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Original Research Article

Investigation of the bioactive constituents and some pharmacological activities of the ethanol extracts of *Acanthospermum hispidum* and *Moringa oleifera*

Femi Taiwo Ojo[1]*, Erkay Özgör[2]*
[1]Cyprus International University, Department of Bioengineering, [2]Cyprus International University, Department of Molecular Biology and Genetics, 99258, Nicosia Mersin 10, Turkey

For correspondence:* ***Email:* *eozgor@ciu.edu.tr;* ***Tel:*** *+90 392 671 11 11*

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Abstract

Purpose: *To determine the bioactive compounds in Moringa oleifera (MO) and Acanthospermum hispidum (AH) with antioxidant, anticancer, antifungal and antibacterial potentials.*
Methods: *Fresh leaves of these plants were collected from Akinmoorin and Awe, Oyo State, Nigeria. Particle-induced x-ray emission (PIXE) technique was performed using a 2.5MeV proton beam (a non-destructive elemental analysis). Phytochemicals were qualitatively analyzed using High-performance liquid chromatography (HPLC), gas chromatographic-mass spectrophotometer (GC-MS) and standard procedures. Disc diffusion method was used to determine the minimum inhibitory concentration (MIC) for fungi and bacteria. Antioxidant properties were carried out by DPPH radical scavenging activity. Cancer cells (MDAMB 231 and HTC 15) were cultivated in the presence of the extracts at body temperature with 5 % CO_2 and 95 % air in 100 % relative humidity. Thereafter, adherent cells were seeded and treated with increasing concentrations of the plant extracts for 48 – 72 h.*
Results: *Phytochemical analysis showed that both plants contain alkaloids, flavonoids, anthraquinones, steroids and terpenoids, saponins, tannins, phenols and glycosides. The MIC for MO on fungi was 21.0 and 12.0 mg/mL for AH. Furthermore, MO MIC for bacteria was 25.25 mg/mL and 9.75 mg/mL for AH. Antioxidant properties revealed MO having 80.02 % and AH, 76 % at 32.00 µL. The ethanolic extracts showed a 50 % reduction in cancer cell viability at 24.5 µg/mL for MO and 16.0 µg/mL for AH, respectively, on MDAMB 231 and HTC 15 cells.*
Conclusion: *Moringa oleifera (MO) and Acanthospermum hispidum (AH) extracts exhibit significant antioxidant, antitumor, antifungal and antibacterial activities. Further studies will require that the active constituents be isolated and formulated into suitable dosage forms.*

Keywords: *Phytochemical screening, Pharmacological activities, Acanthospermum hispidum, Moringa oleifera*

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INTRODUCTION

Medicinal plants are sources of new drugs and it is estimated that more than 250,000 flowering plant species are included in this group [1]. *Moringa oleifera* (MO) and *Acanthospermum hispidum* (AH) are herbs that are considered safe when compared to synthetic medicines that are

regarded as unsafe to humans and the environment [2,3]. *Moringa oleifera* is a fast-growing, drought-resistant tree of the family Moringaceae, native to tropical and subtropical regions of South Asia. It is widely cultivated for its young seed pods and leaves used as vegetables and for traditional herbal medicine [1]. Every part of Moringa is used for certain nutritional and medicinal purposes [4]. *Acanthospermum hispidum*, (Bristly starbur) is an annual plant in the family *Asteraceae*, originally native to America. It has naturalized in many places like Europe, Asia and Africa. The plant is well adapted to light-textured soils but also grows well in heavy-textured soils [5]. Both seeds and leaves contain phenolic acids that are allelopathic to other plants [3].

A study comparing relative antimicrobial activity of MO seed extracts against bacteria and fungi revealed that *P. multocida and B. subtilis* were the most sensitive strains [6]. It has also been reported that aqueous extracts of leaf, fruit and seed of MO act as an antioxidant. During a study reporting the antioxidant properties of freeze-dried Moringa leaves from different extraction procedures [2,4], It was also reported that the major bioactive compounds of phenolic, such as quercetin and kaempferol are responsible for its antioxidant activity [6]. Anticancer activity of MO has been reported. Among bioactive compounds from MO, niazimicin (an MO leaf thiocarbonate) was the most potent. Furthermore, niazimicin also inhibited the tumor promoter teleocidin B-4-induced Epstein-Barr Virus (EBV) activation.

This study aims to investigate the bioactive compounds of *Moringa oleifera* and *Acanthospermum hispidum* and further corroborate their antioxidant, antitumor, antifungal and antibacterial activities.

EXPERIMENTAL

Plant materials

Fresh leaves of *Moringa oleifera* and *Acanthospermum hispidum* were collected between August and September 2021, from SPED International Secondary School, Akinmoorin and Awe town in Oyo State, Nigeria, where they were growing as ornamental plants. They were kept and identified at the Herbarium of Biology Department, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria, by Professor of Plant Science, Professor ATJ Ogunkunle. The voucher numbers were LHO653 (*Acanthospermum hispidum*) and LHO654 (*Moringa oleifera*).

The leaves were washed with tap water to remove adhering dust particles, rinsed with distilled water and air-dried in the laboratory at ambient temperature (30 ± 2 °C) for 5 days. Thereafter, these leaves were cut into small pieces to increase their surface area.

Extraction

Extraction was carried out by macerating 100 g of each leaf in 80 % ethanol using the Soxhlet extractor apparatus. Extraction was continuous until the color of the solvent in the siphoning tank turned colorless [1,7]. The solvent was evaporated under reduced pressure using the rotary evaporator. Different concentrations of the ethanolic extracts were obtained at 1, 2, 4, 6, 8 and 10 mg/mL in a 2 mL tube. The extracts were then distilled and concentrated under low pressure in the rotary flash evaporator to yield the residue. This procedure was repeated with distilled water. The solution from each extract was filtered and concentrated under low pressure in a rotary flash evaporator. After the crude extracts were collected and weighed, they were stored in the refrigerator at 4 ^{o}C for further phytochemical analysis.

Particle-induced x-ray emission (PIXE) technique

Particle-induced X-ray emission (PIXE) technique was performed using a 2.5 MeV proton beam obtained from the Centre for Energy Research and Development (CERD)-Ion Beam Analysis (IBA) [8]. This technique gives elemental concentrations of both *M. oleifera* and *A. hispidum.*

Phytochemical analytical procedures

Phytochemicals were identified via High-performance liquid chromatography (HPLC), Mass Spectrometer (MS) and Qualitative Analysis (QA), and the results were compared. The MS start time was 3 mins, the end time was 35 mins and the solvent cut time was 3 mins. Identification was based on Mass Spectral matching with the standard compounds in the National Institute of Standards and Technology (NIST) library, while identification of analogs and isomers was based on the molecular structure and calculated fragmentation patterns [2,7]. The essential chemical constituents were identified by comparing the mass spectra obtained with the reference spectra of compounds in the mass spectra library of NIST. Quantitative HPLC analysis of each compound was calculated according to its peak area.

Determination of secondary metabolites

With slight modifications where necessary, standard procedures were followed in the screening to identify secondary metabolites (active components) of these leaves in the extracts [2,3,7]. The metabolites screened were alkaloids, flavonoids, anthraquinones, steroids and terpenoids, saponins, tannins, phenols and glycosides.

Evaluation of antimicrobial activity

Antibacterial and antifungal activities were carried out using disc diffusion method [9]. Nutrient Agar was prepared for bacterial isolates while potato dextrose agar was prepared for fungi isolates following manufacturer's specifications. The selections include *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and *Staphylococcus aureus* for bacterial species, while *Candida albicans, Fusarium oxysporum, Penicillium sclerotigenum* and *Aspergillus niger* for fungi species. Bacteria cultures were obtained from the Institute of Microbial Technology, Lautech Teaching Hospital, Osogbo, Nigeria. All fungal cultures were obtained from the Department of Microbiology, Obafemi Awolowo Teaching Hospital, Ile Ife, Osun State, Nigeria.

Minimum inhibitory concentration (MIC)

Bacterial inoculum was prepared by growing cells in Mueller–Hinton broth (Himedia) for 24 h at 37 ºC. Filamentous fungi were grown on Potato dextrose agar slants at 28 ºC for 10 days and the spores were collected using sterile double-distilled water and homogenized. Test cultures were swabbed on top of the media and allowed to dry for 10 mins. A specific amount of extracts was then added to each disc. The loaded discs were placed on the surface of the medium and left for 30 mins at room temperature for compound diffusion. Negative control was prepared using sterile water. The plates for bacteria were incubated for 24 h at 37 ºC and 48 to 72 h at 28 ºC for fungi. Zones of microbial inhibition were recorded in millimeters, and the experiment was repeated thrice [6,10]. The purified ethanolic extracts were identified using spectroscopic methods, and mass spectroscopy. Studies on the isolated extracts' minimum inhibitory concentration were carried out in accordance with the industry-reference standards for bacteria and filamentous fungi [11,12]. Each medium in 10-well plates received the necessary amounts of extracts (1, 2, 4, 6, 8 and 10 mg/mL). From each well, an inoculum was introduced. Ketoconazole was used as a positive control for fungi, while streptomycin, was the positive control for bacteria. The plates were incubated for bacteria for 24 h at 37 ºC and for fungi for 48 to 72 h at 28 ºC. The lowest extract concentration at which there is no discernible growth after an incubation period is known as the MIC.

Antioxidant assay

The mixture of 35.23 mL of ethanolic solutions of MO and AH extracts (maximum dissolved content) and 2 mL of freshly made 10^5 M DPPH radical solution was incubated at 37 °C for 30 mins while absorbance reduction of the mixture was observed at 520 nm (As). Since DPPH radicals have a maximum absorption at 520 nm, the color of the sample changed from purple to pale yellow during this reduction process by the antioxidant. Every day, at the same wavelength, blank samples made of 32.00 µL of ethanol extracts prepared in the aforementioned DPPH radical solution were monitored (Ab). Sterile water containing DPPH served as positive control (Ac). The experiment was conducted three times. The comparatively stable DPPH radical was frequently employed to evaluate a compound's capacity as a hydrogen donor or free radical scavenger. Extract with radical scavenging capacity reduce DPPH radical using a donor hydrogen atom to DPPH free radical, based on an electron transfer or hydrogen atom to DPPH radical, antioxidant chemical and DPPH interact to form 1-1, diphenyl-2-picrylhydrazyl [13,14]. Percentage DPPH radical scavenging activity (I) was calculated using Eq 1.

$$I (\%) = (Ac-As/Ac)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Assessment of cytotoxic properties

The cytotoxicity was determined by following previously reported methods [14-16]. Plant extract was analyzed for cytotoxicity through brine shrimp lethality assay, sea urchin eggs assay, hemolysis assay and MTT assay (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide using tumor cell lines. Breast cancer cell line (MDAMB 231 and MDAMB 468) and colon cancer cell lines (HCT 15 and HCT 116) were used. Cells (1,000 cells/well) were seeded in 20 well plates containing medium with varying concentrations of plant extracts (1, 2, 4, 6, 8 and 10 mg/mL). The cells were cultivated at 37 ºC with 5 % CO_2 and 95 % air in 100 % relative humidity. Thereafter, adherent cells were seeded and treated with increasing concentrations of plant extracts for 48 – 72 h. The MTT reagent was added and the absorbance was determined at 560 nm after 72 h. Cisplatin was used as

control. After various durations of cultivation, an aliquot of 100 mL of medium containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide was loaded in the plate. The cells were cultured for 4 h, thereafter, the solution in the medium was removed. An aliquot of 100 mL of DMSO was added to the plate, which was shaken until the crystals were dissolved. Cytotoxicity of each sample was expressed as a half-maximal inhibitory concentration (IC_{50}) value. The IC_{50} value is the concentration of the test sample leading to 50 % inhibition of cell growth that was averaged from three repeated experiments.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Data were obtained in triplicates from all experiments. Student's *t*-test for unpaired data was applied. Comparison was performed using ANOVA followed by Levene's Test. Differences between means were considered significant at $p < 0.05$.

RESULTS

PIXE data

Results obtained by PIXE technique for both *M. oleifera* and *A. hispidum* is shown in Table 1.

Table 1: Particles-induced x-ray emission results of *M. oleifera* and *A. hispidum*

Element	*Moringa oleifera* (ppm)	*Acanthospermum hispidum* (ppm)
Mg	3837.3±223.3	3538.8±206.0
Si	1475.0±42.5	1332.4±37.2
P	2661.2±72.4	1808.7±62.0
S	8772.6±60.5	5072.6±47.7
Cl	1412.9±39.7	1188.0±3O.7
K	17053.9±56.2	16908.4±50.7
Ca	14467.7±63.7	12567.7±61.6
Mn	71.3±5.4	32.6±5.1
Fe	113.5±4.3	329.7±6.6
Cu	3.7±1.7	5.0±1.7
Zn	15.3±14.3	11.4±2.2
Rb	29.2±14.3	25.1±10.8
Sr	ND*	89.2±20.8
Ba	ND*	662.0±162.9

Note: *ND: Non-detected

Phytochemical composition

The GC-MS results for both *M. oleifera* and *A. hispidum* are shown in Table 2. These results show that both plants contain pharmacologically active substances which include alkaloids, flavonoids, anthraquinones, steroids, terpenoids, saponins, tannins, phenols and glycosides [5].

Antimicrobial activity

Results of antimicrobial activity are shown in Table 3.

Total antioxidant capacity

Table 4 reports the results of the percentage of DPPH radical scavenging activity for both plant extracts. The phytochemical screening showed the presence of flavonoids in the leaf extract. Flavonoids are most commonly known for their antioxidant activity. There is a significant amount of tannins which is effective in protecting the kidneys and have shown potential antiviral, antibacterial and antiparasitic effects.

Cytotoxicity activity

Figure 1 shows non-cancerous cells being active in the dotted lines while cancerous cells showed lethal action outside the dotted lines by the extracts of *M. oleifera* and *A. hispidum* on HCT 15. In Tables 5, both *M. oleifera and A. hispidum* showed lethal action against breast cancer cells in compliance with the IC_{50} recommended. Though, normal cells were not affected. Furthermore, *M. oleifera and A. hispidum* showed lethal action against human colon cancerous cells in lines with the IC_{50} recommended. Normal cells were not affected, as seen in Figure 1 and Figure 2. Results revealed that the two plant extracts possessed an *in vitro* anticancer activity against the two selected cancer cell lines. Extracts showed approximately 50 % reduction in cancer cell viability at 24.5 µg/mL for *M. oleifera* and 16.0 µg/mL for *A. hispidum* on MDA-MB-231 compared to standard reference which had 17.65 µg/mL, while for on HTC-15, extracts showed approximately 50 % reduction in cancer cell viability at 22.2 µg/mL for *M. oleifera* and 14.0 µg/mL for *A. hispidum* compared to control which had 15.80 µg/mL

Table 3: Mean inhibition zone diameter of the ethanol extracts against bacteria and fungi (mm)

Organism	*Moringa oleifera (mm)*	*Acanthospermum hispidum (mm)*
Pseudomonas aeruginosa	31.0±0.90	6.0±0.57
Escherichia coli	25.0±0.53	10.0±0.57
Staphylococcus aureus	23.0±0.71	12.0±0.71
Salmonella typhi	22.0±0.90	11.0±0.55
Aspergillus niger	26.0±0.61	12.0±0.55
Candida albicans	22.0±0.51	14.0±0.71
Fusarium oxysporum	16.0±0.71	12.0±0.71
Penicillium sclerotigenum	20.0±0.81	10.0±0.65

Table 2: GC-MS data for *Moringa oleifera* and *Acanthospermum hispidum* extracts

Peak S/N	Compound Name	Retention time (S)	Composition (%)	Molecular formula	Molecular weight (g/mol)	Pharmacological activity
***Moringa oleifera* extracts**						
1	5-hydroxymethylfurfural	18.217	6.46	$C_6H_6O_3$	126.11	Anti-anemic
2	Benzeneacetonitrile,4-hydroxy-	26.199	2.85	C_8H_7NO	133.15	NAR
3	Phenol-4-(3-hydroxy-1-propenyl)-2-methoxy	33.211	1.34	$C_{10}H_{12}O_3$	180.20	NAR
4	n-hexadecanoic acid	37.346	17.36	C16H32O2	256.43	CNS, Antioxidant
5	Hexadecanoic acid, ethyl ester	37.521	1.44	C18H36O2	284.48	Antioxidant
6	Octadec-9-enoic acid	38.547	40.27	$C_{18}H_{34}O_2$	282.47	Cardiovascular antimicrobial
7	Octadecanoic acid	38.647	5.78	$C_{18}H_{36}O_2$	284.48	Antimicrobial
8	Cis-11- Hexadecenal	39.535	1.91	$C_{16}H_{30}O$	238.42	Antimicrobial
9	Eicosanoic acid	39.635	1.68	$C_{20}H_{40}O_2$	312.54	CNS
10	Sulphurous acid isohexyl pentyl ester	40.304	9.27	$C_{11}H_{24}O_3S$	236.37	NAR
11	Glycerol-1- palmitate	40.417	1.77	$C_{38}H_{19}O_4$	330.51	Antimicrobial
12	Prop-2-one, 1-(4-iospropoxy-3-methoxyphenyl)	40.767	2.27	$C_{13}H_{18}O_3$	222.28	NAR
13	Benzeneaacetic acid, 4-hydroxy -3-methoxy-methyl ester	40.917	1.59	$C_{10}H_{12}O_4$	196.20	NAR
14	n-propyl 11 octadecenoate	41.737	6.02	$C_{21}H_{40}O_2$	324.55	NAR
***Acanthospermum hispidum* extracts**						
1	Thiophene, 2-methoxy-5- methyl-	10.754	1.71	C_6H_8OS	128.19	NAR
2	4H- pyran-4-one, 2,3dihydro-3,5-dihydroxy-6-methyl	14.808	2.06	$C_6H_8O_4$	144.13	Antioxidant
3	Benzeneacetonitrile,4-hydroxy-	26.225	2.52	C_8H_7NO	133.15	NAR
4	E, Z-8, 10-dodecadine-1-ol	35.894	1.56	$C_{12}H_{22}O$	182.31	Antimicrobial
5	n-hexadecanoic acid	37.337	24.98	$C_{16}H_{32}O_2$	256.43	CNS, Antioxidant.
6	Hexadecanoic acid	37.527	4.02	$C_{18}H_{36}O_2$	284.48	Antioxidant.
7	Phytol	38.328	2.58	$C_{2O}H_{40}O$	296.54	Hepatoprotective, Antioxidant Antimicrobial
8	9,12,15- octadecatrienoic acid, (z,z,z)	38.565	47.33	$C_{13}H_{30}O$	278.44	Antimicrobial CNS, Hepatoprotective Anti-inflammatory
9,	9, 12, 15- octadecatrienoic acid. (z,z,z), ethyl ester	38.653	11.66	$C_{20}H_{34}O_2$	306.49	NAR
10	Hexadecanoic acid, 2 hydroxyl- 1- (hydroxymethyl) ester	40.423	1.60	$C_{19}H_{38}O_4$	330.51	Antioxidant anti-inflammatory

NAR = No Activity Recorded; CNS: Central nervous system

Table 4: DPPH inhibitory activity of *Moringa oleifera* and *Acanthospermum hispidum* ethanol extract

Concentration (µg/mL)	*Moringa oleifera* (%)	*Acantospermum hispidum* (%)
0	0	0
20	63.17	46.30
40	65.20	51.35
60	72.16	55.92
80	78.14	70.02
100	80.02	76.00

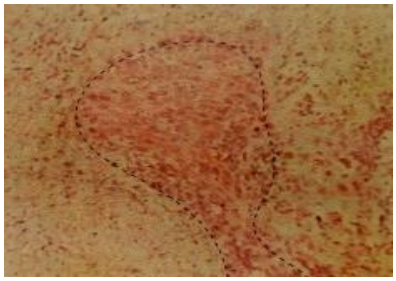

Figure 1: Viable cell lines in the dotted line

Table 5: Effect of *Moringa oleifera, Acanthospermum hispidum* and control on MDA-MB-231 and HTC-15 cell lines

Cell line	Doses (mg/mL)	MO (µg/mL)	AH (µg/mL)	Cisplatin (µg/mL)
MDA-MB-231	1.0	18.0±1.61	5.0±1.05	9.0±0.11
	2.0	22.0±2.54	10.0±1.84	15.0±1.32
	4.0	24±2.34	16.0±1.46	18.0±1.51
	8.0	28.0±2.52	23±2.54	22.0±1.61
	10.0	30.0±2.53	26±2.61	24.0±1.81
HTC-15	1.0	16.0±1.61	3.0±1.05	8.0±0.11
	2.0	20.0±2.54	9.0±1.84	13.0±1.32
	4.0	22±2.34	14.0±1.46	16.0±1.51
	8.0	25.0±2.52	20±2.54	19.0±1.61
	10.0	28.0±2.53	24±2.61	23.0±1.81

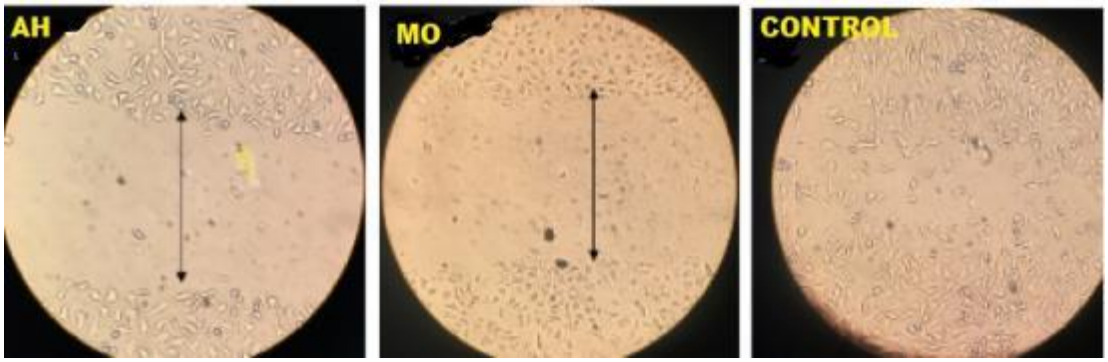

Figure 2: Inhibition of human breast cancer cell line, MDA-MB-231

DISCUSSION

It is a known fact that both organic and inorganic nutrients are required for biochemical processes in humans (heterotrophic creature). Most nutritional requirements are obtained from diets and adequate amount of these elements are essential for health to combat deficiencies that occur in some disease conditions. These essential elements occur in these plants in sufficient quantities [5]. Since these products have been proven to be beneficial to health, it is possible to use purified forms from these plants. The efficacy of some of the detected elements and compounds have not been previously evaluated. The MIC for fungi was defined as the lowest extract concentration, showing no visible fungal growth after incubation time and the minimum inhibitory concentration for bacteria were determined as the lowest concentration of the extract inhibiting the visual growth of the test cultures on the agar plate. It was observed that *M. oleifera* had higher inhibition zone diameter when compared to *A. hispidum* [7]. Hence *M. oleifera* possesses higher bioactive compounds with antimicrobial activity than *A. hispidum*. Nevertheless, in areas where *M. oleifera* is not available, *A. hispidum* could be used.

In this study, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to confirm the antioxidant properties of these two plants. The result of reduction of DPPH radicals showed changes in color from purple to pale yellow indicating the scavenging activity of both *M. oleifera and A. hispidum*. Thus, from the experiment, it was

concluded that *M. oleifera and A. hispidum* have significant antioxidant capacity. Based on *in vitro* studies, the phytochemical components of *M. oleifera and A. hispidum* leaves may be used in treating cancer [16]. A significant number of dead cells were observed upon extract treatment compared with control. There was a significant reduction in cell proliferation particularly by *M. oleifera* extract while normal cells were not affected in the process. Niazimicin is a bioactive compound found in *M. oleifera* leaves which have anti-cancer activity. Its extract has shown potential cytotoxic effect on human cancer cell lines [17,18]. Also, *A. hispidum* leave extracts were reported to have effect on carcinogen metabolizing enzymes. Its leaf is a potential source of polyphenols with potential hypoglycemic activity. Anthraquinones present in both plants possess stringent, purgative, moderate anti-tumor and bactericidal activities [4,5].

CONCLUSION

Both leaves possess antibacterial and antifungal activities against bacterial and fungal strains. They also possess potent antioxidant activity with MO being higher than AH. Cytotoxic activity reveals that the two plant extracts have *in vitro* anti-cancer activity against the selected cancer cell lines without any noticeable toxic effects thus providing a potential in the prophylaxis of cancer treatment irrespective of the cancer type. The findings thus substantiate the use of *A. hispidum* and *M. oleifera* extract as antioxidant, antitumor, antifungal and antibacterial agents. Further studies will require that the active constituents be isolated and formulated into suitable dosage forms.

DECLARATIONS

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Ethical approval

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Conceptualization, data curation, formal analysis, investigation, methodology, resources, writing, review and editing of the original draft was done by Femi Taiwo Ojo. Project administration, supervision, visualization, resources, methodology, writing, review, editing and validation were carried out by Erkay Özgör.

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