **6**: 1559-1563.

Grygier, A., Chakradhari, S., Ratusz, K., Rudzińska, M., Patel, K. S., Lazdiņa, D., *et al*. (2022). Seven underutilized species of the Fabaceae family with high potential forindustrial application as alternative sources of oil and lipophilic bioactive compounds. *Industrial Crops and Products*, **6**(11): 186-191.

Habib, M. R., Nikkon, F., Rahman, M., Haque, M.E., and Karim, M.R. (2007). Isolation of stigmasterol and beta-sitosterol from methanolic extract of root bark of *Calotropis gigantea (Linn)*. Pakistan *Journal of Biological Sciences*. **10**(22): 4174–4176.

Jain, P.S. and Bari, S.B. (2010). Isolation of Lupeol, Stigmasterol and Campesterol from Petroleum Ether extract of woody stem of *Wringhtia tinctoria AsianJjournal of Plant Science* **9**(3): 163-167.

Kwaji, A., Adamu, H.M., Chindo, IY., & Atiko, R. (2018). Isolation, characterization and biological properties of betulin from Entada africana Guill. and Perr. (Mimosaceae). *Journal of Applied and Advanced Research*, **2**(3): 28-31.

Luo, X., Pires, D., Aínsa, J. A., Gracia, B., Mulhovo, S., Duarte, A.,*et al.*(2011). Antimycobacterial evaluation and preliminary phytochemical investigation of

selected medicinal plants traditionally used in Mozambique. *Journal of Ethnopharmacology*, **137**(1): 114–120.

Lev, E. and Amar, Z. (2008)."Fossils" of practical medical knowledge from medieval Cairo. *Journal of Ethnopharmacology*. **119**: 24–40.

Mohammed Y.H., Ghaidaa J.M., and Imad H.H. (2016), Analysis of bioactive chemical compounds of *Nigella sativa* using gas chromatography-mass spectrometry. *Journal of Pharmacognosy and Phytotherapy.* **8**(2): 8-24.

Mohammad, I.K.G. and Choudhary, M.I. (2011). In-vitro Pharmacological Investigations of Aerial Parts of *Indigofera heterantha*, *Journal of Medicinal Plants Research.* **5**(24): 5750-5753.

Nariya, P. (2014). Isolation and characterization of phytosterols from Cordia macleodii (hook f. and thomson) bark by chromatographic and spectroscopic method. *Asian Journal of Pharmaceutical and Clinical Research*, **7**: 86–88.

Newman, D. J. (2022). Natural products and drug discovery. *National Science Review*, **9**(11): 1-14.

Nicolas, A. M., Alvine, M. N., Gaëtan, N. B and Dongmo, M. (2021). Bioguided isolation, structure elucidation and evaluation of new antimicrobial flavonoid from *Carica papaya* leaves. *Journal of Pharmacognosy and Phytochemistry*; **10**(1): 32-39.

Ngoci S.N., Mwendia C.M and Mweniki C. G. (2011), phytochemical and cytotoxicity testing of *Indigofera lupatana*, *Journal of plant and animal science* **11**: 1364-1373.

Omar H.A., Ali, H.A., Yahya S.Y., Mohammed A.and Ezghayer. (2022). GC-MS Analysis of Hexane Fraction of *Indigofera Suffruticosa* Plant Naturally Growing in Iraq.**20**(4): 393-396.

Pullaiah, (2006). Encyclopedia of world medicinal plants, Regency Publications, New Delhi, **3**(11): 43-45.

Prashant, T., Bimlesh, K., Mandeep, K., Gurpreet, K. and Harleen, K. (2011). Phytochemical Screening and extraction: A Review. *Internationale Pharmaceutica Sciencia*. **1**(1): 98 106.

Taj, U. R. Rahman, (2014). Isolation and characterization of a novel ester from seeds of *Indigoferaheterantha*(Wall) *Journal of Natural Product.* **7**: 104-112.

Uddin, G., Rehman, T.U., Arfan, M., Liaqat, W., Mohammad, G., and Choudhary, M.I. (2011). Phytochemical analysis, antifungal and phytotoxic activity of seeds of medicinal plant *Indigofera heterantha* Wall. *Middle-East J.Sci. Res.* **8**: 603-605.

Vijisaral and S. Arumugam, (2014), "GC-MS analysis of bioactive constituents of Indigofera suffruticosa leaves," *Journal of Chemical and Pharmaceutical Research.* **6**(8): 294 - 300.

Warrier, P.K.., Numbiar, V.P.K., and Ramankatty, (2007). Indian medicinal plant" published by Orient longman private limited chenni. **3**: 210-213.