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DETECTION AND ANTI-INFLAMMATORY ACTIVITY OF COMPOUNDS FROM *Amblygonocarpus andongensis* PLANT AND ITS ENDOPHYTIC FUNGUS (*Aspergillus niger)*

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Abstract

Endophytic fungi are microorganisms residing within healthy tissues of host plants, and are capable of producing several bioactive compounds that are identical or similar to those from their host plants. The aim of this work was to carry out a comparative study of bioactive compounds from *Amblygonocarpus andongensis* and its endophytic fungus. Plant leaf material was sampled from Bakori Local Government, Katsina State, Nigeria and identified at the herbarium unit of Ahmadu Bello University Zaria were number ABU4167 was deposited for future reference. Fungal isolation, identification and extraction were conducted following standard laboratory procedures. Extracts obtained were subjected to Thin Layer Chromatography and Liquid Chromatography-Mass Spectrometry analyses as well as anti-inflammatory assay using diclofenac sodium as positive control. The TLC and LCMS analyses conducted on the extracts revealed presence of diversity of compounds in which some are chemically related. Both plant and fungal extracts were found to display a higher anti-inflammatory activity due to lower IC_{50} value of 1.770 and 1.911 µg/ml respectively relative to diclofenac sodium (IC_{50} value 4.731 µg/ml). This research for the first time reported the anti-inflammatory activity of *Amblygonocarpus andongensis* leaf and its endophytic fungus extracts as well as relative number of bioactive compounds present in them.

Keywords: *Amblygonocarpus andongensis*; *Aspergillus niger*; Anti-inflammatory; TLC; LC-MS

INTRODUCTION

Endophytic fungi refer to polyphyletic group of unique microorganisms that reside in healthy tissues of host plants without causing apparent damage (Chatterjee *et al*., 2019; Singh *et al*., 2021). A wide variety of these microorganisms colonize plants' roots from bulk soil (Taniguchi *et al*., 2023). Enzymes including cellulase, laccase, pectinase and xylanase are then secreted that break down cell walls, and alter the physical and structural properties of the host cell wall, allowing them to penetrate and multiply within the host plant tissues (Jha *et al*., 2023) through xylem (Pandey *et al*., 2022). This results to symbiotic relationship in which the endophytes are called symbionts (Bibi *et al*., 2020)**.** During this association, endophytic fungi help the host plants in mitigating biotic and abiotic stresses by developing a "holobiont" (Ji *et al*., 2022), a combination of a host with its microorganisms in which there is a union of the host nuclear genes with that of microbial gene to form a "hologenome".

(Roughgarden, 2023). Consequently, the microorganisms and their host are intimately interconnected in terms of their development, metabolism, physiology and behavior (Roughgarden, 2023) in which the fungi become the accessory organs of the host plant (Ji *et al*., 2022)In addition to developing a holobiont, endophytic fungi produce bioactive substances such as phenols, polyketides, saponins and alkaloids according to Jha *et al.* (2023), to help the host plants in mitigating these challenges. Evidence has shown that endophytic fungi have developed the ability to produce new, same or similar bioactive substances as those originated from their host plants (Wen *et al*., 2022; Ahmed *et al*., 2023)**,** particularly when the plant has pharmaceutical importance (Omomowo *et al*., 2023). Taxol, a chemotherapeutic drug was isolated from *Taxomyces andreanae*, an endophytic fungus from *Taxus brevifolia*, a taxol producing plant (Aboobaker *et al*., 2019).

Among the varieties of endophytic fungi, species of the genus *Aspergillus* have garnered significant attention for their capacity to biosynthesize a broad range of bioactive compounds (Frisvad & Larsen, 2016; He *et al*., 2020). *Aspergillus niger*, a well-known member of this genus, has been isolated from various ecological niches and has shown notable chemical diversity, including the production of various secondary metabolites with a broad spectrum of applications in agriculture, food, and pharmaceutical industry (Yu *et al*., 2021; Wei *et al*., 2022).

Amblygonocarpus andongensis is a tree widely distributed in the savanna region of Africa belonging to the family *Mimosaceae*, and is known for its medicinal uses in the treatment of ulcers, diarrhea, diabetes, hypertension, wounds and inflammation (Rampart *et al*., 2021; Baponwa *et al*., 2022).

In an effort to conduct a relative study of bioactive compounds from endophytic fungus and its host plant, *Amblygonocarpus andongensis* leaf and its isolated endophytic fungus, *Aspergillus niger* were investigated.

MATERIALS AND METHODS

Materials

Petroleum ether and n-hexane were purchased from Chemiz Chemicals, Malaysia, ethyl acetate and methanol were purchased from Systerm and R & M Chemicals Malaysia respectively. Corning 96 well

plates**,** Aluminium precoated TLC plates with silica gel (GF254, 20×20 cm, 0.25±0.03 mm thickness and 8±2 µm≥80% particles size) were purchased from Shanghai Shengya Chemical Co. Ltd, Gonghe New Road, Shanghai. Weighing balances (Mettle Toledo AB54) and (Mettler- Toldeo PB303-S/FACT TOP-PAN) were used for weighing purposes at Microbiology Departments ABU, Zaria and Metabolomic Laboratory, INBIOSIS, UKM, Malaysia. Absorbance readings for DPPH assay were taken using micro plate reader (iMark™/13390) at Metabolomic Laboratory, INBIOSIS, UKM, Malaysia. Diclofenac Na used in this research was obtained from Metabolomic Laboratory, INBIOSIS UKM, Malaysia.

Sampling and Authentication of Plant Material

Fresh and healthy leaves of *Amblygonocarpus andongensis* plant were collected in a sterile plastic bag from Bakori local government zone, Katsina State, (GPS coordinates: 11° 33′ 20.12″ N, 7° 25′ 27.08″ E), brought to the laboratory and processed within 24 h (Kuo *et al*., 2021). The fresh leaf was authenticated by Mal. Namadi, a botanist at the Herbarium Unit, Biological Sciences Department, Ahmadu Bello University, Zaria where the Voucher Number (ABU4167) was deposited for future reference.

Fungal Isolation and Identification

The fresh leaf of *Amblygonocarpus andongensis* plant was thoroughly washed with running tap water to remove dust and debris then immersed in 70% ethanol for 10 s, followed by soaking in 4% sodium hypochlorite for 30 s and finally washed with sterile water. Small pieces were then cut using sterile forceps under aseptic condition and inoculated in Potato Dextrose Agar (PDA) plate supplemented with antibiotic (streptomycin) and incubated at 28±1 °C for 7 days. Pure colony was transferred on PDA plate and preserved at 4 to 5 °C with proper labelling and sub cultured from time to time (Qureshi *et al*., 2019). Fungal identification was conducted at Microbiology Laboratory, Ahmadu Bello University Zaria, Kaduna State, Nigeria. A drop of lacto phenol cotton blue (LPCB) was placed on a clean microscope slide. Mounted needle was used gently to remove a small portion of the colony and placed in the lacto phenol cotton blue drop. The mycelium was spread very well on the slide with the aid of two mounted needles after which it was covered with a cover slip. Excess liquid was wiped with the help of applying a gentle pressure around a cover slip placed between two filter papers. The prepared slide was examined under the microscope using × 4 ×10 and × 40 objectives (Obum-Nnadi *et al*., 2022). The isolated fungus, *Aspergillus niger* was coded **L4** and kept at 4 to 5 °C until use.

Extraction

Extraction of Plant Material

The shade dried and powdered plant leaf material (450 g) was percolated in methanol (2,250 ml) at room temp and allowed to stay for 18h. The leaf extract coded **F1** was then filtered, dried under vacuum on a rotary evaporator and kept in refrigerator until use for analysis (Ebbo *et al*., 2020).

Extraction of Fungal Material

Small portion of fungus on PDA plate was transferred to conical flask containing Czapek Dox broth media (100 mL). This was placed on a shaker at 100 revolutions per minute at room temperature for 7 days to form fungal seed culture. Fungal seed culture (1 mL) was transferred into 25 Erlenmeyer flasks containing sterilised D-mannitol media (200 mL) using sterilised syringe for incubation. The flasks were covered with sterilised bungs and kept static for 28 days at 28±1 °C and regularly observed for recording observations. Metabolite extraction was achieved by separating the mycelium from the culture broth with sterile Whatman® filter paper. The fermentation broth was extracted separately three times in equal amounts (200 mL) of ethyl acetate. The combined ethyl acetate layers were concentrated at reduced pressure and allowed the remaining solvent to evaporate by air drying at room temperature to further concentrate the extract. The fungal extract was coded **F5** and stored at 4 °C until further use (Chatterjee *et al*., 2020).

Thin Layer Chromatography (TLC) of the Extracts

TLC was conducted according to the method describe by Ginovyan *et al*., (2020). A spot of the extract prepared was placed on aluminium pre-coated thin layer chromatography plate using capillary tube and allowed to dry. The plate was developed in appropriate solvent involving petroleum ether (100%) and n-hexane:Ethyl acetate gradient (70:30, 50:50 and 30:70%). The retention factor (R_f) value was calculated using the following relation:

$$R_f \text{ value} = \frac{Distance\ moved\ by\ the\ sample}{Distance\ moved\ by\ the\ solvent}$$

Liquid Chromatography Mass Spectrometry (LCMS) of the Extracts

The LCMS analysis was performed using Thermo Scientific C18 column (AcclaimTM Polar Advantage II, 3 x 150mm, 3um particle size) on an UltiMate 3000 UHPLC system (Dionex) consisting of 4 sections (solvent tray, pump, autosampler and column compartment). Gradient elution was performed at flow rate of 0.4 mL/min and 40 °C column temperature using H_2O + 0.1% Formic Acid (A) and 100% Acetonitrile (B) with 22 min total run time. The injection volume of sample was 5 µl. The gradient started at 5% B (0-3 min); 80% B (3-10 min); 80% B (10-15 min) and 5% B (15-22 min). The equipment was optimized before the experiment by analyzing a standard solution (caffeine). Peak was identified by means of retention time. Mass spectrometric analysis was conducted using mass spectrometer (MicroTOF-Q III 8228888.20450 Bruker Daltonic) equipped with an electrospray ion source (ESI) operating both in positive and negative switching modes. MS/MS detection was achieved by using gaseous N_2 which served as nebulized gas to create a mist of charged droplets. Nebulization was achieved at 2.0 bar, 300 °C and dry gas flow rate 8.0 l/min. The scan mode for mass signal detection started at 50 *m/z* and ended at 1500 *m/z*. The voltage for ion spray and collision cell RF were set at 4500 V and 200.0 V, respectively.

Anti-inflammatory Assay of the Extracts

The anti-inflammatory assay was performed following the method described by Senadeera *et al*. (2021), with minor modifications. Each test sample (10 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to obtain a stock solution with a concentration of 10,000 µg/mL. Serial dilutions were conducted directly in a 96-well microplate to achieve concentrations of 1000, 500, 250, 125, 62.5, 31.25, and 15.625 µg/mL. Diclofenac sodium was used as the positive control and prepared at the same concentration series. The reaction mixture was composed of 2.8 mL phosphate-buffered saline (PBS, pH 6.4) and 0.2 mL fresh hen's egg albumin. Aliquots of 2 mL from each test sample or control at the respective concentrations (15.625–1000 µg/mL) were gently mixed with the reaction mixture. The mixtures were incubated in a water bath at 37 ± 2 °C for 15 minutes, followed by heating at 70 °C for 5 minutes. After cooling to room temperature, absorbance was measured at 660 nm using a microplate reader (iMark™/13390). Phosphate-buffered saline was used as the blank, and the positive control (diclofenac sodium) was processed in the same manner. All experiments were conducted in triplicate. The percentage inhibition of protein (albumin) denaturation for each test sample was calculated using the following formula:

$$\% \, Inhibition = 100 \times \left[\frac{1 - Vt}{Vc}\right]$$

Where: Vt = Absorbance of the test sample at 660nm, Vc = Absorbance of control at 660nm.

The percentage inhibition of protein denaturation (% inhibition) was converted to activity of inhibition concentration at 50 percent (IC_{50}) using SPSS statistical software. The graph of % inhibition was plotted against concentration (µg/mL).

STATISTICAL ANALYSIS

All tests were carried out with three replicates for each group of samples. Data from the study were analysed using one-way analysis of variance (ANOVA), and variant means were separated post-hoc using the least significant difference (LSD) method. Statistical package for social sciences (SPSS) version 20 was used for analyses. Significance was accepted at $P < 0.05$. Results were presented as means ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Extraction

The percentage yield and appearance of the extracts are presented in Table 1.

Table 1: Yield, Percentage Yield and Appearances of the Extracts

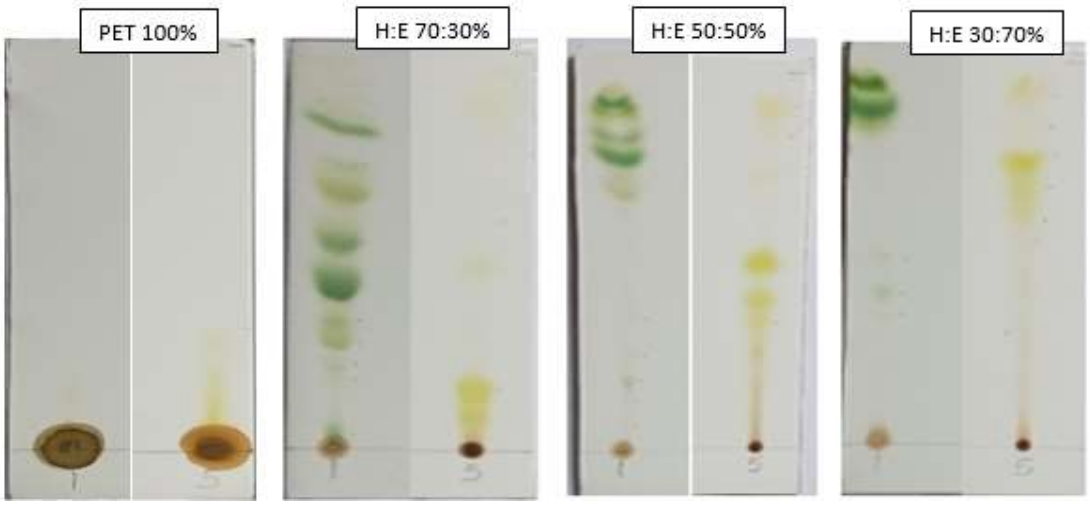
S/N	Fraction	Appearance	Yield	% Yield
1	F1	green solid	89,390mg/450g	19.86
2	F5	Yellowish-green solid	7,870mg/25L	-

F1: leaf extract; F5: fungal extract

Fractions F1 and F5 were found to appear as green and yellowish-green solids respectively. The percentage yield of the leaf was found to be 19.86% suggesting presence of insignificant amount of organic compounds in the plant material.

TLC Analysis

The TLC result presented in Table 2 below shows the number of spots and R_f values in different solvent system used while spots appearances are presented in Figure 1.



1: Fraction F1 (leaf extract); 5: fraction F5 (fungal extract)

Figure 1: Comparative TLC analysis of fractions F1 and F5 showing different compounds with different profiles.

Table 2: TLC Analysis of Fractions F1 and F5

Fraction	Solvent system	No. of spot (s)	R_f Value(s)
F1	PE (100%)	2	0.15, 0.89
	H:E (70:30)	13	0.03, 0.17, 0.21, 0.26, 0.31, 0.40, 0.52, 0.63, 0.71, 0.78, 0.85, 0.90, 0.97
	H:E (50:50)	11	0.19, 0.26, 0.35, 0.63, 0.67, 0.71, 0.76, 0.81, 0.87, 0.90, 0.93
	H:E (30:70)	7	0.32, 0.38, 0.47, 0.80, 0.83, 0.91, 0.94
F5	PE (100%)	3	0.09, 0.19, 0.28
	H:E (70:30)	5	0.08, 0.15, 0.45, 0.57, 0.92
	H:E (50:50)	6	0.34, 0.41, 0.50, 0.73, 0.84, 0.91
	H:E (30:70)	5	0.59, 0.67, 0.75, 0.83, 0.91

PE: Petroleum ether; H:E: n-Hexane:Ethyl acetate

Fraction F1 was found to produce 2 spots while fraction F5 produced 3 in PE (100 %) with different R_f values. A number of spots with different R_f values were observed in H:E (70:30) in which fraction F1 produced 13 with better separation whereas fraction F5 displayed 5. Fractions F1 and F5 yielded 11 and 6 spots respectively in H:E (50:50). Seven (7) and five (5) spots were observed in the case of fractions F1 and F5 respectively in H:E (70:30) solvent system. The comparative TLC analysis of the 2 fractions in H:E (30:70) has revealed presence of 2 spots for each at R_f values 0.83 and 0.91. This could be due to the presence of same or chemically related compounds in both samples.

LC-MS Analysis

The LC result from LCMS analysis presented in Figure 2 shows the number of compounds at different retention time while the MS result (Figures 3a and 3b) indicates compounds and their fragmentation pathways. Fraction F1 produced fewer number of compounds (43) relative to fraction F5 (50) in the LC analysis in which 2 sets of chemically related compounds at retention times 8.7 and 9.0 were observed (Figure 2). The comparative MS analysis has displayed a fragments at m/z 611 for eluted compounds at retention time 8.7 (Figure 3a) from which for each there were successive formation of fragments at m/z 589 and 567. Similarly, the fragments at m/z 230 for eluted compounds at retention time 9.0 (Figure 3b) proceeded with successive formation of fragments at m/z 229 and 212. The similarities of fragmentation pathways displayed by the MS analysis could be suggestive of the fact that fractions F1 and F5 contained similar compounds or moieties. Moreover, the ability for the fungi and plant to produce the same or similar compounds could be associated with their ability to share genetic information through formation of holobiont during their symbiotic relationship.

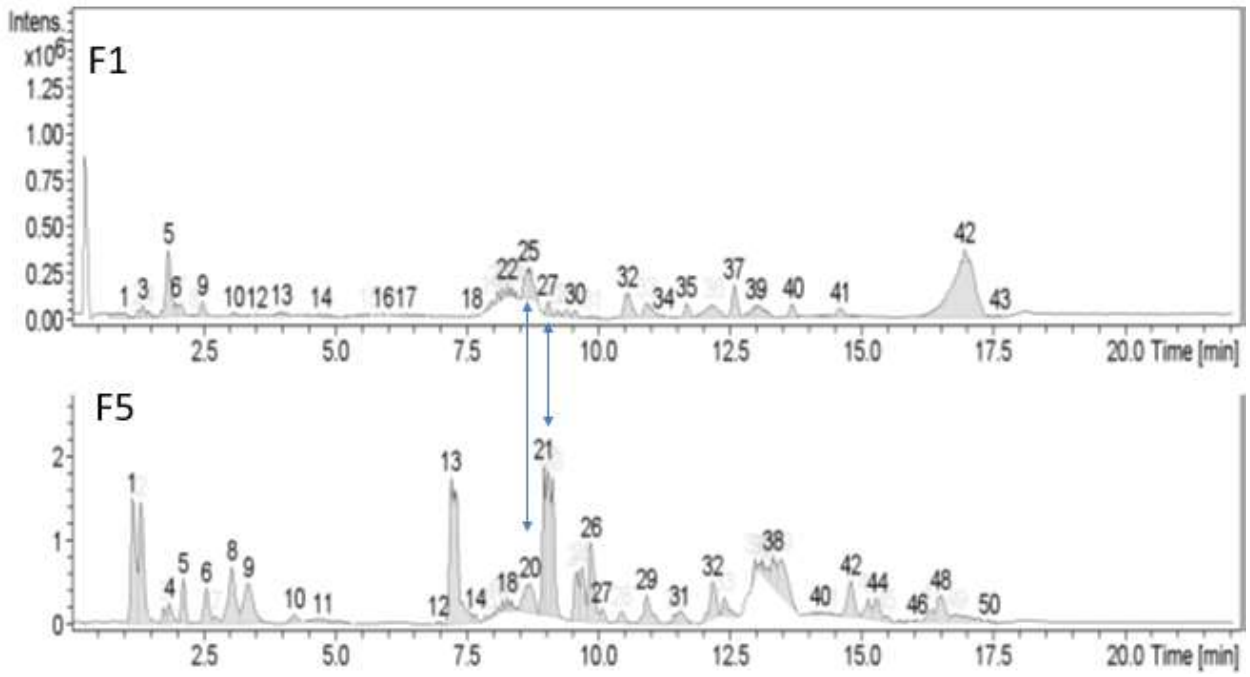


Figure 2: Comparative LC analysis of fractions F1 and F5 showing diversity of compounds at with retention time.

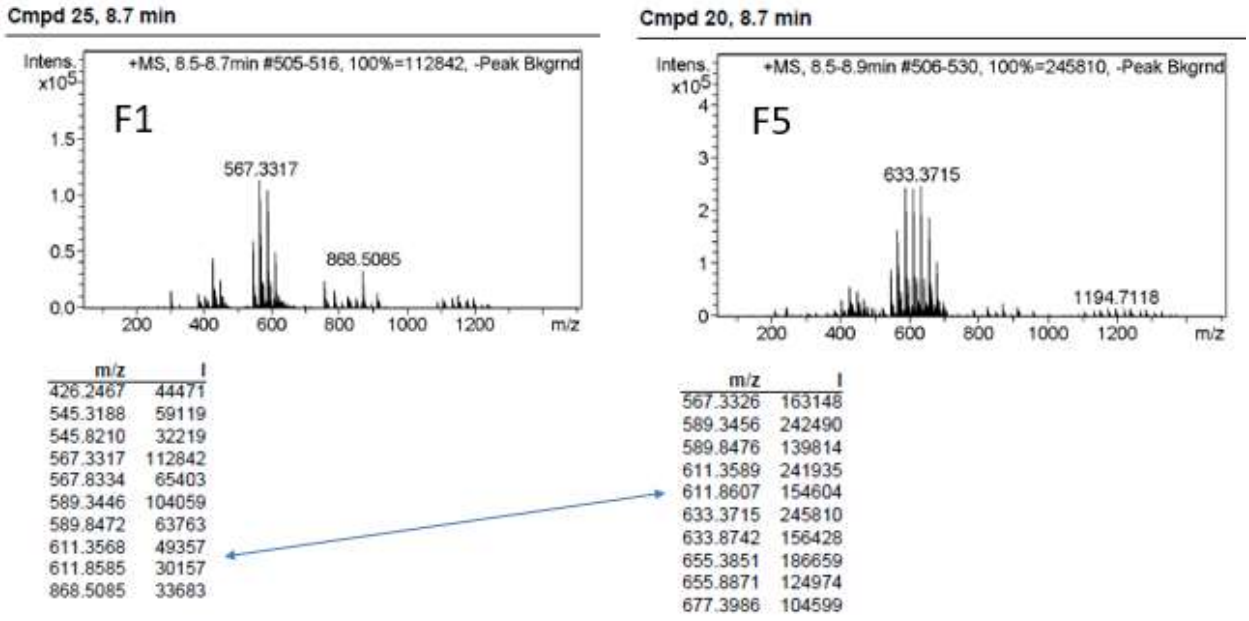


Figure 3a: Comparative MS analysis of fractions F1 and F5 showing the molar masses and fragmentation pathways of chemically related compounds eluted at the same retention time (8.7 min).

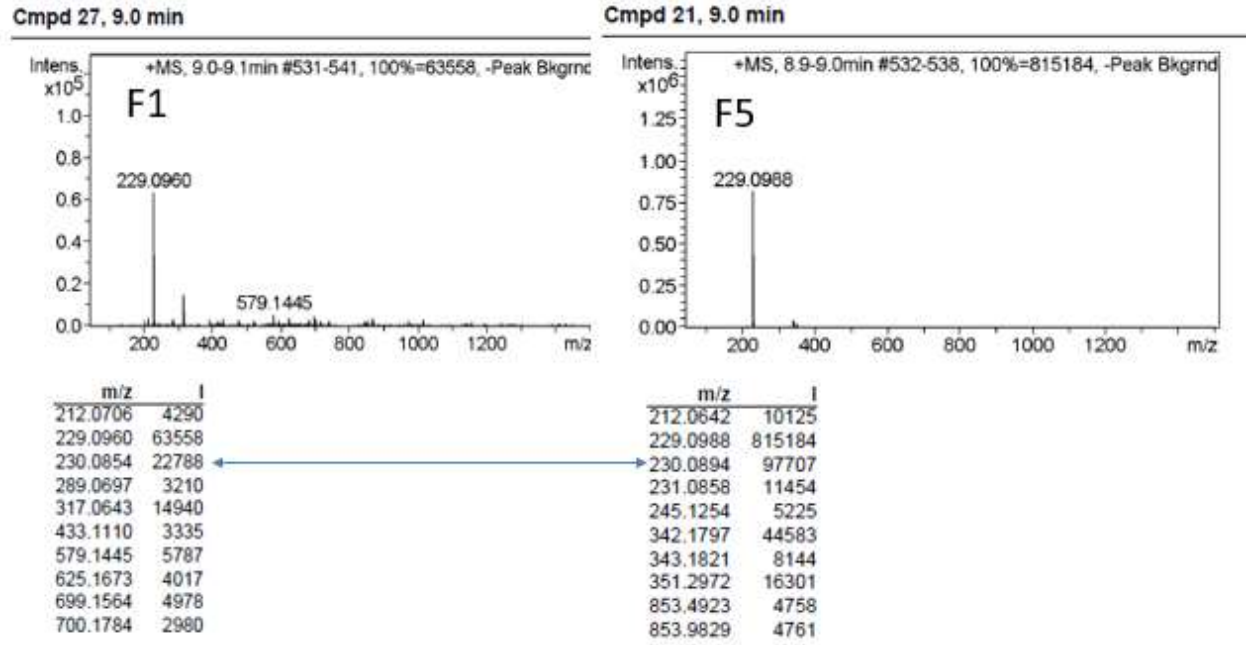


Figure 3b: Comparative MS analysis of fractions F1 and F5 showing the molar masses and fragmentation pathways of chemically related compounds eluted at the same retention time (9.0 min).

Anti-inflammatory Assay

The anti-inflammatory activity results are presented in Table 3. Both fractions F1 and F5 demonstrated anti-inflammatory activity with F1 slightly more active than F5 by displaying lower IC_{50} value of 1.770 µg/ml (the lower the value the higher the activity). The comparative anti-inflammatory activity of the 2 fractions with that of reference standard (Diclofenac Na) indicated that their abilities to inhibit albumin denaturation are more than twice as that of the positive control. This highlights the potential of these extracts as an alternative sources of anti-inflammatory agents.

Table 3: Comparative Percentages Albumin Denaturation Inhibition of Fractions F1 and F5 and Positive Control.

Conc. (µg/ml)	F1	F5	Diclofenac Na
1000	165.2401	120.0261	139.1516
500	106.2165	102.4657	87.7696
250	94.3910	90.74872	69.9200
125	92.7103	81.48742	64.2807
62.5	86.7571	76.96702	61.8406
31.25	78.0803	75.48361	61.0206
15.625	75.1341	66.50296	59.3298
7.8125	70.5173	66.03187	58.1047
IC_{50}	1.770	1.911	4.731

CONCLUSION

Both plant and fungal extracts produced diversity of compounds with fungal extract (F5) displaying more in comparison to leaf extract (F1). However, both extracts were discovered to contain similar compounds as revealed by the LC-MS analysis. The anti-inflammatory activities revealed by both extracts were found to be higher than that of diclofenac Na (positive control). This research for the first time suggests anti-inflammatory activity of *Amblygonocarpus andongensis* leaf and its endophytic fungus extracts as well as relative number of bioactive compounds present in them.

Recommendation

This research is recommending further work towards isolating and identifying compounds from both *Amblygonocarpus andongensis* leaf and its endophytic fungus especially the similar compounds revealed by the LCMS analysis.

Conflict of Interest

The authors declare no conflict of interest.

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Declaration of Generative AI in Scientific Writing

During the preparation of this work, the authors used ChatGPT 3.5 in order to assist in refining the clarity, coherence, and improve academic tone to polish sentence structure where appropriate. All content was carefully reviewed and edited by the authors to ensure originality, accuracy, and adherence to the academic standards of the field. The final responsibility for the content in publication remains entirely with the authors.

Credit Authorship Contribution Statement

Gali Nuhu Umar: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing- original draft, Writing- review & editing. **Muhammad Sani Sallau**: Methodology, Validation, Resources, Supervision, Writing- review & editing. **Hamisu Ibrahim**: Methodology, Validation, Resources, Supervision, Writing- review & editing. **Habiba Iliyasu Atta**: Methodology, Validation, Resources, Supervision, Writing- review & editing. **Nurkhalida Kamal**: Conceptualization, Methodology, Validation, Resources, Supervision, Funding acquisition, Writing-review & editing.

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