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Research Article

Phytochemical and Biological Studies of *Helichrysum acutatum* DC

Funsho Oyetunde-Joshua[1], Roshila Moodley[1,*], Hafizah Cheniah[2], Rene Khan[3]

ABSTRACT

Helichrysum acutatum from the Asteraceae family is a shrub indigenous to Southern Africa. The plant is used in traditional medicine as an enema for newborn babies. This study aimed to isolate and identify the bioactive constituents from *H. acutatum*. In addition, the crude extracts and isolated compounds were tested for their antioxidant, antibacterial and cytotoxic activities. The phytochemical investigation afforded the known compounds stigmasterol, stigmasterol glucoside, and caffeic acid. The antioxidant activity of the ethyl acetate extract showed higher activity compared to other extracts, ascorbic acid and butylated hydroxytoluene. Antibacterial profiling of all the extracts showed no activity against Gram-negative and Gram-positive bacterial strains. The cytotoxic activity of the crude extracts was assayed *in vitro* against two human cancer cell lines, liver hepatoblastoma (HepG2) and colorectal adenocarcinoma (Caco-2). The human embryonic kidney cell line (Hek-293) was used as the non-transformed control. The plant extracts showed insufficient antiproliferative or cytotoxic activity to the tumour and regular cell lines tested, which signifies suitable for human consumption. Overall, this plant has better antioxidant activity than other plants in the genus, which needs further exploration.

Key words: Antioxidant, Caffeic acid, Cytotoxicity, Magnetic resonance.

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INTRODUCTION

Plants are a storehouse for different therapeutic molecules and have played a significant and crucial role in modern drug discovery and development. The search for safe, effective, and affordable health care has made many trusts traditional medicines. People believe these medicines are relatively safe for use from a natural source. Also, the search for new molecular compounds in treating and managing diseases has resulted in drug leads from natural products. Natural products account for more than 50% of modern drugs used clinically, with some possessing the ability to inhibit cancer cells.[1]

Oxidative stress has been implicated in degenerative diseases such as Parkinson's, Alzheimer's, and cancer. Oxidative stress occurs when there is a shift in the production and removal of reactive oxygen species (ROS), favouring the production rather than the deduction. The National Cancer Institute (NCI)[2] defined ROS as unstable molecules containing oxygen that quickly react with other molecules in the cell. They are free radicals and could be referred to as oxygen radicals. Examples include peroxide, superoxide, hydroxyl radicals, and singlet oxygen species.[3] The build-up of ROS in the cell may cause damage to the DNA, RNA, proteins, and even cell death. The body's imbalance of antioxidants and free radicals leads to oxidative stress. Antioxidants react with free radicals in the body and terminate their chain reaction.

Helichrysum species possess antimicrobial, antifungal, antiviral, and antioxidant activities. There are approximately 600 species in the genus *Helichrysum* (Asteraceae). Compounds including chalcones, diterpenes, phloroglucinol, and its derivatives have been isolated from the aerial parts and roots of some *Helichrysum* species.[4] Traditionally, some species have found use in treating diarrhoea, wounds, colds, coughs, and respiratory tract infections.[5] Essential oils from Helichrysum species have been used, with the most widely used essential oil being from *Helichrysum italicum* (Roth) G. Don.[6] *Helichrysum acutatum* DC. is a perennial woody herb with a flowering stem that grows in grasslands; Hillard (1983)[7] classified it morphologically into group 21. Other plants in this group are *H. dasymallum* Hilliard and *H. oreophylum* Klatt. Although *H. acutatum* DC is widely sold in the muthi market, there is no literature on its ethnopharmacology relevance. The locals in the market provided anecdotal information on its use in traditional medicine as an enema for newborns. Bohlmann and Abraham (1979)[8] investigated the plant's roots and isolated fourteen compounds, including chalcones and diterpenes. In the current study, the roots of *Helichrysum acutatum*, widely sold at the muthi market in Durban, were investigated to establish a rationale for its traditional use by the locals as no biological studies have previously been done on the plant.

MATERIALS AND METHODS

Plant material

Plant material (root) of *H. acutatum* was purchased from Berea muthi market. The taxonomist in the School of Life Sciences, UKZN, authenticated the sample, and a voucher specimen (18271 01 900600) was deposited in the ward herbarium. The plant was air-dried and then crushed with a metal mortar and pestle to a smaller fragment for extraction.

General experimental procedures

Infra-red spectra were obtained using Perkin Elmer Spectrum 100 FT-IR spectrometer with universal ATR sampling. NMR spectra (^{1}H, ^{13}C, and 2D) were recorded on Bruker Avance^{III} 400Hz spectrometer, using deuterated chloroform, methanol or DMSO

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at room temperature, with TMS as the internal standard. Column chromatography was carried out using Merck silica gel 60 (0.040-00063mm) as a stationary phase and solvents of different polarities as a mobile phase. The separated fractions were examined using Merck 20 cm × 20 cm silica gel 60 F254 aluminium sheets for TLC. The TLC plates were first visualised under UV (254 and 366 nm) before spraying with 10% H_2SO_4 in methanol (MeOH) solution, followed by heating for the second visualisation. High-resolution mass spectra (HR-MS) were recorded on the Waters Micromass LCT Premier TOF-MS instrument. All reagents were of analytical grade and were sourced from either Merck (Darmstadt, Germany) or Sigma (St. Louis, USA) chemical companies.

Extraction and isolation

The roots of *H. acutatum* (1.3 kg) were oven-dried, crushed, and extracted sequentially with organic solvents of varying polarities, starting with the least polar to the most polar solvent in the order; DCM, EtOAc, and MeOH with the aid of a mechanical shaker. Each solvent was filtered and concentrated under reduced pressure using a rotary evaporator to give 5.4g of DCM, 4.5 g of EtOAc, and 20 g of MeOH extracts.

The DCM extract was subjected to separation on a silica gel column, with hexane and EtOAc as solvents using gradient elution to give 50 fractions of 100 mL monitored on TLC. Fraction 18 gave compound 1 (20 mg) as white flakes. The MeOH extract was purified on a packed silica gel column using hexane: EtOAc as the mobile phase, like the DCM extract. Fraction 20 gave compound 1, and fractions 40-45 were purified by washing with MeOH to give compound 2 (5 mg), an off-white powder. The EtOAc extract was fractionated on silica gel and eluted sequentially with hexane and EtOAc, starting from 100% hexane that was stepped by 10% to 100% EtOAc. A total of 40 fractions of 100 mL were obtained. Fractions 26-28 gave compound 3 (15 mg), a orange powder.

Antioxidant activity

The DPPH radical scavenging ability and the ferric reducing antioxidant power (FRAP) were determined using established methods.[9,10] Butylated hydroxytoluene (BHT) and ascorbic acid served as positive controls. All experiments were carried out in triplicate.

Antibacterial susceptibility test

Three Gram-positive indicator bacteria, *Bacillus subtilis* ATCC 6653, methicillin-resistant *Staphylococcus aureus* ATCC 43000 and *Mycobacterium smegmatis* mc^2 155 and four Gram-negative indicator bacteria, beta-lactam-resistant *Escherichia coli* ATCC 35218, multidrug-resistant *Pseudomonas aeruginosa* ATCC 27853, extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* ATCC 700603 and the quorum sensing indicator *Chromobacterium violaceum,* were employed to evaluate the antibacterial activity. Three crude extracts of *H. acutatum* and two pure compounds were subjected to antibacterial screening using the agar well diffusion method.[11] The test samples were dissolved in MeOH to a final concentration of 20 mg/mL for the crude extracts and 10 mg/mL for the compounds.

Cytotoxicity testing

Cell culture

Cryopreserved cells were rapidly thawed in the incubator and centrifuged. The cell pellet was recovered and propagated to 100% confluency in a 25 mL tissue culture flask with the addition of CCM (complete culture medium), which consists of 500 mL DMEM, supplemented with 1% L-glutamine, 1% penicillin-streptomycin-fungizone and 10% FCS, in a humid environment (5% CO_2, 37^{0}C). The media was removed, and the cells were washed thrice with PBS. Trypsin was added to the flasks with Caco-2 and HepG2 cells to remove those that had adhered to the flasks. The flask with Hek-293 was agitated to remove the cells that had attached to it. The cells were resuspended in 2 mL for CCM and counted using the trypan blue method.

Sample preparation

Stock solutions of crude extracts were prepared by dissolving in DMSO and diluted with CCM to a concentration of 10 mg/ml, and eight different concentrations (0-5000 µg/mL) for the MTT assay were prepared from the stock. The final concentration of DMSO in each stock was less than 0.5%.

MTT assay

The viability of Caco-2, HepG2, and Hek-293 cells after exposure to varying concentrations of test samples for 24 h was evaluated using the MTT (tetrazolium salt reduction) assay. 2 × 10^5 cells in CCM were seeded in 96-well microplates and incubated at 37^{0}C in 5% CO_2 overnight for adherence of cells to the plate. The medium was removed, and 100 µL of test samples prepared in CCM at varying concentrations were added to each well. The wells receiving only media served as the control. Treatment was done in triplicate for each test sample at 24 h. After 24 h, the medium was removed, 20 µL of MTT salt in CCM at a concentration of 5 mg/mL, and 100 µL PBS was added and incubated for 4 h at 37^{0}C. After that, the MTT solution was removed, and the formazan crystals formed solubilised with 100 µL DMSO. The cell growth inhibition by tested samples was measured using a Bio-Tek µQuant plate spectrophotometer (Winooski, Vermont, United States) at 570 nm. Results were presented as percentage cell viability.

$$\% \text{ cell viability} = \frac{\text{average OD of treated cells}}{\text{average OD of control cells}} \text{ x } 100$$

Analysis of mitochondrial membrane potential (MMP)

An increase in depolarization of the mitochondrial membrane with a subsequent decrease in mitochondrial membrane potential could activate pro-apoptotic factors. JC-10, a water-soluble dye, was used to probe the mitochondrial membrane potential in this assay. Cells were seeded at 2 × 10^4 into each well and allowed to adhere to the plate. IC_{80} and IC_{50} concentrations were prepared from the stock and added to each well. After 24 h, treatment media was removed and stored for other assays. Each well received 25 µL of JC-10 dye and 50 µL PBS before incubation at 37^{0}C for 1 h in the dark. After 60 min of incubation, the JC-10/PBS solution was removed, and 80 µL PBS was added before the plates were read.

ATP quantification

The intracellular ATP levels were monitored using a CellTiter-Glo® reagent (Promega) prepared according to the manufacturer's instructions. After taking the reading of the plate used for the mitochondrial membrane potential assay, 50 µL of PBS was added to each well, followed by 25 µL of CellTitre-Glo® reagent. Next, a luminescent reading was done on a Modulus™ microplate luminometer (Turner Bio-Systems, California, USA).

LDH release assay

LDH is a stable cytoplasmic enzyme found in the plasma of living cells. Release of LDH from the cytoplasm to the surrounding cell culture due to loss of the plasma-wall integrity can be used to quantify cell viability and necrosis *in-vitro*. 50 µL of the treatment media from the mitochondrial membrane potential assay was pipetted into a 96-well plate. Thereafter, 25 µL of the assay buffer (iodonitrotetrazolium (INT)

chloride, nicotinamide adenine dinucleotide (NAD) sodium salt, and lithium lactate) was added. Plates were incubated at room temperature in the dark for 30 min. The reaction was stopped by adding a 12.5 µL stop solution (acetic acid). LDH was quantified by measuring the absorbance at 500 nm using a Biotek µQuant spectrophotometer (Winooski, Vermont, United States).

Statistical analysis

Data were exported to Microsoft Excel for analysis and processed on GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, California, United States). All data were normalised to the untreated control, and the student's t-test was used to determine statistically significant differences ($P < 0.05$). All data were expressed as mean ± SD ($n = 3$).

RESULTS

Identification of isolated compounds

Three compounds were isolated and elucidated from the roots of *H. actutatum* (Figure 1). These include a sterol, a sterol glycoside, and a phenolic acid. The DCM and MeOH extract of the root yielded stigmasterol (compound 1). The spectral data compared well to that published in the literature for this compound.[12] The MeOH extract produced stigmasterol glucoside (compound 2), as confirmed by literature.[13] The EtOAc extract yielded caffeic acid (compound 3). The ^{1}H NMR and ^{13}C NMR spectral data are consistent with cinnamic acid.[14] except for resonances in the aromatic region for three protons at δ_H 7.05 (H-2), 6.94 (H-6), and 6.79 (H-5), indicating an ABX ring system. The downfield chemical shift (δ_H 7.54 (H-7) and 6.23 (H-8)) of deshielded alkenyl hydrogens means unsaturation, and the high coupling constant (15.9 Hz) indicates a trans arrangement. The HSQC spectrum showed correlations between the carbons at δ_C 115.1, 116.5, and 122.8 and the protons at δ_H 7.05, 6.79, and 6.94, respectively. Compound 3 was therefore identified as caffeic acid, confirmed by literature data.[15]

Antibacterial activity

The plant extracts were tested at 0.5 and 1 mg against three Gram-positive bacteria and four Gram-negative bacteria, while the two phytocompounds were tested at 0.25 and 0.5 mg due to the limited mass of phytocompounds. No antimicrobial activity was observed against all three Gram-positive and all four Gram-negative indicator organisms with extracts and isolated phytocompounds (Table S1).[16]

Antioxidant activity

The roots' crude DCM, EtOAc, and MeOH extracts were subjected to antioxidant testing using DPPH (Figure S1) and FRAP (Figure S2). The radical scavenging ability was in the order of BHT > EtOAc > ascorbic acid > MeOH > DCM. For the FRAP assay, the activity of the reference standards swapped and the order of reducing potential was ascorbic acid > EtOAc > BHT > MeOH > DCM.

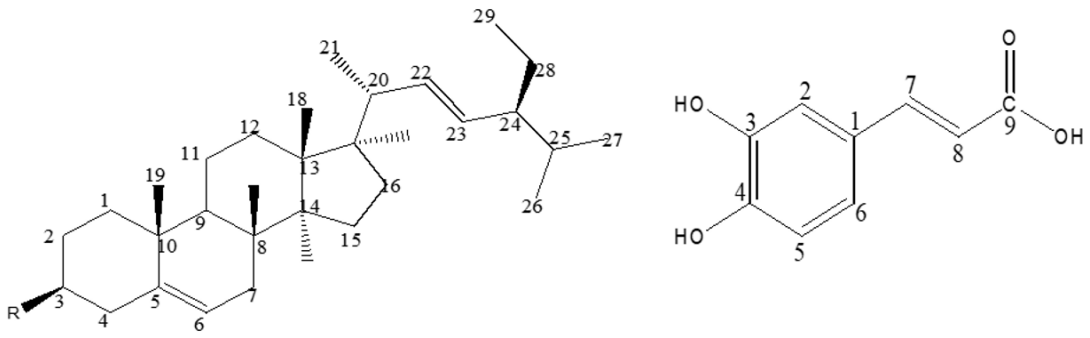

Figure 1: Structures of compounds 1 (stigmasterol), 2 (stigmasterol glucoside) and 3 (caffeic acid) isolated from *Helichrysum acutatum*.

Table 1: Analysis of the results obtained for the cytotoxicity assays (LDH, MMP and ATP) after treatment with the IC_{80} and IC_{50} concentrations of the extracts (DCM, EtOAc and MeOH) using the cell lines, Caco-2, HepG2 and Hek-293.

Cell Lines	Assay	Extracts at IC_{80} concentration			Extracts at IC_{50} concentration		
		DCM	EtOAc	MeOH	DCM	EtOAc	MeOH
Caco-2	*LDH*	increase	increase	increase	increase	increase	increase
	MMP	decrease	decrease	decrease	decrease	decrease	decrease
	ATP	increase	increase	increase	decrease	decrease	decrease
HepG2	*LDH*	decrease	decrease	decrease	decrease	increase	decrease
	MMP	increase	increase	increase	increase	increase	increase
	ATP	decrease	decrease	slight decrease	decrease	decrease	decrease
Hek-293	*LDH*	no change	decrease	decrease	no change	decrease	increase
	MMP	decrease	increase	increase	increase	increase	increase
	ATP	increase	increase	increase	increase	increase	increase

LDH - *Lactate dehydrogenase*, MMP – Mitochondrial membrane potential, ATP - Adenosine triphosphate, DCM – Dichloromethane, EtOAc – Ethyl acetate, MeOH – Methanol.

Cytotoxicity testing

MTT assay

The different crude extracts were exposed to the tumour cell lines (Caco-2 and HepG2) and the normal human kidney cell line, Hek-293, for 24 h, using the MTT assay to evaluate their cytotoxicity (Figure S3). Treatment with 50.12 µg/mL DCM extract decreased cell viability from 100% (control) to 89% in HepG2, 76% in Caco-2, and 62% in Hek-293. At 501.19 µg/mL cell viability decreased 14% for HepG2, 8% for Caco-2, and 52% for Hek-293. IC_{50} values (in µg/mL) were 126.9, 165.55, and 699.6 for Caco-2, HepG2, and Hek-293, respectively.

Exposure to the EtOAc extract (50.12 µg/mL) stimulated cell growth from 100% (control) to 105% and 114% in HepG2 and Hek-293, respectively, while there was no change in Caco-2. Differently, at 100 µg/mL, there was a sharp decrease in cell viability to 53% for Caco-2 and a slight decrease to 101% for HepG2 and Hek-293. Between 100-501.19 µg/mL, no significant reduction in cell viability was observed in Caco-2 and Hek-293, but a decrease was recorded for HepG2 from 101% (100 µg/mL) to 77% (501.19 µg/mL). The results showed that the extract does not affect Hek-293 compared to Caco-2 and HepG2.

Treatment with the MeOH extract produced a stimulatory effect at a 50.12 µg/mL concentration in HepG2, increasing viability from 100% (control) to 114%. The exact concentration decreased viability in Caco-2 from 100% (control) to 87%; no change in viability was observed in Hek-293. Between 50.12-1000 µg/mL, no significant difference in viability was observed across all three cell lines. However, a sharp decrease in cell viability was observed in Caco-2 and HepG2 after 1000 µg/mL.

LDH release assay

The quantification of LDH released was used to determine cytotoxicity and necrosis. IC_{80} and IC_{50} concentrations of the DCM extract (Figure S4) increased LDH released by 1.27 and 1.38-fold in Caco-2; 1.33 and 1.35-fold decrease ($P < 0.05$) was observed for HepG2; 1.06 and 1.04-fold increase for Hek-293 cell lines. Treatment with IC_{80} and IC_{50} concentration of the EtOAc extract caused a 1.36 and 1.65-fold increase ($P < 0.05$) in LDH released in Caco-2; a 2.0-fold decrease for IC_{80} and 1.14-fold increase for IC_{50} in HepG2; and 1.67 and 1.61-fold decrease ($P < 0.05$) in Hek-293 cell lines. A 1.22 and 1.93-fold increase in LDH released was observed in Caco-2 after exposure to IC_{80} and IC_{50} concentrations of the MeOH extract; a 1.20 and a 1.14-fold decrease was observed for HepG2 at IC_{80} and IC_{50} treatments, and a 1.49-fold decrease for IC_{80} treatment and 1.22-fold increase for IC_{50} treatment was observed for Hek-293.

Mitochondrial membrane potential (MMP) assay

Depolarising the mitochondria led to the release of pro-apoptotic proteins, probed using JC-10 dye. Exposure of the different cell lines to the $IC_{80,}$ and IC_{50} values of the DCM extract (Figure S5) showed a 1.35 and 1.33-fold decrease ($P < 0.05$) in mitochondrial membrane potential in Caco-2, 1.42 and 1.82-fold increase ($P < 0.05$) in HepG2, and 1.09 and 2.22-fold increase ($P < 0.05$) in Hek-293 cell lines. Treatment with IC_{80} and IC_{50} concentrations of the EtOAc extract resulted in 1.49 and 1.34-fold decrease ($P < 0.05$) in Caco-2, 1.44 and 2.51-fold increase ($P < 0.05$) in HepG2, and 1.32 and 2.33-fold increase ($P < 0.05$) in Hek-293 cell lines. The IC_{80} and IC_{50} values of the MeOH extract caused a 1.47-fold decrease ($P < 0.05$) for Cac0-2, 1.64 and 2.09 increase in HepG2, and 1.96 and 2.55-fold increase ($P < 0.00001$) in Hek-293 cell lines. Treatment with the MeOH extract produced similar results to the EtOAc extract. The general trend was decreased mitochondrial membrane potential in Caco-2 and increased HepG2 and Hek-293 cell lines for all tested samples.

ATP assay

The amount of intracellular energy (ATP) was used to quantify cell viability and mitochondrial function. ATP was increased by 1.14-fold in Caco-2 after exposure to the IC_{80} value of the DCM extract (Figure S6) but decreased by 1.72-fold ($P < 0.05$) with the IC_{50} treatment. For the same extract, a 1.20 and 1.67-fold decrease was observed in HepG2 with the IC_{80} and IC_{50} therapies, respectively, while a 3.25 and 1.79-fold increase ($P < 0.05$) was observed in Hek-293 cell lines. Treatment with IC_{80} and IC_{50} concentrations of the EtOAc extract increased ATP by 1.22-fold and decreased it by1.39-fold ($P < 0.05$) in Caco-2, respectively. In HepG2, a 1.18 and 2.56-fold decrease in ATP was observed at IC_{80} and IC_{50} concentrations of the EtOAc extract, respectively. In contrast, there was a 3.40 and 1.62-fold increase ($P < 0.05$) in Hek-293 at IC_{80} and IC_{50} values, respectively. Exposure to the IC_{80} concentration of the MeOH extract increased ATP by 1.17-fold in Caco-2 cells and decreased it by 1.28-fold ($P < 0.05$) at the IC_{50} concentration. ATP was reduced by 1.09 and 1.43-fold in HepG2 cell lines at IC_{80} and IC_{50} values of MeOH extract, while a 3.32 and 1.71-fold increase was observed in Hek-293, respectively.

DISCUSSION

The phytochemical investigation of the roots of *H. acutatum* led to the isolation of a sterol (stigmasterol),[12] a sterol glucoside (stigmasterol glucoside)[13] and a phenolic compound (caffeic acid).[15] Diterpenes, chalcones and phloroglucinol have been reported from the roots and aerial parts of *H. acutatum,* but these three compounds have not previously been isolated from the plant.[7] Caffeic acid is naturally present in many fruits, and this compound and its derivatives have been known to possess antioxidant, anticancer and antibacterial activities. These activities are mainly attributed to the free phenolic acid that has high bioavailability and good water solubility, the position of the OHs in the catechol moiety and the double bond in the carbonic chain.[17]

This study showed *H. acutatum* to have relatively good antioxidant activity compared to the standards. The potent antioxidant activity demonstrated by the EtOAc extract could be due to the presence of caffeic acid, which has been reported to be an excellent free radical scavenger.[18] Other *Helichrysum* species said to have good antioxidant activity include *H. longifolium* DC, which demonstrated good radical scavenging activity,[19] *H. teretifolium*, (L.) D. Don and *H. arenarium* (L.) Moench.[20]

H. acutaum demonstrated poor antibacterial activity. Our results are consonant with the findings for the chloroform extract from *H. acutatum* by Lourens *et al.* (2011)[21] that demonstrated low antibacterial activity against Gram-positive bacteria and yeast strains, and the acetone extracts from *H. acutatum, H. glomeratum* Klatt and *H. piloselfum* that showed no effect against both Gram-negative and Gram-positive bacteria.[22] However, some plants in the genus have shown promising antimicrobial activity, such as the acetone extract from *H. candolleanum* H. Buek, *H. herbaceum* (Andrews), *H. melanacme* (DC), *H. psilolepis* Harv., *H. rugulosum* Less., *H. simillimum* DC and *H. umbraculigerum* Less. that significantly inhibited microorganism proliferation with MIC values of 0.10 mg/Ml.[22,23] In addition, *H. caespetitum* DC. Harv. showed good activity against four WHO *N. gonorrhoea* strains (F, O, N, G) within the range 0.037-0.33 mg/ mL.[24]

The cytotoxicity of the plant extracts using the MTT assay showed that they are less toxic against the two tumour cell lines (Caco-2 and HepG2) and the standard cell line (Hek-293) with IC_{50} values within the range of 126-4533 µg/mL and 165-4719 µg/mL for Caco-2 and HepG2 cell lines, respectively. A previous study showed the chloroform: methanol extract (1:1) of *H. acutatum* to have cytotoxic activity against the cancerous cell line, MCF-7.[21] This shows the selective cytotoxicity of *H. acutatum.* Plants in the genus that demonstrated good cytotoxic activity include

H. petiolare Hilliard and B.L. Burtt., which were cytotoxic to B16F10 and MeWo skin melanoma cell lines in a dose-dependent manner.[25] The results from our study suggest that *H. acutatum* is potentially safe for human consumption due to its low cytotoxicity toward the standard cell line tested. Despite showing relatively good antioxidant activity, the EtOAc extract demonstrated poor cytotoxic activity towards the two tumour cell lines tested. This contrasts with the results obtained for the EtOAc extract of the flowers of *H. plicatum* DC. that showed good antioxidant activity and cytotoxicity against K562 and PC3 cell lines with IC_{50} values of 25.9 and 39.2 µg/mL, respectively.[26]

Mitochondria have been known to play an essential role in maintaining cell health and could be used to monitor cell viability.[27] A decrease in the mitochondrial membrane potential has been reported to be the first step in apoptosis, and apoptosis has been reported to require an increase in energy.[28] In cytotoxicity evaluations, cells either undergo an apoptotic or necrotic death.[29] In necrosis, cells swell, lose membrane integrity, and release their intracellular content into the external environment. LDH, a soluble enzyme found in the cell cytoplasm, is released when the cell membrane is compromised. The amount of LDH released can be used to quantify cell death.

At the IC_{80} concentrations, all three extracts showed an increase in LDH release, a decrease in the mitochondrial membrane potential and increased ATP levels in Caco-2 cell lines (Table 1). While at the IC_{50} concentrations, increased LDH release, decreased mitochondrial membrane potential, and decreased ATP levels were observed for the same cell lines (Table 1). Generation of ATP takes place in the mitochondria, a disruption in mitochondrial membrane potential could lead to depletion of intracellular ATP, and this was the case after treatment of Caco-2 tumour cell lines with IC_{50} concentrations of the tested extract. These changes are concentration-dependent, and depletion of cellular ATP has been shown to switch cell death from apoptosis to necrosis. Apoptosis is a programmed cell death that requires energy (ATP), while necrosis is accidental cell death that does not require energy.[29] The effect of concentration on the mode of cell death was observed when MCF-7 was treated with the aqueous extract of *Lepidium sativum* Linn. extracts: apoptosis was induced in the cell when treated with 25 and 50% of the extract, but at a higher concentration of 75%, necrosis was induced.[30] At both concentrations, the plasma membrane was disrupted, leading to the release of LDH in Caco-2 cell lines. The high increase in LDH release observed for some extracts indicates the extent of membrane damage, while those with slight increases in the release of LDH indicate minimal membrane damage (Table 1).

Exposure of HepG2 cell lines to the IC_{80} and IC_{50} concentrations of the different extracts caused no depolarisation in mitochondrial membrane potential, nor was the plasma membrane disrupted, but a depletion in ATP levels was observed. The cytotoxic activity of the extract towards HepG2 was through depletion of intracellular energy without effect on the plasma membrane.

The results show that the plant can initiate apoptotic and necrotic cell death, depending on the concentration and the cell lines. Some plant extracts that have decreased mitochondrial membrane potential with subsequent cytochrome C release for apoptotic pathway initiation are *Murraya koenigii* (L.) Spreng, *Annona reticulate* L., *Moringa oleifera* Lam., *Hibiscus sabdariffa* L., *Lablab purpureus* (L.), and *Euphorbia hirta* Linn.[31]

CONCLUSION

Three compounds were successfully isolated from the roots of *H. acutatum*, and this study is the first report of these compounds from the plant. The findings show *H. acutatum* extracts to have better antioxidant activity than antibacterial and anticancer activity for the tested microbes and cell lines. This study has established a toxicity profile and scientific basis for using *H. acutatum* as an antioxidant in traditional medicine and confirms its safety for human consumption. The study also highlights the lack of antibacterial activity of the species compared to others in the genus, which are well known for their use in traditional medicine in treating different infectious diseases.

AUTHOR CONTRIBUTIONS

Funsho Oyetunde-Joshua**:** Conceptualization, investigation, data curation, formal analysis, writing - original draft preparation. Roshila Moodley: Conceptualization, supervision, validation, writing – review and editing, funding acquisition. Hafizah Cheniah: methodology, data curation. Rene Khan: methodology, data curation.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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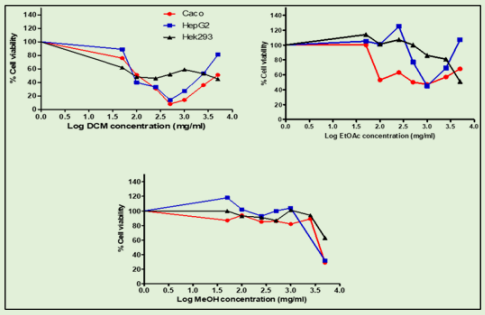
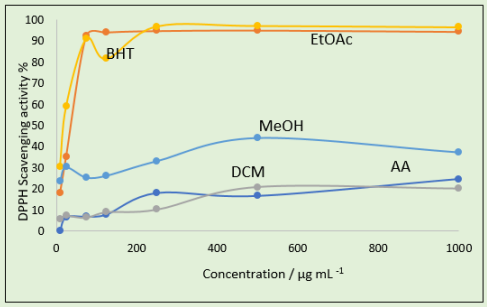
GRAPHICAL ABSTRACT

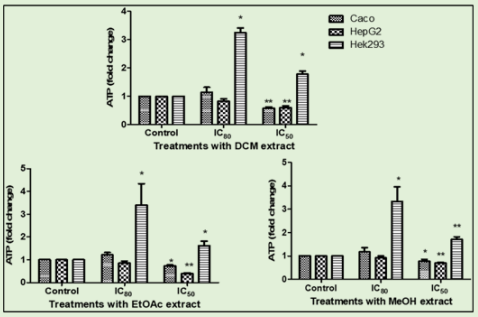


Effect of different concentrations of *H. acutatum* extracts (DCM - dichloromethane, EtOAc - ethyl acetate, and MeOH – methanol) on the viability of Caco-2, HepG2 and Hek293 by the MTT assay.



DPPH radical scavenging activity (mean ± SD, n = 3) of selected *H. acutatum* extracts (DCM - dichloromethane, EtOAc - ethyl acetate, and MeOH – methanol).

***Helichrysum acutatum* DC.**



Effect of IC_{80} and IC_{50} concentrations of *H. acutatum* extracts on ATP levels of Caco-2, HepG2 and Hek-293 cell lines. Significant differences between means at * $p < 0.05$ and ** $p < 0.01$. DCM - dichloromethane, EtOAc - ethyl acetate, and MeOH - methanol.

Analysis of the results obtained for the cytotoxicity assays (LDH, MMP and ATP) after treatment with the IC_{80} and IC_{50}concentrations of the extracts (DCM, EtOAc and MeOH) using the cell lines, Caco-2, HepG2 and Hek-293.

Cell Lines	Assay	Extracts at IC_{80} concentration			Extracts at IC_{50} concentration		
		DCM	EtOAc	MeOH	DCM	EtOAc	MeOH
Caco-2	LDH	increase	increase	increase	increase	increase	increase
	MMP	decrease	decrease	decrease	decrease	decrease	decrease
	ATP	increase	increase	increase	decrease	decrease	decrease
HepG2	LDH	decrease	decrease	decrease	decrease	increase	decrease
	MMP	increase	increase	increase	increase	increase	increase
	ATP	decrease	decrease	slight decrease	decrease	decrease	decrease
Hek-293	LDH	no change	decrease	decrease	no change	decrease	increase
	MMP	decrease	increase	increase	increase	increase	increase
	ATP	increase	increase	increase	increase	increase	increase

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