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Investigation of Therapeutics Benefits of *Dioscorea bulbifera* in Breast Cancer

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ABSTRACT

Aim: To investigate the therapeutics benefits of Dioscorea bulbifera in breast cancer. Materials and Methods: Extract of Dioscorea bulbifera prepared by using Soxhlet extraction method. The various experimental models were performed as, In silico Docking Study and Pass Online study, In vivoNMU induce breast cancer in female wistar rats for 30 days, In vitro MDA-MB 231 breast cancer cell line study and hemolytic activity. All studies were compared with standard drug Tamoxifen. Results: Molecular docking studies showed higher binding affinity and more number of interactions of Diosbulbin B with respective receptors as compared with the standard like Doxorubicin, Fluvistrant, Mefepristone, Onapristone. In vitro NMU induced cancer model revealed that the estrogen and progesterone levels decreases in blood serum in test group as compared to disease control group and standard group after 30 days of study. In vitro MDAMB 231 cell treated with Dioscoreabulbifera (10, 50, 100, 200, 500 mg/ml) showed significant decreased in cell count and cell viability, which indicates cytotoxic effect of hydro alcoholic extract of Dioscorea bulbifera. Conclusions: From the findings of the performed models we can conclude that hydroalcoholic extract of Dioscorea bulbifera shows anti-cancer activity which can work by aromatase inhibiting activity as it reduce estrange and progesterone levels and might be useful as a better and safer herbal alternate of breast cancer.

Keywords: Anti cancer activity; Breast cancer; *Dioscorea bulbifera*; NMU induced breast cancer model; Cell line study; Hemolytic activity; *In silico* docking study

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INTRODUCTION

Cancer is a general term used to refer to a condition where the body's cells begin to grow and reproduce in an uncontrollable way. These cells can then invade and destroy healthy tissue, including organs. Cancer sometimes begins in one part of the body before spreading to other parts. Cancer is a common condition and a serious health related problem. More than one in three people can develop some form of cancer during their lifetime. There are more than hundred different types of cancers are there but the most common types of the cancers are Breast cancer, Cervical cancer, Prostate cancer, Lung cancer, Mouth cancer [1]. There ‘’are different types of breast cancers such as ductal carcinoma, infiltrating ductal carcinoma, lobular carcinoma, invasive lobular carcinoma, inflammatory breast cancer, etc. Early “breast cancer often does not cause symptoms. Therefore regular breast exams and mammograms are important, so cancers that don't have symptoms may be found” earlier. Various ‘’types of the treatments were used for breast cancer such as hormone therapy, radiation therapy, chemotherapy, targeted drug therapy, mammography, ultrasound, etc. [2] Targeted therapy drugs that targets the specifically targeted genes and makes some kind of changes in genes and helps in cancer cells.’’ For eg. Drugs that target HER2, including trastuzumab(Herceptin), pertuzumab(Perjeta), lapatinib(Tykerb), PARP Inhibitors, Angiogenesis drugs [3]. The ‘’use of the plant-derived natural products for medicinal benefits has played an important role in almost all the people on earth. In Indian system of medicine (ISM), Ayurveda, Siddha, Unani and Homeopathic system provide health care for large part of the population, ISM is vas and its review revealed several plants which can be used for the drug development [4]. Since 1961, several anticancer drugs have been made available on the market that trace their

origins to plants such as Taxol(Paclitaxel) [4], Navelbine [4], and Gemzar(Gemcitabine) [5], Oncovin(Vincristine) [6] etc are allopathic drugs used in cancer disease but have serious side effects and also they are very costly as compared to herbal medicines and also have very less side effects so we choose herbal drug for the management of cancer disease" i.e. Dioscorea bulbifera(Varahikand). *Dioscorea bulbifera* "is one of the unique medicinal plants among 600 species in the family Dioscoreaceae which has found its importance in traditional medicine throughout the world. Aglycons of its steroid saponins like diosgenin have gained attention as precursors in the synthesis of sex hormones, cardiatonic glucosides, fertility control compounds, corticosteroids and anabolic" agents [7]. It "also possess the various activities like anti-tumor, anti-diabetic, anti-inflammatory, gastroprotective, anti-oxidant, etc types of" activity [8]. Dioscorea bulbifera contains Diosbulbin B as chemical constituent which is used in synthesis of corticosteroids which is responsible for management of breast cancer [9]. Research herb possess the anti-oxidant property by acting on free radicals like DPPH and OH ions so it can be used in management of cancer disease [10,11].Mixture of different parts of the plants i.e leaf, roots and bulbs were used in this study for the management of breast cancer.

It is reported that *Dioscorea bulbifera* possess the anti tumor activity so it can be used in management of breast cancer [8]. To complete this investigation of *Dioscorea bulbifera* various experimental models were used, such as *In Silico* method (Docking study) from that we can come to know about the binding affinity of the drug towards the respective receptors, *In vitro* model (Hemolytic Assay) from that we can come to know about the dose of the drug as well the cytotoxic effect of the drug, *In vivo* (N methyl nitrosoureinduce model), from that we can come to know about the pharmacological effects of the drug.

MATERIALS AND METHODS

Collection and authentification

The plant used in this study was acquired from local area near Porbandar city. Authenticity of this plant was confirmed by Mrs. Trupti Marakna, Assistant Professor, School of Science, RK University by comparing their morphological characters with the description mentioned in different standard texts.

Pharmacognostic studies

Morphological charecterization of crude herb was done and compared with available literature.

Extraction (Figure 1)

Figure 1: Method performed for extraction

Extraction of Diosbulbin B from *Dioscorea bulbifera* was done by Soxhlet extraction (continuous hot extraction) process. Plant was collected from local area near Porbandar region and was dried in open atmosphere. Pulverisation of different parts of plants was prepared. Weighed powder was placed in thimble and measured amount of extract solvent (70%V/V methanol) was added. Evaporation of extract solvent will be done using water bath. % yield will be measure.

Experimental models

***In silico* model**

Pass online study: Structure of Diosbulbin B was downloaded from website https://pubchem.ncbi.nlm.nih.gov/ and saved as pdf file format. This file format does not open in PASS online software. Therefore, it was converted to .mol

file format by using OpenBable GUI 2.3.2 software. This file was uploaded in PASS online software and predicted biological activity profile for Diosbulbin B was obtained.

Docking protocol: For "docking, ligands and proteins are required in .pdbqt file format. They were prepared for docking using AutoDock Tool 1.5.6 (ATD). Also, grid box sizes were decided using the same programme. Finally, molecular docking was done using AutoDock Vina 1.0 and further steps for docking were followed as" under [12,13].

In vitro model

Hemolytic assay [14]:

Blood was collected by retroorbital method from animals in tube containing anti-coagulant. Blood solution was prepared by washing six times with tris buffered solution (TBS), containing 6.05 gm tris HCL, 8.76 gm NaCl in 800 ml distilled water and pH 7.6 of solution was adjusted by 1M HCl. Following last wash, red blood cells (RBC) were diluted to 1/10 of their volume with TBS. The assay was performed by mixing 0.3 ml of RBC solution with 1.2 ml of crude extract; 1.2 ml of distilled water was set as positive control and 1.2 ml of TBS as a negative control. The mixture were kept vortexed, left for 2h at room temperature, and then centrifuged at 4000 RPM for 10 min at 40°C. Absorbance of supernants were measured at 541 nm in UV visible spectrophotometer. The percentage hemolysis of each fraction was calculated using expression below (Figure 2).

$$\text{Hemolytic activity} = \frac{Abs(sample) - Abs(negative\ control)}{Abs(positive\ control) - Abs(negative\ control)} \times 100$$

Figure 2: Remi Centrifuge

Cell line Study [15]:

Approximately 10,000 cells were plated per well in a 96 well plate and the plate was incubated at 37 degree Celsius in a CO_2 incubator. Thereafter the cells were treated with the different concentrations of the compound. Each concentration was tested in triplicates. The plate was then incubated at 37 degree Celsius in a CO_2 incubator.

Thereafter, the cell viability upon 48 hours of drug treatment was assessed using MTT assay.

20 microliter of 5mg/ml MTT solution were added to each well after 46 hours of drug exposure and the plate were incubated at 37 degree Celsius in a CO_2 incubator for 2 hours.

The reduction of MTT by the viable cells leads to the formation of formazon. The medium were removed from the wells and 100 microliter of DMSO was added to dissolve the formazon crystals and absorbance of the dissolved formazon was taken at 570 nm using plate reader. Percent cell viability was calculated by equating the absorbance of the control well to 100 percent viability performed in Perd University, Ahmedabad.

***In vivo* model**

N- methyl nitrosourea (NMU) induce cancer model [16]:

Female wistar rats at 21 days of age were selected for experiment. NMU (50 mg/kg i.p.) were injected on 1, 7, 14, 21 days alternately through left and right abdominal wall. The MNU was always dissolved immediately before use in 0.9 % NaCl adjusted to pH 4 with acetic acid. The solubility of NMU in water at room temperature was 1.4 % (w/v). The experiment was terminated on the 30th day of the animals' age. At the end of 30 days of study blood were collected from animals by retroorbital method and serum was separated by centrifugation. Then serum was subjected for the analysis of estrogen and progesterone level by ARCHITECT *i* system.

Biochemical parameters:
Serum: Estrogen, Progesterone
Assay Procedure for estrogen: The system automatically performs the following actions:
Dispenses 80µL of sample and 75µL of Ancillary Pack Reagent into a cuvette and incubates for 4.5 minutes at 37°C. Dispenses 75µL of Lite Reagent and incubates for 2.75 minutes at 37°C.
Dispenses 100µL of Solid Phase Reagent with 25µL of Ancillary Well Reagent and incubates for 5.5 minutes at 37°C. Separates, aspirates, and washes the cuvette with wash 1 and dispenses 300 µL of Acid Reagent and Base Reagent each to initiate the chemiluminescent reaction.

Calculation of results:
The system reports serum estradiol results in pg/mL (common units) or pmol/L (SI units), depending on the units defined when setting up the assay. Conversion formula is 1 pg/mL = 3.67 pmol/L

Assay procedure for progesterone:
Load the ARCHITECT Progesterone Reagent Kit on the ARCHITECT*i* System. Verify that all necessary assay reagents are present. Ensure that septums are present on all reagent bottles. The minimum sample cup volume is calculated by the system and is printed on the Order list report. No more than 10 replicates may be sampled from the same sample cup. To minimize the effects of evaporation verify adequate sample cup volume is present prior to running the test. If using primary or aliquot tubes, use the sample gauge to ensure sufficient patient specimen is present. ARCHITECT Progesterone Calibrators and Controls must be mixed thoroughly by low speed vortex or inversion prior to use. To obtain the recommended volume requirements for the ARCHITECT Progesterone Calibrators and Controls, dispense a minimum of 200 µL of each calibrator or a minimum of 150 µL of each control into each respective sample cup. Load samples and press run.

List of chemical and instruments needed:

Instruments	Equipments
Heating mental	Micro pipette and micro tips
Cooling centrifuge	EDTA tubes
Weighing balance	Eppendorf
Spectrophotometer	Capillary
ARCHITECTi System	Small plastic beakers
CO2 incubator	Tuberculin syringe
Plate Reader	Oral feeding needle
Laminar flow hood	Dissection Box

Chemicals:
Water, methanol, diethyl ether, DMSO etc. 5.6 Statistical Analysis Method To checking the significance of data, following statistical tests were performed;
ANOVA: to see variability within all the groups.
Tuckey's test: for the same purpose mentioned in above test.
INSTAT software: to derive all the statistical terms like Standard Error of Mean (SEM),
ANOVA, *P*-value, Degree of freedom, Standard deviation, etc

RESULTS

Pharmacognostic evaluation of Hydroalcoholic extract of *Dioscorea bulbifera*
Extract of crude drug prepared by Soxhlet extraction method using methanol and water as solvent. After completion of three cycles solvent in RBF is evaporated and 27.37% W/W yield is obtained having dark brown colour and semisolid consistency (Table 1).

Table 1: Pharmacognostic evaluation of extracts of *Dioscorea bulbifera*

Extract	% W/W yield	Colour	Consistency
Hydroalcoholic extract of *Dioscorea bulbifera*	27.37%	Dark Brown	Semi solid

Phytochemical screening of hydroalcoholic extract of *Dioscorea bulbifera*
The extracts obtained from Soxhlet were subjected to various chemical tests to determine the presence of phytochemical constituents like favanoids, terpenoids, steroid, saponin, cardiac glycosides. The result is reported below (Table 2)

Table 2: Phytochemical screening of extracts of *Dioscorea bulbifera*

Type of extract	Name of Compound	*Dioscorea bulbifera*
Hydroalcoholic extract	Cholesterol	-
	Alkaloid	-
	Flavanoid	+++
	Terpenoid	+++
	Cardiac glycoside	++
	Steroid	++
	Saponin	+

+ indicates the presence of phytochemical constituents; - indicates the presence of phytochemical constituents

Pass online study for activity prediction:

Predicted pharmacological activities of diosbulbin B using Pass online software revealed that Diosbulbin B might be active (Pa) 93.6 % as BRAF expression inhibitor, 82.6 % apoptosis inducer, 74.2% as antineoplastic, 59% TNF expression inhibitors, 48% chemopreventive as cancer treatment. (Table 3)

Table 3: Activity determination Study of *Dioscorea bulbifera* using PASS Online Software

Pa	Pi	Activity
0,936	0,001	BRAF expression inhibitor
0,826	0,006	Apoptosis agonist
0,742	0,019	Antineoplastic
0,590	0,014	TNF expression inhibitor
0,569	0,017	AR expression inhibitors
0,480	0,016	Chemopreventive

Pa – Probability of activation; Pi – Probability of inactivation

In silico* study- Docking study of *Dioscorea bulbifera:

From docking study of *dioscorea bulbifera*, we come to know about the binding affinity of drug towards the respective receptor. If drug is having more binding affinity then it requires less energy for binding. From the below result of binding affinity of Diosbulbin B with the estrogen protein/receptor, there was significant decrease in binding affinity as compared to Doxorubicin as standard drug. There was no any kind of significant difference of binding affinity of Diosbulbin B as compared to Fluvestrant as standard drug (Table 4) (Figures 3 and 4).

Table 4: Docking study of Diosbulbin B with estrogen receptor

Mode	Estrogen			
	Diosbulbin B	Doxorubicin	Temoxifene	Fluvestrant
1	-12.4	-9.5	-7.3	-9.9
2	-12	-9.3	-7.1	-9.3
3	-11.1	-9	-7	-9.3
4	-10.4	-8.9	-7	-9.3
5	-10.2	-8.7	-6.9	-9
6	-10.2	-8.6	-6.8	-9
7	-10.2	-8.5	-6.7	-9
8	-10.1	-8.4	-6.7	-9
9	-10.1	-8.4	-6.6	-9

Binding affinity of Diosbulbin B decreases with the progesterone receptor as compared to Doxorubicin, Mefepristone and Onapristone (Table 5).

Table 5: Docking study of Diosbulbin B with progesterone receptor

Mode	Progesterone			
	Diosbulbin B	Doxorubicin	Mefepristone	Onapristone
1	-12.3	-11.1	-10.3	-10.6
2	-11.8	-10.7	-10	-9.7
3	-11.8	-10.3	-9.9	-9.2
4	-11.8	-10.3	-9.8	-9.2
5	-11.6	-10.1	-9.8	-9.1
6	-11.5	-9.9	-9.6	-9.1
7	-11.5	-9.8	-9.5	-9.1
8	-11.4	-9.7	-9.4	-9
9	-11.9	-9.6	-9.3	-8.9

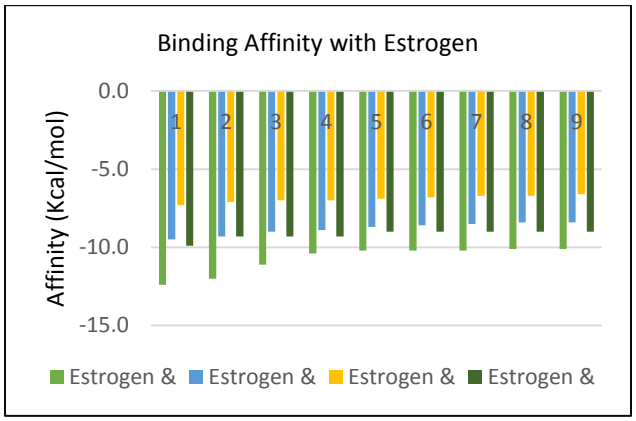

Figure 3: Binding affinity of Diosbulbin B with estrogen receptor

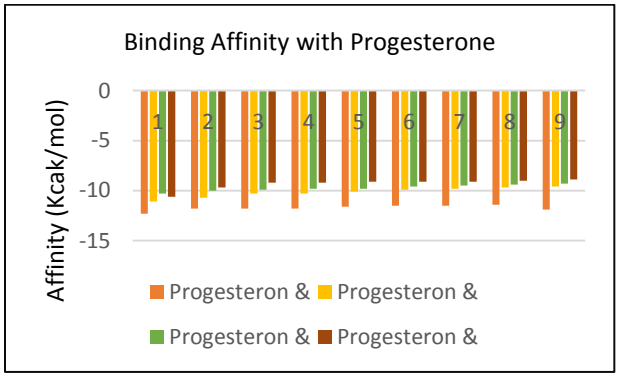

Figure 4: Binding affinity of Diosbulbin B with progesterone receptor

From the below result, it is observed that there is no any kind of difference observed in binding affinity of Diosbulbin B with the Her2 receptor (Table 6) (Figure 5).

Table 6: Docking study of Diosbulbin B with Her2 receptor

Mode	Her2	
	Diosbulbin B	Doxorubicin
1	-11.2	-11
2	-11	-10.4
3	-10.9	-10.4
4	-10.8	-10.1
5	-10.5	-9.9
6	-10.4	-9.5
7	-10.1	-9.5
8	-9.9	-9.3
9	-9.7	-9.3

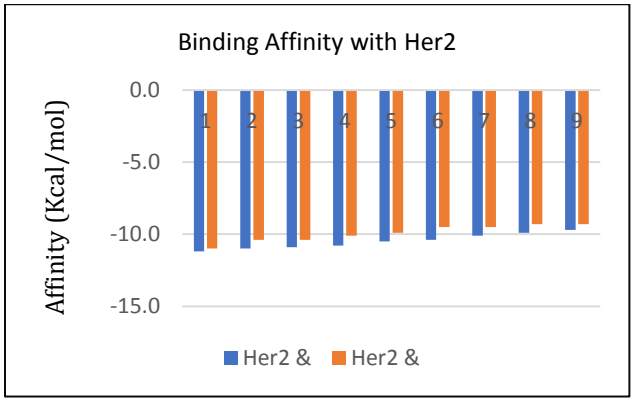

Figure 5: Binding affinity of Diosbulbin B with Her2 receptor

From the below result, it is observed that Diosbulbin B on aromatase having same binding affinity as compared to Exemestane and Testolactone. There was decrease in binding affinity of Diosbulbin B on aromatase as compared to Doxorubicin and Letrozole (Table 7) (Figure 6).

Table 7: Docking study of Diosbulbin B with aromatase

Mode	Aromatase				
	Diosbulbin B	Doxorubicin	Exemestane	Letrozole	Testolactone
1	-11.4	-10.4	-11.2	-7.7	-11.5
2	-10.7	-10.2	-10.6	-7.6	-10.7
3	-10.7	-10	-10.6	-7.6	-10.5
4	-10.5	-9.9	-10.5	-7.4	-10.4
5	-10.4	-9.8	-10.4	-7.4	-10.4
6	-10.2	-9.8	-10.3	-7.3	-10.4
7	-10.1	-9.8	-10.2	-7.3	-10.1
8	-10	-9.8	-9.9	-7.2	-9.9
9	-10	-9.8	-9.6	-7.2	-9.9

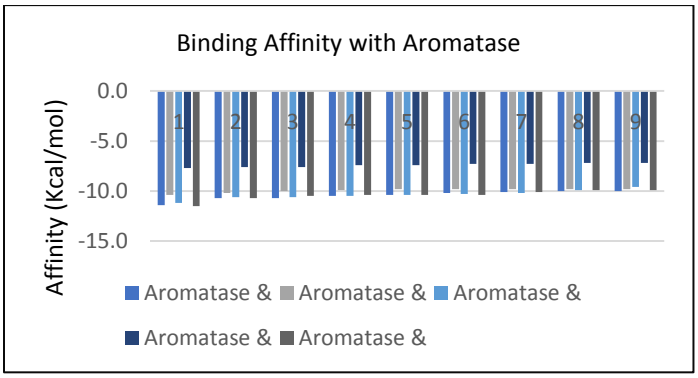

Figure 6: Binding affinity of Diosbulbin B with aromatase

Effects of hydroalcoholic extract of *Dioscorea bulbifera* on cell viability (Hemolytic activity):

Haemolytic activity of extract was done to know the cytotoxic effect of *Dioscorea bulbifera*, by collecting blood from animals by retroorbital method. Serum was separated by cooling centrifuge and different solution of drug was prepared and absorbance of sample was taken by UV-visible spectrophotometer. As the absorbance of sample increases there are more chances of haemolysis, which indicates cytotoxic activity of test samples of different concentration (Table 8).

Table 8: Hemolytic activity of extracts of *Dioscorea bulbifera*

Concentration(mg/ml)	Absorbance (λ_{max} 541 nm)	% haemolysis
50	1.954	36%
200	2.573	54%
500	3.552	82%

Effects of Hydroalcoholic extract of *Dioscorea bulbifera* on MDAMB 231 breast cancer cell line:

The breast cancer cell lines MDA-MB-231 were used to evaluate cytotoxic effects of Hydroalcoholic extract of *Dioscorea bulbifera. MDA-MB-231* cancer cell line is helpful to understand important role in control of growth and proliferation of breast tumor cells. MDA-MB-231 cancer cell may serve as a model for native human breast cancer cell line as they express high estrogen receptors and adenosine receptor. Overexpression of these receptors results in activation of Adenosine A2B receptors mediate a stimulation of adenylyl cyclase, but are in addition coupled to a Ca2+ signal. The Ca2+ response requires activation of phospholipase C and appears to be mediated via Gq/11 pathway. Further work will be required to understand the role of the A2B adenosine receptors in tumor growth and development (Figure 7).

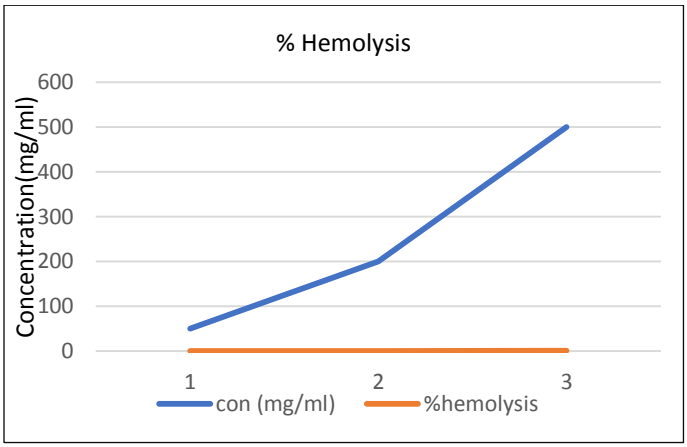

Figure 7: Hemolytic activity of extracts of *Dioscorea bulbifera*

% cell viability:

Different concentration of hydro alcoholic extract of *Dioscorea bulbifera* were subjected to determined % cell viability study using MDA-MB-231 at 8, 24 and 48 hours. There were concentration dependent decreased in cell viability was observed in treatment of hydroalcoholic extract of *Dioscorea bulbifera* (Table 9) (Figures 8-13).

Table 9: %cell viability of hydroalcoholic extract of *Dioscorea bulbifera*

Concentration (mg/ml)	% Cell Viability		
	8 hrs	24 hrs	48 hrs
Negative control	-	-	-
10	106.65	50.24	43.79
50	105.61	49.62	26.97
100	100.58	48.47	24.72
200	87.22	35.2	15.82
500	64.83	12.28	6.72

IC50:

The IC50 is the concentration of an inhibitor where the response (or binding) is reduced by half. This quantitative measure indicates how much of a particular drug or other substance is needed to inhibit a given biological process by half. Different concentration of hydro alcoholic extract of Dioscorea bulbifera at different time cost were subjected to determined IC5o value using Graphpad prism 6 software at 8, 24 and 48 hours. There were dependent time cost decreased in IC5o value was observed in treatment of hydroalcoholic extract of *Dioscorea bulbifera*. (Table 10)

Table 10: IC50 of hydroalcoholic extract of *Dioscorea bulbifera*

Time cost	IC50 (Calculated using graphpad Prism 6)
8 hrs	1925 mg/ml
24 hrs	6.054 mg/ml
48 hrs	0.885 mg/ml

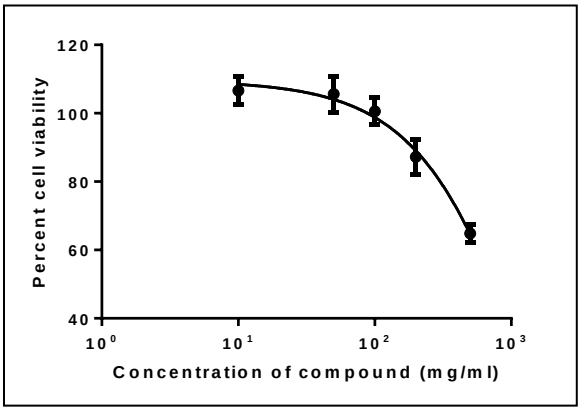

Figure 8: Growth curve for MDA-MB-231 cells at 8 hrs

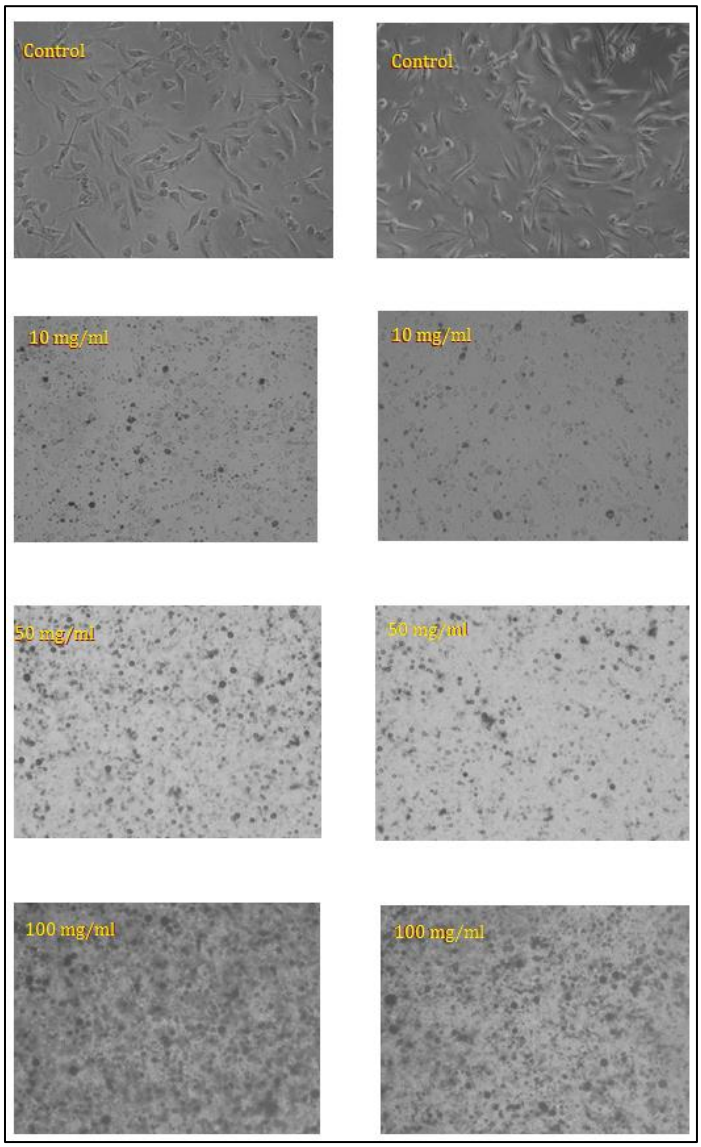

Figure 9: Photomicrograph of MDA-MB-231 cells at 8 hrs

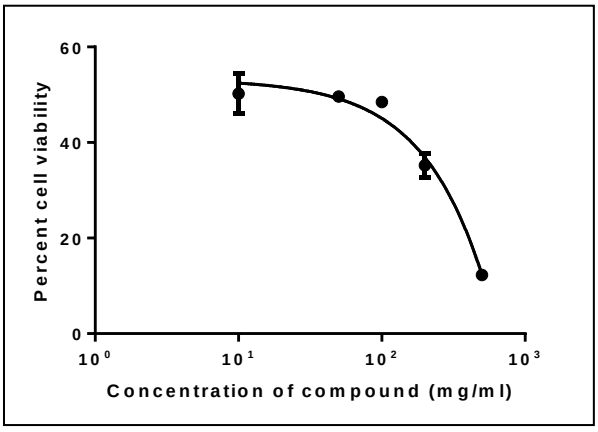

Figure 10: Growth curve for MDA-MB-231 cells at 24 hrs

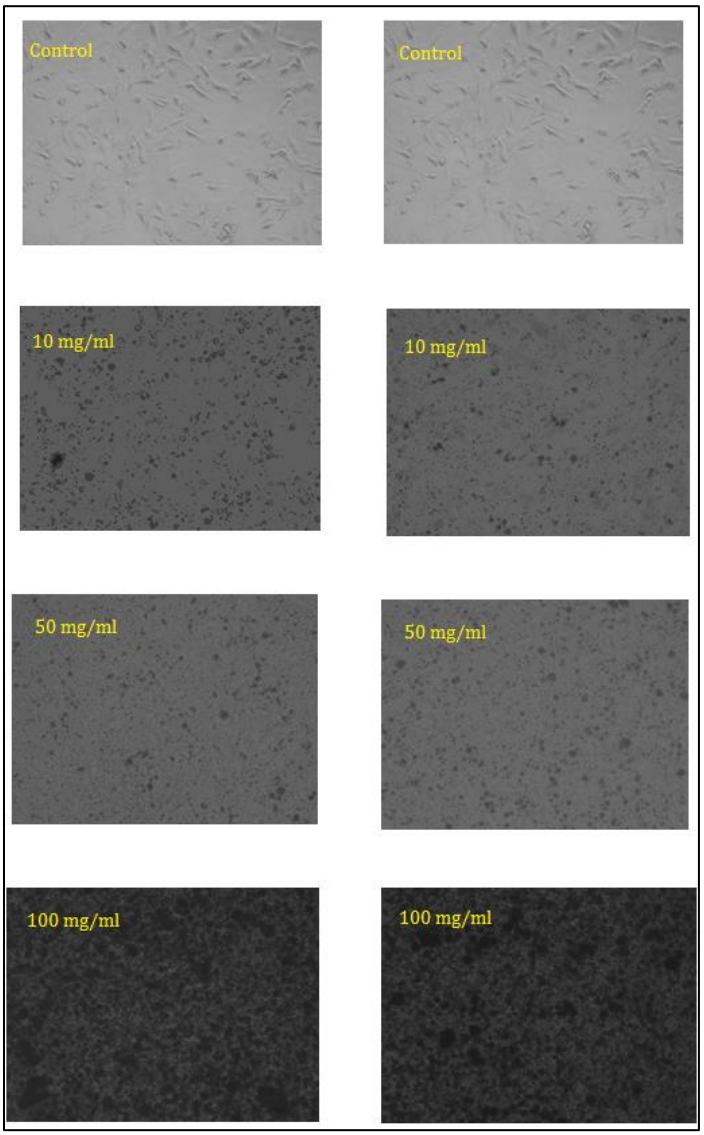

Figure 11: Photomicrograph of MDA-MB-231 cells at 24 hrs

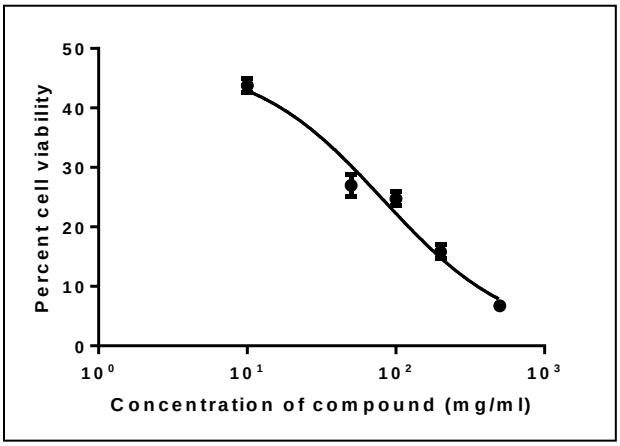

Figure 12: Growth curve for MDA-MB-231 cells at 48 hrs

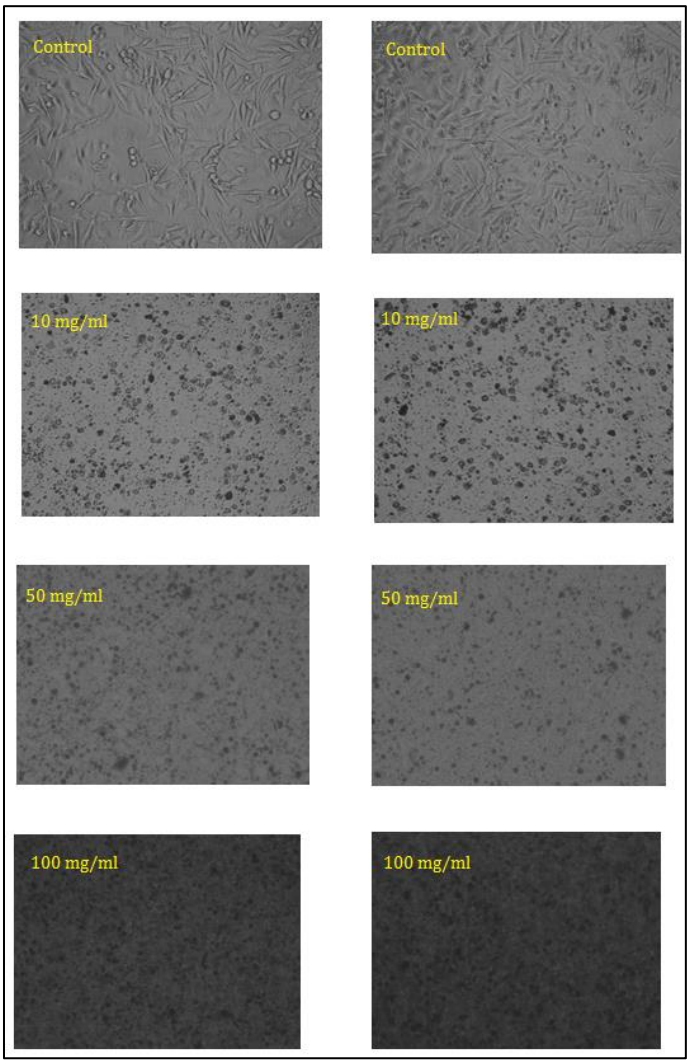

Figure 13: Photomicrograph of MDA-MB-231 cells at 48 hrs

Effects of Hydroalcoholic extract of *Dioscorea bulbifera* on Estrogen level:

Estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium. Increase estrogen levels in body indicate that there are more chances of breast cancer in human. There was a significant increase in estrogen level in disease control group (74.54 ± 3.10) as compared to normal control group (41.36 ± 1.11). There was significant decrease in estrogen level in standard Tamoxifen(20mg/kg) group (58.76 ± 2.40), HEDB (200mg/kg) group (57.17 ± 2.32), HEDB (500mg/kg) group (59.49 ± 1.64) as compared to disease control group (Tables 11 and 12) (Figure 14).

Table 11: Beneficial effect of *Dioscorea bulbifera* on Estrogen levels at 15th day in NMU induce breast cancer Wistar female rats

Groups	Estrogen level on 15th day (pg/ml)						Average ± SEM
Normal	42.07	40.01	41.01	43.03	40.04	42.05	41.36 ± 1.11
Disease	75.02	79.52	71.47	73.03	77.36	70.89	74.54 ± 3.10#
Standard Tamoxifen (20 mg/kg)	55.25	58.23	59.63	63.24	57.58	58.67	58.76 ± 2.40*
HEDB (200mg/kg)	58.02	56.32	59.36	53.27	60.23	55.85	57.17 ± 2.32**
HEDB (500mg/kg)	59.32	61.32	57.56	57.17	60.34	61.23	59.49 ± 1.64***

- Significant increase in estrogen level in disease control group as compared to normal control group; ** indicate significant decreased in estrogen level in standard treated group as compared to diseases control group (level of significance p<0.001≈significant.); *** indicate significant decreased in estrogen level in treatment group as compared to diseases control group (level of significance p<0.001≈highly significant).

Table 12: Beneficial effect of *Dioscorea bulbifera* on Estrogen levels after 30 days in NMU induce breast cancer Wistar female rats

Groups	Estrogen level after 30 days (pg/ml)						Average ± SEM
Normal	42.07	40.01	41.01	43.03	40.04	42.05	41.36 ± 1.11
Disease	95.03	99.05	97.05	96.03	98.04	95.05	96.70 ± 1.49#
Standard Tamoxifen (20 mg/kg)	48.07	47.01	50.23	55.55	49.04	49.09	49.83 ± 2.74**
HEDB (200mg/kg)	45.77	47.93	49.06	48.55	49.04	47.09	47.90 ± 1.17***
HEDB (500mg/kg)	44.77	43.06	41.66	42.55	45.04	44.09	43.52 ± 1.21***

- Significant increase in estrogen level in disease control group as compared to normal control group; ** indicate significant decreased in estrogen level in standard treated group as compared to diseases control group (level of significance p<0.001≈significant.); *** indicate significant decreased in estrogen level in treatment group as compared to diseases control group (level of significance p<0.001≈highly significant.)

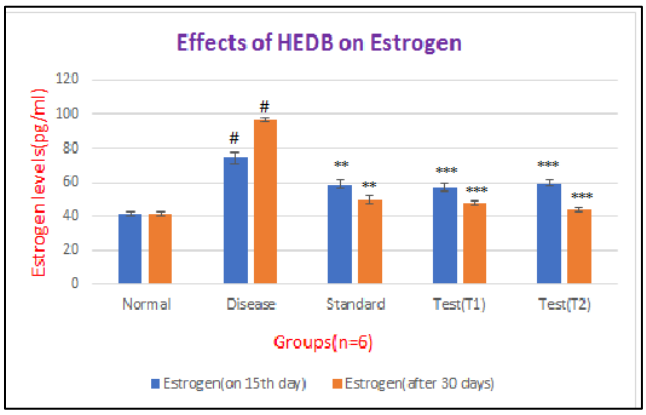

Figure 14: Estrogen levels in hydroalcoholic extract of *Dioscorea bulbifera*

n=6 and results were shown as mean ± SEM; Control group received distilled water; Disease control group received NMU (50mg/kg, i.p.) in respective group; Standard control group received Tamoxifen (20mg/kg, p.o.) in respective group; Test (T1) group received HEDB(200mg/kg, p.o.) (Hydro alcoholic extracts of *Dioscorea bulbifera)*; Test (T2) group received HEDB (500mg/kg, p.o.) (Hydro alcoholic extracts of *Dioscorea bulbifera)*

- Significant increased in estrogen level in disease control group as compared to normal control group; ** indicate significant decreased in estrogen level in standard treated group as compared to diseases control group (level of significance p<0.001≈significant.); *** indicate significant decreased in estrogen level in treatment group as compared to diseases control group (level of significance p<0.001≈highly significant.)

Effects of hydroalcoholic extract of *Dioscorea bulbifera* on progesterone level:

Progesterone is an ovarian steroid hormone that is essential for normal breast development during puberty and in preparation for lactation and breastfeeding. Progesterone decreases the cell proliferation. As the level of progesterone increases there are more chances of breast cancer. There was significant increase in progesterone levels in disease control group (36.62 ± 1.12#) as compared to normal group (9.68 ± 0.37). There was significant decrease in progesterone level in standard group (21.08 ± 2.41**), HEDB (200 mg/kg) group (20.14 ± 1.75***) and HEDB (500 mg/kg) group (19.08 ± 2.45***) as compared to disease control group (36.62 ± 1.12#). (Tables 13 and 4) (Figure 15)

Table 13: Beneficial effect of *Dioscorea bulbifera* on Progesterone levels at 15th day in NMU induced breast cancer Wistar female rats

Groups	Progesterone level on 15th day (pg/ml)						Average ± SEM
Normal	9.2	10.3	9.5	9.7	10	9.4	9.68 ± 0.37
Disease	38.23	36.3	34.6	36.7	37.5	36.4	36.62 ± 1.12#
Standard Tamoxifen (20 mg/kg)	19.4	22.5	20.5	25.7	20	18.4	21.08 ± 2.41**
HEDB (200mg/kg)	18.36	20.38	23.25	19.23	21.32	18.32	20.14 ± 1.75***
HEDB (500mg/kg)	15.3	20.32	16.35	19.65	22.32	20.54	19.08 ± 2.45***

- Significant increase in progesterone level in disease control group as compared to normal control group; ** indicate significant decreased in progesterone level in standard treated group as compared to diseases control group (level of significance p<0.001≈significant.); *** indicate significant decreased in progesterone level in treatment group as compared to diseases control group (level of significance p<0.001≈highly significant)

Table 14: Beneficial effect of *Dioscorea bulbifera* on Progesterone levels after 30 days in NMU induce breast cancer Wistar female rats

Groups	Progesterone level after 30 days (pg/ml)						Average ± SEM
Normal	9.2	10.3	9.5	9.7	10	9.4	9.68 ± 0.37
Disease	>40	38.2	37.3	39.2	37.5	38.6	38.16 ± 0.70#
Standard Tamoxifen (20 mg/kg)	9.4	9.5	9.5	11.7	10	9.4	9.91 ± 0.82**
HEDB (200mg/kg)	9.4	9.5	9.5	9.2	10	9.4	9.5 ± 0.24***
HEDB (500mg/kg)	9.3	10.5	9.5	9.7	10	9.4	9.73 ± 0.41***

- Significant increase in progesterone level in disease control group as compared to normal control group; ** indicate significant decreased in progesterone level in standard treated group as compared to diseases control group (level of significance p<0.001≈significant.); *** indicate significant decreased in progesterone level in treatment group as compared to diseases control group (level of significance p<0.001≈highly significant.)

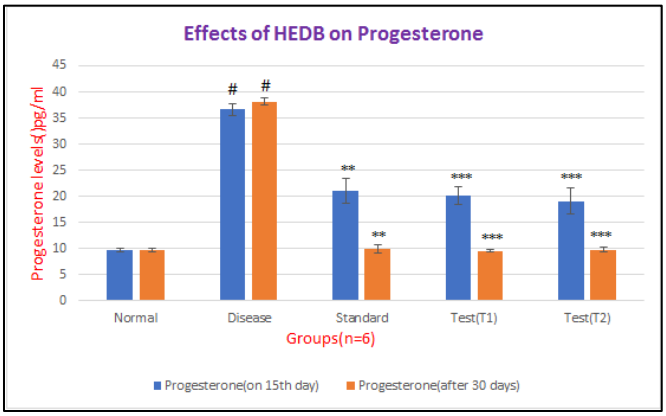

Figure 15: Progesterone levels in hydroalcoholic extract of *Dioscorea bulbifera*

n=6 and results were shown as mean ± SEM; Control group received distilled water; Disease control group received NMU(50mg/kg) in respective group; Standard control group received Tamoxifen (20mg/kg) in respective group; Treatment (1) control group received HEDB(200mg/kg); Treatment (2) control group received HEDB(500mg/kg)

- Significant increase in progesterone level in disease control group as compared to normal control group; ** indicate significant decreased in progesterone level in standard treated group as compared to diseases control group (level of significance p<0.001≈significant.); *** indicate significant decreased in progesterone level in treatment group as compared to diseases control group (level of significance p<0.001≈highly significant)

DISCUSSION AND CONCLUSION

In silico study of Diosbulbin B was carried out to know the binding affinity of Diosbulbin B with the respective receptors/proteins. The receptors/proteins involved in study are estrogen, progesterone, Her2, Aromatase. *In vitro* Haemolytic activity of extract was done to know the cytotoxic effect of *Dioscorea bulbifera*, as the absorbance of sample increases there are more chances of haemolysis, which indicates cytotoxic activity of test samples of different concentration. Different concentration of hydro alcoholic extract of *Dioscorea bulbifera* were subjected to determined % cell viability study using MDA-MB-231 cell line at 8, 24 and 48 hours. There were concentration dependent decreased in cell viability was observed in treatment of hydroalcoholic extract of *Dioscorea bulbifera*. The results of NMU induce breast cancer revealed that, Estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium. Increased estrogen levels in body indicate, that there are more chances of breast cancer in human. There was a significant increase in estrogen level in disease control group (74.54 ± 3.10 pg/ml) as compared to normal control group (41.36 ± 1.11 pg/ml). There was significant decrease in estrogen level in standard Tamoxifen(20mg/kg) group (58.76 ± 2.40, pg/ml), HEDB (200mg/kg) group (57.17 ± 2.32, pg/ml), HEDB (500mg/kg) group (59.49 ± 1.64, pg/ml) as compared to disease control group. As the level of progesterone increases there are more chances of breast cancer. There was significant increase in progesterone levels in disease control group (36.62 ± 1.12$^{\#,}$ pg/ml) as compared to normal group (9.68 ± 0.37, pg/ml). There was significant decrease in progesterone level in standard group (21.08 ± 2.41**), HEDB (200 mg/kg) group (20.14 ± 1.75*** pg/ml) and HEDB (500 mg/kg) group (19.08 ± 2.45*** pg/ml) as compared to disease control group (36.62 ± 1.12$^{\#}$ pg/ml). Thus at the end of 30 days trial, results indicated that *Dioscorea bulbifera* was effective in breast cancer management by decreasing estrogen and progesterone levels in blood serum and also decreases the cell viability of MDA-MB 231 cells as concentration and time points increases. These data indicted hydroalcoholic extract of *Dioscorea bulbifera* has therapeutic effect in breast cancer by working on adenosine receptor.

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