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INDUCTION OF APOPTOSIS ON BREAST ADENOCARCINOMA AND SUPPRESSION OF PATHOGENIC BACTERIAL GROWTH BY ANTIOXIDANT RICH TUBER EXTRACT OF *DIOSCOREA BULBIFERA*

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Abstract

Nature derived product drew a lot of interest because of their potential nutritional and therapeutics value. Most natural compounds are now processed and developed as possible antioxidative, antimitotic, antimicrobial, anti-inflammatory, antiangiogenic, and anti-carcinogenic pharmaceutical agents. Indeed, several natural products have been used as lead compounds in the development of most potent physiologically relevant chemicals for therapeutic application. In the current study antioxidant and anticancer potential of tuber extract of *Dioscorea bulbifera* have been determined as well as its antimicrobial potential against pathogenic bacteria. The IC50 value for the extract was found to be 55µg/ml for MCF-7 and 75µg/ml for MD-AMB-231 human breast cancer adenocarcinoma whereas minimum inhibitory concentration (MIC) value of the methanolic extract against S. aureus was 0.19 mg/ml and minimum bactericidal concentration (MBC) was 1.56 mg/ml. Further to identify compounds responsible for antioxidant, anticancer and antimicrobial activity GCMS analysis of the extract was conducted. Histopathological and haematological assay were carried out on animal model to identify any toxic effect of extract on vital organ system. This study may be used to provide the groundwork for the creation of novel nature-derived drugs whose

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biological activity can be determined by further purification, characterization, and validation of the active phytochemicals and their mechanism of action.

Keywords: *Dioscorea bulbifera*, breast cancer, natural therapeutics, phytochemistry, toxicity

Introduction

Breast cancer is the most prevalent cancer in women, accounting for one out of every four cases and one out of each six deaths, and is the most common ailment with high majority of across globe (Bray *et al*., 2021). The global prevalence of cancer incidence and mortality is rapidly increasing due to changes in lifestyle (Bray *et al*., 2021). Conventional chemotherapeutic drugs cause serious toxicity and adverse effects like hepatotoxicity, nephrotoxicity, cardiomyopathy, neuropathy, being also immunosuppressive (Yu *et al*., 2018). Furthermore, cancer cells may acquire drug resistance after chemotherapy treatment. As a result, higher dosages are required to produce a tumoricidal impact comparable to the first dosage. Higher doses are frequently associated with a greater risk of serious adverse effects (Zheng *et al*., 2017). Hence, further research into safer and more effective chemoprevention and therapy is definitely required to increase cancer care efficiency and reduce treatment costs. Cancer chemoprevention using natural phytochemical substances is a novel approach to preventing, delaying, or curing cancer. Natural therapeutics over chemotherapies have several advantages including reducing toxicity caused by chemotherapeutics drugs, and decreasing the incidence of chemotherapy resistance by cancer cells (Lin *et al.,* 2020).

Similarly, emergence of drug resistance in cancer cell is a major threat to public health and civilization across the world (Larsson and Flach, 2022). Plant extracts have long been utilized in traditional medicine as a safe, efficient, and natural treatment for a variety of ailments and disorders. Several plant-derived compounds were shown to exhibit antibacterial action (Newman and Cragg, 2012). The fundamental benefit of adopting plant-derived phytochemicals for legitimate medical purpose is that they do not have the negative side effects that synthetic drugs do (Abuga *et al*., 2021).

Dioscorea bulbifera, often known as air yam, belongs to the family of *Dioscoraeceae* being known for having important therapeutic potential. It contains polyphenols and organic acids, with some of them acting as potential antioxidants. Diosgenin, a steroidal saponin, is also present in the tuber. The plant consists of a number of active compounds that can be used to treat cancer (Mainasara *et al*., 2021). The plant also contains active phytochemicals with antimicrobial potential (Adeosun *et al*., 2016). A very few reports have been published for anticancer and antimicrobial activity of this plant.

The present study was designed to evaluate the anticancer potential of *Dioscorea bulbifera* against breast adenocarcinoma as well as antimicrobial activity of the

methanolic and acetone extracts against two gram positive bacteria and five gram negative bacteria.

Materials and methods

Collection and Extraction of Plant Material

Tuber samples of *D. bulbifera* were collected from Gandhmardhan, Western Odisha (20.8739° N, 82.8428° E). The plant species were identified with the help of regional flora books (Haines, 1961) and the help of a taxonomist. The successive hot extraction using petroleum ether, chloroform, ethyl acetate, acetone, methanol, and water was followed as described in Abubakar and Haque (2020).

Antioxidant activity

Total phenolic content

The total phenolic content of both the raw and boiled tuber powder samples was determined by the modified Folin-Ciocalteau method (Wolfe *et al*., 2003) with little modification. Briefly, 0.5 ml of diluted extract (1 mg/ml) was mixed with 2.5 ml of 10 time diluted Folin-Ciocalteau reagent. To this mixture 2ml of 7.5% of Na_2CO_3 (w/v) solution was added after incubation of mixture at room temperature for 5 min. Then reaction mixture was again incubated at 40 ℃ for 30 min followed by cooling to room temperature. The absorbance of reaction mixture at 765nm was measured with UV-visible spectrophotometer (Bio-Rad SmartSpec 3000). The total phenolic content of the samples was expressed as mg of gallic acid equivalents/100g of dry mass.

Total flavonoid content

The total flavonoid content of both the raw and boiled tuber powder samples was carried out using the method of Ordonez *et al*. (2006). Briefly, to the 1 ml of aliquots, 4 ml of distilled water and 0.3 ml of 5% sodium nitrite solution were added. After 5 minutes, 0.3 ml of 10% aluminum chloride was added and after 6 minutes, 2 ml of 1M sodium hydroxide was added. The final volume was adjusted to 10 ml. The intensity of orange yellowish color appeared was measured at 510nm. The total flavonoid content was expressed as mg of quercetin equivalents/100g of dry mass.

DPPH radical scavenging assay

The effect of the extracts on DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was estimated using the method of Liyana-Pathiranan and Shahidi (2005). The optical density of the reaction mixture was measured at 517nm with spectrophotometer (Bio-Rad SmartSpec 3000) where BHT (Butylated hydroxytoluene) was used as reference. The DPPH radical scavenging potential of the samples was calculated by the following equation:

$$\text{DPPHRSA (\%)} = \frac{\text{OD of Control} - \text{OD of Sample}}{\text{OD of Control}} \times 100 \qquad (1)$$

where OD is the optical density; Control is the DPPH radical+methanol; Sample is the DPPH radical+ sample extract/standard.

Anova single factor was used to check the significance of difference between the DPPH values of the six solvent extracts.

Evaluation of antibacterial activity and determinations of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Agar well diffusion was used to test antibacterial activity of *D. bulbifera* extracts (Dubey and Padhy, 2013). The agar well diffusion technique was used to test the antibacterial potential of plant extracts with a 25 mg/ml concentration using isolated bacterial strains that demonstrated resistance to a maximum number of antibiotics. MIC and MBC of the tuber extracts were determined based on a micro broth dilution technique using 96well microtiter plates. Additionally, The MBC value was determined by sub-culturing bacteria from each well of the microplate on a nutrient agar plate at a dilution level.

GC-MS analysis

The GC-MS analysis of the methanolic extract of *D. bulbifera* was carried out using the methods of Uthirapathy *et al*. (2021). The sample was analyzed by Clarus 500Gc-Ms Perkin Elmer using the Column type-elite-5 (5% diphenyl 95% dimethyl polysiloxane). The following parameters were used: column dimension - 30m×0.32mm; carrier gas-helium - 1ml per 1min; column temperature - 50°C up to 285°C; temperature of injector and detector - 290°C; injection mode - split; volume injected: 0.5µl of solution (20µg/ml); run time - 30min; transfer line temperature - 230°C; scan range - 40-450 amµ; Electron ionization technique.

The mass spectrum was taken using a mass detector-turbo mass gold Perkin Elmer and the components were identified by comparing the mass spectra of compounds with available NIST (Lemmon *et al*., 2010) and Wiley (Oberacher *et al*., 2012) mass spectra libraries. The quantitative composition was obtained by peak area normalization.

Anticancer activity

Cell culture and reagents

All chemical reagents and media used for cell culture were procured from Sigma-Aldrich and Invitrogen. Both the breast cancer cell line MCF 7 and MDAMB-231 were obtained from the cell repository of the National Centre for Cell Science Pune, Maharashtra.

Anticancer evaluation by using MCF-7 and MDAMB-231 cell lines

The *in vitro* cell proliferation assay for anticancer activity was conducted by using two human breast cancer adeno carcinoma, MCF7 and MDAMB-231. In brief, cells were seeded at a density of 4×10^3 cells per well in 96 culture plate supplemented with complete medium (MEM, DMEM) and 1% penicillin/ streptomycin, in a CO_2 incubator with temperature at 37 °C and 5% CO_2. The methanolic extract of *Dioscorea bulbifera* was added to cell medium with increasing concentrations from (6.25 to 100 µg/ml) and incubated for 72 h. The

sulphorhodamine B (SRB) assay was performed to study viability of cells and absorbance was read at a wavelength of 495nm using a Bio Red 96 well plate reader (BioRed- India). The IC_{50} value for the extract was calculated from the plate reader data by using an online IC_{50} value Calculator (AAT Bioquest, Inc., Sunnyvale, CA, USA) (Saluja *et al*., 2020; Meher *et al*., 2021c; Patel *et al*., 2021).

DAPI staining for apoptosis detection

4',6-diamidino-2-phenylindole (DAPI) fluorescence stain was used to study apoptotic activities with fluorescence microscopy. MDAMB-231 cells were grown on 6-well plates and were treated with the methanolic extracts of *Dioscorea bulbifera* at a concentration of 50 µg/ml for 72 h. After incubation, the plates were fixed with 3% formaldehyde and washed with Phosphate buffered saline (PBS), stained with DAPI having a concentration of 10µg/ml, and washed after 5 minutes by using PBS to remove the unbound stain. A fluorescent microscope was used to capture the images (Nikon Eclipse Ts2R-FL). The morphology of cells was used to identify apoptotic cells (*e.g*. nuclear condensation), membrane bleb development, and apoptotic bodies, in comparison to untreated cells (Meher *et al*., 2021a; Pradhan *et al*., 2022).

Acridine Orange and ethidium bromide staining for detection of apoptosis

Apoptotic cells were visualized by Acridine Orange (AO) and ethidium bromide (EtBr) staining method with fluorescence microscopy. MDAMB-231 cells were grown on 6-well plates and were treated with the methanolic extracts of *Dioscorea bulbifera* at a concentration of 50 µg/ml for 72 h. After incubation, 6 well plates were fixed in 3% formaldehyde and washed with PBS, stained with AO with a concentration of 3mg/ml and washed after 5 minutes by using PBS to remove the unbound stain. Images were captured using a fluorescent microscope (Nikon Eclipse Ts2R-FL). Apoptotic cells were identified based on the stain taken by the cells. The cells stained with green represent both live and pre-apoptotic cells stained with AO, cells stained with EtBr and AO having mixed red-green, displayed membrane blabbing forming apoptotic bodies as well as nuclear condensation (Meher *et al*., 2021b; Behera *et al*., 2022).

Toxicity evaluation

We have performed the acute and subacute toxicity of the methanolic extract of *Dioscorea bulbifera* tuber. The study was conducted on albino rats of Wister strain, according to OECD guidelines 423 and 407 respectively. The animal experiment was conducted at the School of Pharmacy, Siksha 'O' Aunsandhan University, and the protocol used was approved by the Animal Ethics Committee (Protocol IAEC/SPS/SOA/18/2019).

Animal protocol

The male and female rats of (180-230 g), aged 6-8 weeks were purchased from Neelachal Tirati, Kolkata, Saha Enterprises (1828/po/Bt/S/15/CPCSEA). Animals were kept in a temperature controlled environment (23 ± 20°C) with a 12 hours light-dark cycle. Food and water were freely available (Burger *et al*., 2005). The control group received water only. Methanolic extract of *D. bulbifera* tuber was

given to the animal by the rubber tubing. There was one control group and three treated groups. Each group contained six animals. All protocols used in this study were approved by the committee on the Ethics of Animal Experiments of School of Pharmaceutical Sciences, Sikshya O Anusandhan (Deemed to be University), Bhubaneswar, Odisha, India (Protocol IAEC/SPS/SOA/18/2019), in compliance with guide for the care and use of laboratory animals published by the existing EU regulation and US National Institute of Health on animal experimentation for drug testing regulation.

Dose level

Methanolic extract of *D. bulbifera* was given to doses level of 200 mg/kg, 400 mg/kg and 800 mg/kg body weight.

Acute toxicity

The acute toxicity study was carried out using male albino Wister rats (180-230g) as per OECD guidelines. Twenty four animals were divided into four groups, each group containing 6 rats, one control group and three treated groups. The control group received water only and each treated group received a single oral dose of extract. After administering the extract, the animals were observed for any changes in the general behavior, physiological activities and survival in 72 hours.

Subacute toxicity

The subacute toxicity study was carried out using male albino Wister rats (180-230g) as per OECD guidelines. Twenty six animals were divided into four groups, each group containing 6 rats, one control group and three treated groups. The control group received water only, whereas the treated group received extracts by rubber tubing for 45 days. After 45 days, the animals were anesthetized with formalin and the animals were sacrificed to collect their blood and organs (liver, kidney) for biochemical and histological analysis.

Blood biochemical analysis

On day 46, the control and treated groups of animals were given an overdose (0.2 ml) of 3.5% formaldehyde. Then, blood was taken from the heart for analysis. The biochemical parameters analyzed from serum were glucose (G), total cholesterol (TC), triglycerides (TG), aspartate amino transferase (AST), alanine amino transferase (ALT), urea (Ur), creatinine (Cr) and total protein.

Histopathology and clinical biochemistry

On day 46, the control and treated groups of animals were given an overdose (0.2 ml) of 3.5% formaldehyde. The vital organs such as liver and kidney were removed and processed for histopathological analysis. Tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin. The tissues were observed under the microscope for toxicity evaluation. The results were subjected to one way Anova using Microsoft excel (Office 2000, Microsoft corporation, Redmond, WA, USA). P values less than 0.05 were considered as significant.

Results and discussion

Antioxidant properties

Total phenolics and flavonoids content of Dioscorea bulbifera tuber

Antioxidants are substances that, present at a low concentration compared with that of an oxidizable substrate in the medium, inhibits oxidation of the substrate (Halliwell, 2007). Under this classification, phenolic compounds, which are derived from the secondary metabolism of plants, protect multiple organs from oxidation and, therefore, phenolic compounds are regarded as natural antioxidants. The total phenolics (TPC) and flavonoids (TFC) content of six solvents extracts were analyzed and values are presented in Table 1. The highest total phenolic content (699.47 µgGAE/100mg) and total flavonoids content (705.43 µgQE/100mg) were estimated in methanolic extract compared to the other solvent extracts. Ghosh *et al*. (2013) also mentioned highest amount of TPC in methanolic extract of *Dioscorea bulbifera* bulb among petroleum ether, ethylacetate, methanol and ethanol extracts. Lowest TPC (50.18 µg GAE/100 mg) content was found in petroleum ether while the lowest TFC (20.53 µg QE/100 mg) content was found in water extracts. TFC content of acetone extract (37.58 µg QE/100 mg), ethylacetate extract (117.53 µg QE/100 mg) and petroleum ether extract (38.46 µg QE/100 mg) were significantly lower than TPC content of acetone extract (111.98±0.09 µg GAE /100 mg), ethylacetate extract (295 µg GAE /100 mg) and petroleum ether extract (50.18 µg GAE/100 mg). However, Chloroform extract contained moderate amount of TPC (151.42 µg GAE/100 mg) and TFC (140.38 µg QE/100 mg). Previously, Bhandari and Kawabata (2004) have reported 166 mg GAE/100 mg of total phenols for the acetone extract of *Dioscorea bulbifera* tuber on the wet weight basis. While, Okwu and Ndu (2006) reported 8.04 mg quercetin equivalent/100g of flavonoid contents of *Dioscorea bulbifera* tuber on the dry weight basis.

Table1. The total phenolics (TPC) and flavonoids (TFC) content of *Dioscorea bulbifera* tuber extracts.

Solvents	TPC (µg GAE/100 mg)	TFC (µg QE/100 mg)
Petroleum ether	50.18±0.35	38.46±0.30
Chloroform	151.42±0.41	140.38±0.17
Acetone	111.98±0.09	37.58±0.06
Ethylacetate	295.23±0.25	117.53±0.25
Methanol	699.47±0.34	705.43±0.30
Water	82.80±0.01	20.53±0.37

DPPH scavenging activity

DPPH scavenging potential of methanolic extract was found to be the highest while acetone, ethyl acetate, chloroform and water exhibited moderate scavenging potential. Petroleum ether extract possessed the lowest DPPH scavenging activity. Ghosh *et al*. (2013) also reported the highest DPPH radical scavenging activity for the methanolic extract of *D. bulbifera*. DPPH scavenging activity of different

solvents was presented in Table 2. There was statistically significant difference between DPPH values of six solvents extract at $p<0.05$. Out of six solvent extracts, methanolic extract showed the highest DPPH inhibition potential. These findings may be due to the phytochemical profile of methanolic extract. GC-MS revealed the presence of antioxidant compounds such as 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl; Oxiraneoctanoic acid; Hexadecanoic acid; 2-hydroxyl (hydroxyl methyl), ethyl, n-hexadecanoic acid; and E,Z-1,3,12-Nonadecatriene in the methanolic extract of *D. bulbifera*. The present findings also support the observation of Murugan and Mohan (2012) who observed similar radical scavenging activity (79.3%) of *Dioscorea* sp. extract with IC_{50} value of 38.33 µg/mL in DPPH assay.

Table 2. Percentage of inhibition of DPPH with different solvent extracts.

Name of solvents	% of inhibition
Petroleum ether	32.97±0.24
Chloroform	63.20±0.08
Ethyl acetate	73.13±0.04
Acetone	84.61±0.02
Methanol	93.81±0.02
Water	57.65±0.03

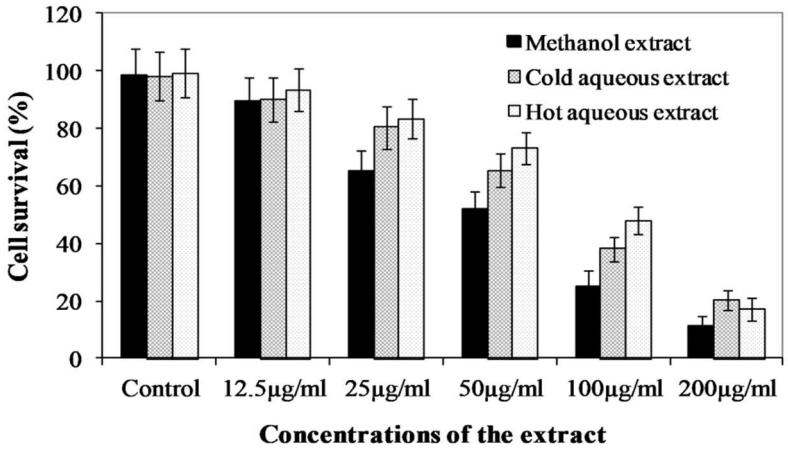
Anticancer activity

Natural products are gaining popularity in cancer therapy because they are regarded to be more physiologically friendly, meaning they are more co-evolved with their target areas and less harmful to normal cells (Mishra and Tiwari, 2011). *Dioscorea bulbifera L.* has been proven as a pharmacopotential natural product, as it was found to be effective against several diseases including diabetes, malaria, inflammation and cancer (Ghosh *et al*., 2015; Cui *et al*., 2016). As a result, it was appropriate to investigate *D. bulbifera* potential as anticancer agents. The goal of this study was to see if methanol and aqueous extracts of *Dioscorea bulbifera* L. were toxic to breast cancer (MCF-7, MDAMB-231) cells, and to identify the anticancer chemicals as an early step in the development of anticancer drugs. Tuber extracts with different solvents were tested to demonstrate inhibition of cancer cells proliferation (MCF-7 and MDAMB-231) at dosages ranging from 12.25 µg/ml to 200 µg/ml. The tuber extracts inhibited cancer cell proliferation in a concentration-dependent manner (Figure 1). The IC_{50} (the concentration of extracts inhibiting 50% of cell death) value of the different extracts was determined and collated in Table 3. The methanolic extract had the lowest IC_{50} value of 55 µg/ml and 75 µg/ml, respectively using MCF-7 and MDAMB-231 cancer cell lines. Previously, Nur and Nugroho (2018) reported IC_{50} value of 115.63µg/ml for the chloroform extract of *D. bulbifera* against T47D breast cell. Similarly, Hidayat *et al.* (2018) showed anticancer activity of *D. bulbifera* tuber extract and demonstrated that ethyl acetate fraction remarkably inhibited the survivability of human colorectal carcinoma, human colorectal adeno carcinoma, and human lung carcinoma. Apoptosis was used to explore the mechanism of cell death in response to the tuber extract.

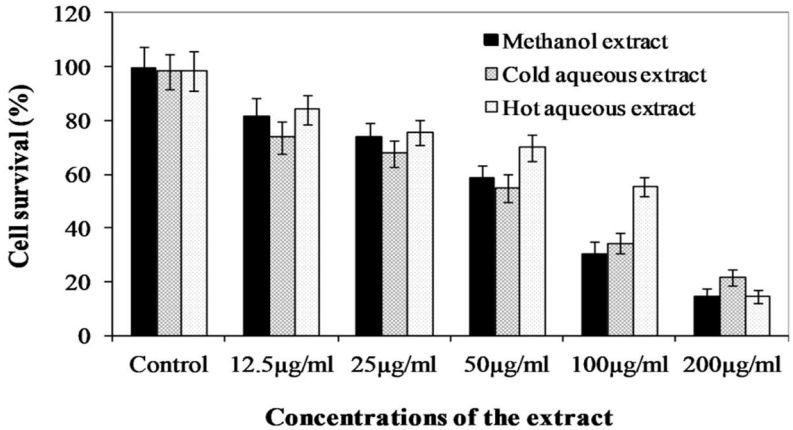
Apoptosis is characterized morphologically by changes in the membrane and nuclear structure of the treated cells. MCF-7 and MDAMB-231 cancer cells treated with 55 µg/ml IC_{50} concentration of methanolic extract revealed induction of apoptosis by staining with DAPI, Acridyne Orange and Ethidium bromide. The treated cells appeared condensed chromatin, membrane blebs, and many shattered nuclei, all of which suggested induction of apoptosis to cancer cells (Figure 2).

Table 3. IC_{50} value (µg/ml) of *D. bulbifera* extracts treated against MCF-7 and MDAMB-231 breast cancer cell lines.

	Methanolic extract	**Cold Aqueous extract**	**Boiled aqueous extract**
MCF-7	55±3.4	72±2.3	105±2.7
MDA-MB-231	75±2.8	86±2.4	126±1.8



a)



b)

Figure1. Inhibition of cancer cells proliferation with increasing concentration of different extracts of *D.bulbifera.* a) MCF-7 cancer cells, b) MDAMB-231 cancer cells.

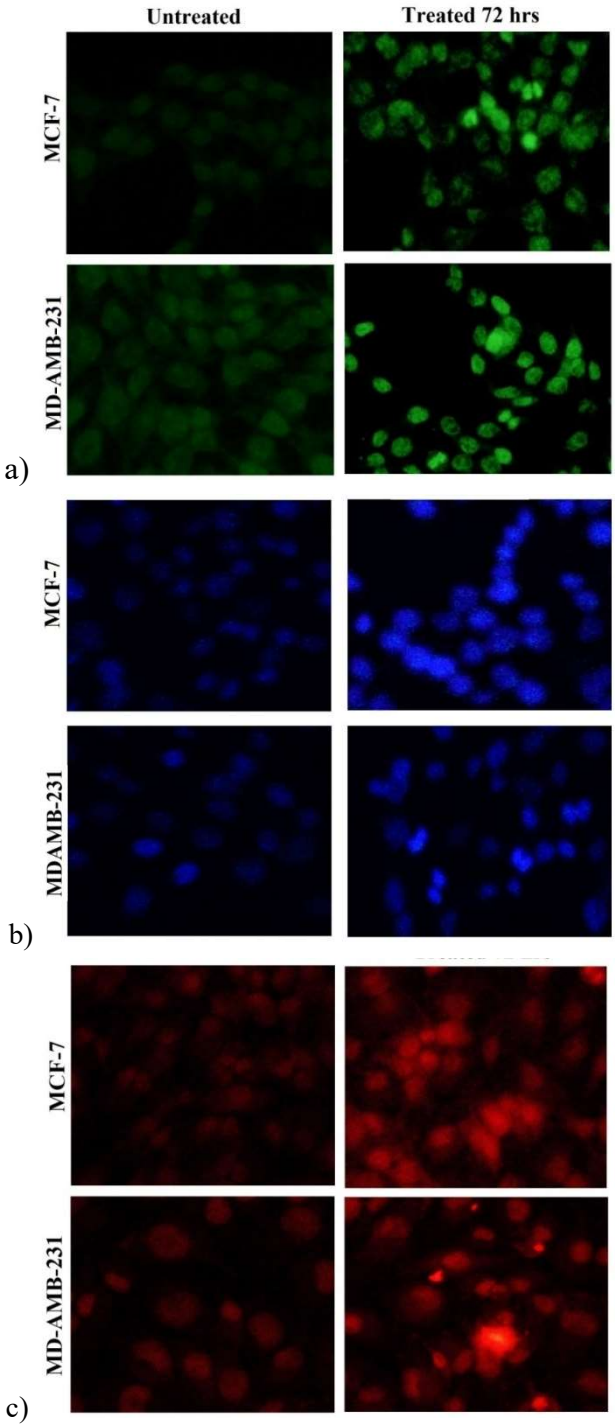


Figure 2. Methanolic extract of *D. bulbifera* treated at IC_{50} concentration against two breast cancer cell lines, showing apoptotic cells, stained with (a) acridine orange, (b) DAPI, (c) ethidium bromide.

Antibacterial activity

The antibacterial activity of six solvent extracts was assessed using the agar well diffusion technique (2 GP and 5 GNs) on separate lawn cultures of five bacterial isolates. The highest zones of inhibition against *S. aureus* (30 mm) and *C. freudii* (30 mm) were recorded using methanolic tuber extract. Similarly, the zone of acetone tuber extract inhibition against *S. aureus* was 27 mm, 25 mm against *E. fecalis,* and 24 mm against *C. freundii*. Compared to the other four solvent extracts, the petroleum-ether and the aqueous extract had less antibacterial activity. All other solvent extracts were shown to have antibacterial activity (Table 4).

Table 4. Antibacterial assay by agar-well diffusion method of hot solvent tuber-extracts of *D. bulbifera* against multi drug resistant MDR strains of bacteria as diameter size of zone of inhibition (mm).

Bacteria	Petroleum ether	Chloroform	Acetone	Ethyle acetate	Methanol	Water	Linezolid/ imipenem (30/10µg/ml)
S. aureus	10.33±0.57	20.83±0.28	26.71±0.46	29.40±	30.7±0.67	29.04±0.94	29
E. fecalis	16±1	15.62±0.54	24.85±0.24	29.7±0.63	28.92±0.88	24.55±0.50	33
A .baumannii	8.33±0.57	12.65±0.56	21.73±0.4	19.36±0.53	27±1	23.62±0.54	31
C. freundii	12±1	19.40±1.50	24.22±0.29	23.29±0.40	30.55±0.50	27.41±0.52	26
K. pneumonniae	11±1	20±1	23.44±0.49	26.40±0.43	22.58±0.52	26.76±0.75	29
P. mirabilis	18±1	18.92±1	25.60±0.52	19.40±0.35	20.11±1.17	23.83±0.80	26
P. aeruginosa	33±0.57	22.03±1	22.06±0.23	25.40±0.35	27.58±0.52	26.83±0.90	29

The maximal bactericidal activity of methanolic and acetone extracts was obtained by determining the MIC and MBC values. *E. fecalis* and *P. aeruginosa* had MIC values of 0.39 mg/mL and *S. aureus* - 0.19 mg/mL. *K. pneumonniae* and *A. baumanni* had MIC values of 0.78 mg/mL and MBC values of 3.12 mg/mL, respectively. *E. fecalis, C. freundii and P. aeruginosa* had MBC values of 1.56 mg/mL respectively. MIC and MBC values of acetone extract were also reported for all microorganisms (Table 5). The present findings are in support of Okigbo *et al*. (2009) who found that the aqueous extract of *D. bulbifera* demonstrated strong antibacterial activity against *E. coli* when tested using the disc-diffusion technique. However, ethanolic extracts were effective only against *C. albicans* and *S. aureus*.

Table 5. MIC and MBC values of the best 2 solvent extracts of *D. bulbifera* against isolated MDR strains (mg/ml).

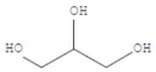
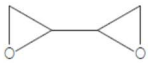
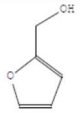
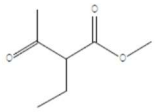
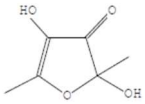
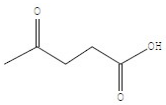
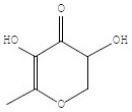
Bacteria	Methanol		Acetone	
	MIC	MBC	MIC	MBC
S. aureus	0.19±0.01	1.58±0.03	1.59±0.03	6.27±0.03
E. faecalis	0.39±0.01	1.59±0.03	1.57±0.01	6.28±0.03
A.baumannii	0.79±0.02	3.14±0.02	3.13±0.02	12.73±0.20
C. freundii	1.58±0.03	6.24±0.02	1.57±0.01	6.26±0.01
K. pneumonia	0.80±0.02	3.14±0.02	3.13±0.007	6.26±0.01
P. mirabilis	1.57±0.01	3.13±0.01	1.57±0.01	6.27±0.02
P. aeruginosa	0.40±0.01	1.57±0.50	6.27±0.20	25.15±0.07

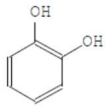
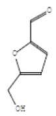
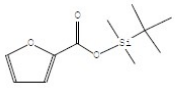
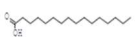
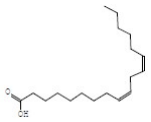
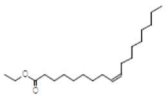
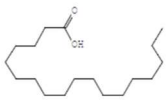
Note: MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

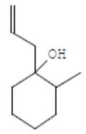
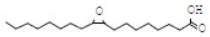
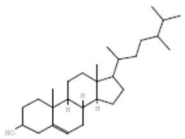
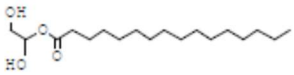
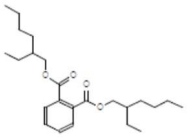
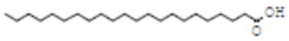
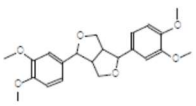
Phytochemical profiling of methanolic extract of D. bulbifera tuber by GC-MS

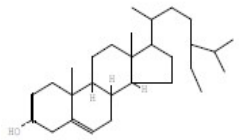
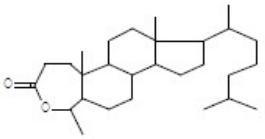
The occurrence of numerous secondary metabolites was registered in the phytochemical analysis of *Dioscorea bulbifera* methanolic extract. The existence of 24 chemicals was observed in the *D. bulbifera* tuber according to GC-MS analysis as presented in table 6. NIST library was utilized to identify the chemical structure. The chemical profiles of the identified compounds with their retention time, percentage peak area, molecular formula, molecular weight, and structure are depicted in Table 6. Presence of similar compounds with some variation has also previously identified (Mainasara *et al*., 2021; Uthirapathy *et al*., 2021). Geographic variation has a significant impact on phytoconstituents of plant. *D. bulbifera* is high in phytochemicals, which may be responsible for the tuber's diverse therapeutic potential.

Table 6. Phytochemicals identified in *Dioscorea bulbifera* tuber methanolic extract.

Peak	Characteristics		Chemical structure
2	Retention time Area Area % MW MF Name	3.42 92004535 1.91 92 $C_3H_8O_3$ Glycerin	
5	Retention time Area Area % MW MF Name	3.685 65728282 1.37 86 $C_4H_6O_2$ 2,2'-Bioxirane	
8	Retention time Area Area % MW MF Name	5.37 93793577 1.95 98 $C_5H_6O_2$ 2-Furan methanol	
10	Retention time Area Area % MW MF Name	5.865 118969443 2.48 144 $C_7H_{12}O_3$ Butanoic acid, 2-ethyl-3-oxo-, methyl ester	
13	Retention time Area Area % MW MF Name	8.34 73624179 1.53 144 $C_6H_8O_4$ 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	
15	Retention time Area Area % MW MF Name	10.41 47838807 1 116 $C_5H_8O_3$ Pentanoic acid, 4-oxo	
20	Retention time Area Area % MW MF Name	13.405 402751972 8.38 144 $C_6H_8O_4$ 4H-Pyran-4-one, 2,3-dihydro- 3,5-dihydroxy-6-methyl-	

22	Retention time Area Area % MW MF Name	15.005 58832705 1.22 110 $C_6H_6O_2$ Catechol	
23	Retention time Area Area % MW MF Name	15.81 99883193 2.08 126 $C_6H_6O_3$ 5-Hydroxy methyl furfural	
28	Retention time Area Area % MW MF Name	27.665 48696877 1.01 226 $C_{11}H_{18}O_3Si$ 2-Furoic acid, TBDMS derivative	
34	Retention time Area Area % MW MF Name	32.81 887452606 18.47 256 $C_{16}H_{32}O_2$ n-Hexadecanoic acid	
42	Retention time Area Area % MW MF Name	35.495 583484649 12.14 280 $C_{18}H_{32}O_2$ 9,12-Octadecadienoic acid (Z,Z)-	
43	Retention time Area Area % MW MF Name	35.56 375280083 7.81 310 $C_{20}H_{38}O_2$ Ethyl Oleate	
44	Retention time Area Area % MW MF Name	35.785 108739037 2.26 284 $C_{18}H_{36}O_2$ Octadecanoic acid	

48	Retention time Area Area % MW MF Name	37.575 56195142 1.17 154 $C_{10}H_{18}O$ (Z)1-Allyl-2-methylcyclohexanol	
49	Retention time Area Area % MW MF Name	37.78 77167718 1.61 298 $C_{18}H_{34}O_3$ Oxiraneoctanoic acid, 3-octyl-, cis (Anticancer, Antioxidant)-	
52	Retention time Area Area % MW MF Name	38.585 62707667 1.31 400 $C_{28}H_{48}O$ Campesterol	
53	Retention time Area Area % MW MF Name	39.34 67209909 1.4 330 $C_{19}H_{38}O_4$ Hexadecanoic acid, 2-hydroxy-1-(hydroxylmethyl) ethyl ester	
55	Retention time Area Area % MW MF Name	39.7 81566186 1.7 390 $C_{24}H_{38}O_4$ Bis(2-ethylhexyl) phthalate	
56	Retention time Area Area % MW MF Name	39.79 66600737 1.39 340 $C_{22}H_{44}O_2$ Docosanoic acid	
57	Retention time Area Area % MW MF Name	39.885 68154486 1.42 386 $C_{22}H_{26}O_6$ 1H,3H-Furo[3,4-c]furan, 1,4-bis(3,4-dimethoxyphenyl) tetrahydro-, [1R-(1.alpha., 3a.alpha.,4.beta.,6a.alpha.)]-	

59	Retention time Area Area % MW MF Name	40.835 49021686 1.02 262 $C_{19}H_{34}$ E,Z-1,3,12-Nonadecatriene (Antioxidant)	
61	Retention time Area Area % MW MF Name	41.25 381687646 7.94 414 $C_{29}H_{50}O$ Gamma -Sitosterol	
62	Retention time Area Area % MW MF Name	41.4 53507871 1.11 416 $C_{28}H_{48}O_2$ 3,4-Seco-5.alpha.-cholestan-3-oic acid, 4-hydroxy-4-methyl-, epsilon.-lactone, (4R)-	

Acute toxicity study

There was no sign and symptoms of toxicity observed in orally treated animal (200-800mg/kg body weight) neither any physiological changes were detected during the entire observation period. As a result, the LD_{50} of *D. bulbifera* methanolic extract might be higher than 800 mg/kg.

Subacute toxicity

Blood biochemical parameters and histopathology of kidney and liver of the control and experimental animals were observed to investigate any side effects to the animal.

Biochemical parameters

To see whether the *D. bulbifera* methanolic extract has any toxicity effects on animals, blood biochemical parameters were compared between treated and untreated groups of animals as can be seen in Table 7. After about 6 week of daily dosages of the extract there was no major signs of change in any blood biochemical parameters between treated and untreated animals (using one way Anova test at $p \leq 0.05$), suggesting no adverse effects on animal. In contrast to the present findings, substantial rise in ALT and AST levels in *D. bulbifera* extract-treated groups compared to the control group was reported with verified hepatotoxicity in rats (Ma *et al*., 2014). It was also reported that Diosbulbin A, Diosbulbin B, Diosbulbin C, Diosbulbin D and 8-epidiosbulbin E compounds of *Dioscorea bulbifera* have hepato-toxicity effect (Ma *et al*., 2014). However, GC-MS evaluation of methanolic extract of *D. bulbifera* demonstrated the lack of any of

these hazardous compounds in our study, which might explain the non-toxic impact of methanolic extract of *D .bulbifera* tuber in treated rats.

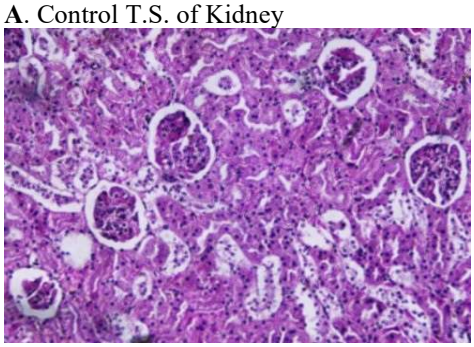
Table 7. Blood biochemical parameters of the control and treated groups of animal with *D. bulbifera* tuber methanolic extract.

Parameters	Group-I (control)	Group-II 200mg/kg body weight	Group-III 400mg/kg body weight	Group-IV 800mg/Kg body weight	Normal range
Glucose (mg/dl)	85.06±0.66	85.53±0.41	84.70±0.41	85.25±0.36	70-110
Urea (mg/dl)	31.99±0.61	32.73±0.33	32.00±0.26	31.82±0.61	15-45
Creatinine (mg/dl)	0.94±0.02	0.93±0.02	0.90±0.01	0.94±0.03	0.5-1.5
Total protein (mg/dl)	6.84±0.02	6.93±0.12	6.84±0.02	6.88±0.11	6.0-8.0
Total cholesterol (mg/dl)	128.0±0.03	128.1±0.06	128.3±0.19	128.3±0.18	140-250
Tri glycerides (mg/dl)	92.05±0.03	91.93±0.36	92.15±0.28	92.25±0.35	25-160
(AST) (IU/L)	32.19±0.24	32.22±0.22	31.98±0.54	32.13±0.39	Up to 46
(ALT) (IU/L)	27.72±0.32	28.26±0.31	28.43±0.31	28.53±0.34	Up to 40

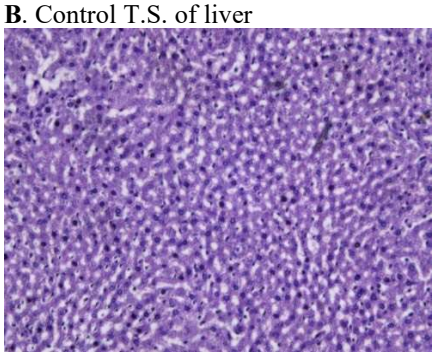
Histopathological studies

The effect of methanolic extract of *D. bulbifera* tuber on kidney and liver histopathological alterations after 6 weeks of therapy is given in Figure 6. When compared to the untreated group, the therapy with daily dosages of 200, 400, and 800 mg/kg body weight for 45 days failed to show any significant differences. Necrosis, infiltration, oedema and conjunction, which are sign of hepatotoxicity, were not observed in the liver cells of the experimental group. The liver showed normal hepatic lobular architecture. The kidneys revealed normal glomeruli, proximal and distal tubules, interstitium, and blood vessels. The histopathological images of kidney and liver of untreated and treated with different doses are shown in Figure 6.

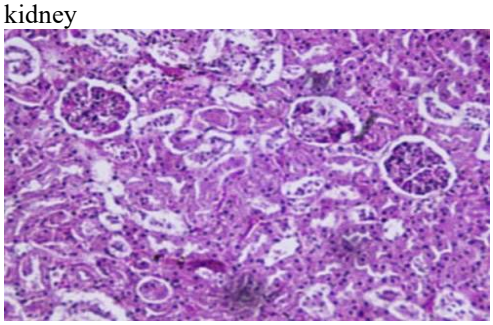
A. Control T.S. of Kidney



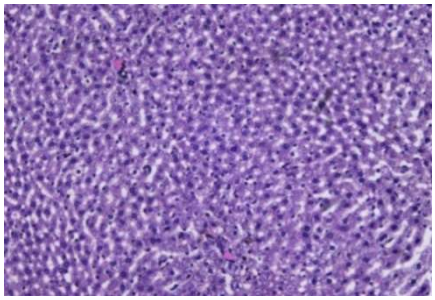
B. Control T.S. of liver



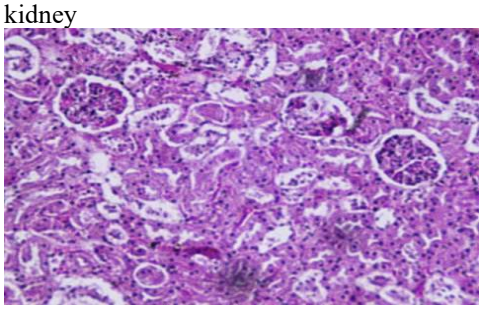
C. Treated (200 mg/kg body weight) T.S. of kidney



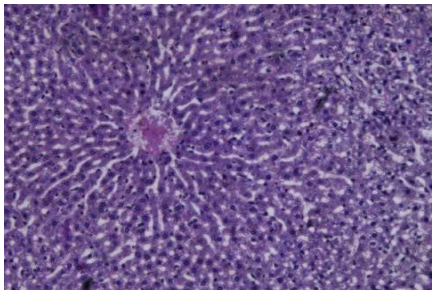
D. Treated (200 mg/kg body weight T.S. of liver



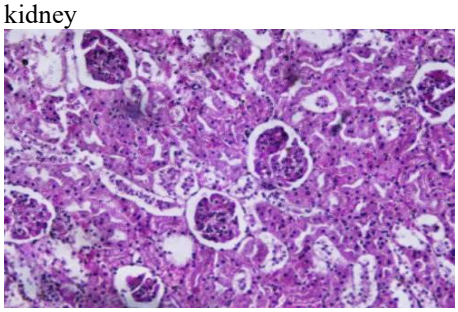
E. Treated (400 mg/kg body weight) T.S. of kidney



F. Treated (400 mg/kg body weight) T.S. of liver



G. Treated (800 mg/kg body weight) T.S. of kidney



H. Treated (800 mg/kg body weight) T.S. of liver

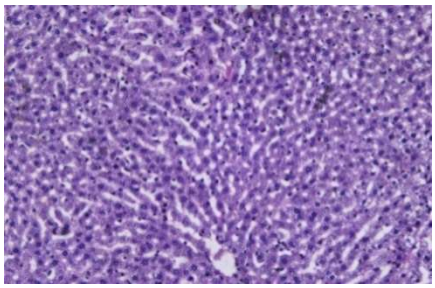


Figure 6. Panels represent H&E staining of paraffin-embedded 5 micron-thick sections of the kidney and liver at magnifications 200x of control and treated animals with increasing dose of *D. bulbifera* tuber methanolic extract.

Conclusions

In conclusion, we summarize the abundance of an existing natural therapeutic agent in *D. bulbifera*. Our findings showed that the extracts in methanolic and cold aqueous solutions were effective against proliferation of both MCF-7 and MDA-MB-231 breast cancer cells. Both extracts showed to induce apoptotic cell death in breast cancer cells. *D. bulbifera* extracts exhibited anticancer ability as well as antimicrobial activities against MDR pathogenic bacteria strain. GC-MS analysis of crude extracts showed the presence of several potential anticancer and antimicrobial compounds. Meanwhile, the extract didn`t show any significant toxicity in *in vivo* experiment. However, determining the molecular mechanism of the active phytochemicals, require more investigations on purification of novel molecules. Our preliminary studies results provide more evidence in favor of *D. bulbifera* potential as an alternative therapeutic treatment for breast cancer as well as against MDR pathogenic bacterial infection.

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