Research Article

In-Vitro Cytotoxicity Screening of the Selected Ethnomedicinal Plants for Their Activity on Breast Cancer

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ABSTRACT

Breast cancer is a life threatening disease amongst the women worldwide, with alarmingly increasing annual rates of reported incidence and death. The chemotherapy is associated with the number of side effects out of which major disadvantage are toxicity to normal cells and poor retention of drug in the cancerous cells. Hence there is a need to find the better alternative drugs for the treatment of breast cancer with fewer side effects. In the recent years, the current approaches have been focused on the use of ethno medicinal plants as a source that could effectively control cancer. In the present study, six ethno medicinal plants namely *Curculigo orchioides, Curcuma longa, Emblica officinalis, Teriminalia bellerica, Teriminalia chebula*and *Withania somnifera*were studied for their use in breast cancer using two different models; brine shrimp lethality assay and MTT assay on MCF7 breast carcinoma cell line. The results suggested that *Withania somnifera* and *Curcuma longa* showed the most significant cytotoxicity, 76.91% and 74.98% respectively on the breast carcinoma cell line at the concentration of 1000 µg/ml. Further *in-vitro* and *in-vivo* studies along with the clinical studies are necessary to establish its use in the treatment of breast cancer.

Keywords: Cytotoxicity, breast cancer, ethno medicinal plants, brine shrimp lethality assay, MTT assay.

INTRODUCTION

Breast cancer causes significant morbidity and mortality amongst women, and metastasis mainly affects outcome of the disease.[1,2]Lack of effective therapeutic strategies for control and treatment of breast cancers, and the huge financial burden placed on individuals and nations mean urgent action must be taken in the fight against breast cancer. Also, the side effects due to conventional chemotherapy have necessitated the search for newer therapies mostly in the form of natural products. In the recent years, interest in the natural products has grown, and in the light of long-term and safe cancer prevention, current approaches have been focused on the use of food and ethnomedicinal herbs as sources of products that could effectively control cancer.[3-5]

Brine shrimp lethality assay was primarily proposed by Michael in the year 1956 and was later developed by Vanhaecke in 1981.[6,7]It is based on the ability to kill the laboratory cultured *Artemianauplii,* brine shrimp. The assay serves as a useful tool for preliminary assessment of toxicity, safety of plant extracts, for the detection of fungal toxins, plant extract toxicity, and cyanobacteria toxins.[8-11] Cell-based assays are often used for screening of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed.[12]In the present study we have aimed to study the cytotoxic effect of the following ethnomedicinal plants (Table 1):

Table 1: Selected ethnomedicinal plants for the cytotoxcity study

Name of the plant	Family	Common Name	Parts Used	Uses
Curculigoorchioides	*Hypoxidaceae*	Kali musli	Rhizomes	Diuretic, anti-cancer, aphrodisiac[13]
Curcuma longa	*Zingiberaceae*	Turmeric	Rhizomes	Anti-inflammatory, antioxidant, anti-cancer[14]
Emblicaofficinalis	*Euphorbiaceae*	Amla	Fruits	Antioxidant, anti-cancer, anti-inflammatory[15]
Teriminaliabellerica	*Combretaceae*	Baheda	Fruits	Blood purifier, anti-cancer, throat diseases[16]
Teriminaliachebula	*Combretaceae*	Harde	Fruits	Fever, cough, astringent, anti-cancer[17]
Withaniasomnifera	*Solanaceae*	Ashwagandha	Roots	Anti-inflammatory, anti-tumour, anti-stress, anti-oxidant[18]

MATERIALS AND METHODS

Collection and authentication of plant material

The selected plant materials were collected from the fields and forest region of Gujarat. They were dried and then crushed into fine powder. The plant material was authenticated by its various morphological and microscopical characters and voucher specimens were deposited at Department of Pharmacognosy, KBIPER, Gandhinagar.

Preparation of plant extracts

20g of the powder of the selected ethnomedicinalplant was taken to prepare its different extracts. Alcoholic, Hydro-alcoholic (70:30- alcohol : water) and aqueous extracts were prepared by maceration of the powdered material for 24 h with respective solvent and refluxing for about 1 h with occasional shaking, consecutively 3 times. At the end the filtrate was concentrated to dryness and stored in an air tight container for further use.

Brine shrimp lethality bioassay

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of the plant extracts.[19-20] Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1 L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each well containing 2.0 ml of brine solution. In each experiment, 0.5ml of the plant extract was added to 2.0 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted. Experiments were conducted along with control (vehicle treated), different concentrations of plant extracts (100, 500 and 1000 µg/ml) of the test substances in a set of three well per dose (n=3). Each concentration was added in triplicates (n=3). LD_{50} values were calculated using Graph pad Prism.

Cytotoxicity assessment: MTT assay

Reagents and Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT), Foetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Modified Eagle's Medium (MEM), Trypsin-EDTA, Anti-biotic solution (Penicillin and streptomycin mixture) and Dimethyl Sulfoxide (DMSO) were obtained from Hi-Media Laboratories Ltd., Mumbai.

Cell line and maintenance

MCF-7 (Human Breast Carcinoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in Minimum essential medium (MEM) (Eagle) with Non-essential amino acids, supplemented with 10% Foetal Bovine Serum (FBS), antibiotics 1 % (penicillin and streptomycin) in an humidified atmosphere of 5% CO_2 at 37 ˚C until confluent. The cells were dissociated with Trypsin – EDTA solution. The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 well micro titre plates (Tarsons India Pvt. Ltd.).

Procedure

The prepared extracts were tested for its cytotoxicity by MTT- assay.[21] MCF7 cells were seeded in their respective culture medium (200 µl, 1 x 104cells/well) in a 96-well plate and incubated at 37 ˚C for 24 h with 5% CO_2supply. After incubation, the control wells were replenished with fresh medium and the test wells were treated with 100, 250, 500 and 1000 µg/ml of extracts. The cells were further incubated for 48 h maintaining the same conditions. After the treatment incubation period, medium in each well was replenished with 200µl of fresh medium plus 20µl of MTT (0.5 mg/ml). The plate was then incubated for 4 h in the same conditions after which the absorbance was measured at 570 nm using ELISA reader. Percentage cytotoxicity was calculated by the following formula:

% Cytotoxicity = [(Ac-At)/Ac)] X 100

Where,

Ac = mean absorbance of the control wells

At = mean absorbance of the test wells

RESULTS

Brine Shrimp Lethality Assay

The results of the brine shrimp lethality assay of the prepared extracts of the selected ethnomedicinal plants at various doses of 100, 500 and 1000 µg/ml are as shown in Table 2. The present data shows that the alcoholic extract of the *Curcuma longa* showed the most significant toxicity with 100 % mortality at the dose of 1000 µg/ml on the brine shrimp larvae. The alcoholic extract of *Terminalia bellerica, Terminalia chebula* and *Withania somnifera*and the hydro-alcoholic extract of *Curcuma longa and Withania somnifera* showed potent to moderate toxicity on the brine shrimp larvae. The extracts of the other plants did not show significant toxicity on the brine shrimp larvae.

LD_{50} values of the plant extracts showing more than 50% mortality in brine shrimp lethality assay were calculated using Graph pad prism. The LD_{50} values of alcoholic extract of *Curcuma longa, Terminalia bellerica, Terminalia chebula, Withania somnifera* were 226.93µg/ml, 906.41 µg/ml, 803.97 µg/ml and 541.82 µg/ml respectively. The LD_{50} values of hydro-alcoholic extract of *Curcuma longa* and *Withania somnifera* alcoholic extract were 555.00µg/ml and 884.68µg/ml respectively.

Table 2: Brine shrimp toxicity of the extracts of the selected ethno medicinal plants

Name of the plant	Percentage Mortality								
	Alcoholic Extract Conc.(µg/ml)			Hydro-alcoholic Extract Conc.(µg/ml)			Aqueous Extract Conc.(µg/ml)		
	100	500	1000	100	500	1000	100	500	1000
Curculigo orchioides	03.3	06.6	36.3	06.6	10.0	33.3	06.6	06.6	16.6
Curcuma longa	26.6	66.6	100.0	10.0	43.3	73.3	00.0	03.3	13.3
Emblica officinalis	13.3	26.7	46.6	03.3	13.3	04.0	06.6	10.0	33.3
Teriminalia bellerica	06.6	16.6	56.6	03.3	20.0	46.6	03.3	13.3	26.6
Teriminalia chebula	03.3	23.3	63.3	06.6	16.6	40.0	03.3	10.0	36.6
Withania somnifera	10.0	43.3	83.3	06.6	23.3	56.6	03.3	16.6	30.0

MTT Assay

The MTT assay for the cytotoxicity assessment of the prepared extracts was carried out at four different concentrations: 100,250,500 and 1000 µg/ml.

The results of alcoholic extract are as shown in Figure 1, the results of hydro-alcoholic extracts are shown in Figure 2 and the results of aqueous extracts are as shown in Figure 3. Vinblastine was used as a standard cytotoxic drug for MCF7 cells.

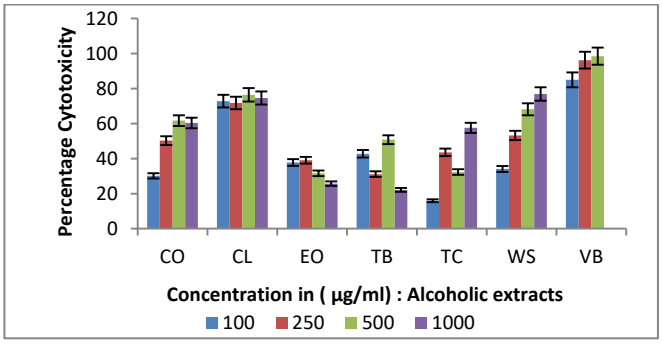

Figure 1: Percentage cytotoxicity of the Alcoholic extracts of the plant extracts. *Curculigo orchioides*(CO), *Curcuma longa* (CL), *Emblica officinalis*(EO), *Teriminalia bellerica*(TB), *Teriminalia chebula*(TC), *Withania somnifera*(WS), Vinblastine (VB).

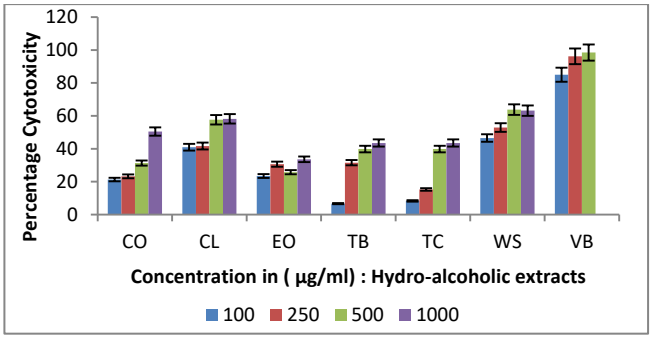

Figure 2: Percentage cytotoxicity of the hydro-alcoholic extracts of the plant extracts. *Curculigo orchioides*(CO), *Curcuma longa* (CL), *Emblica officinalis*(EO), *Teriminalia bellerica*(TB), *Teriminalia chebula*(TC), *Withania somnifera*(WS), Vinblastine (VB).

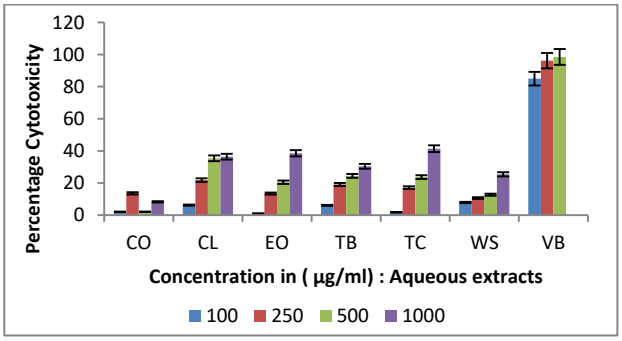

Figure 3: Percentage cytotoxicity of the Aqueous extracts of the plant extracts. *Curculigo orchioides*(CO), *Curcuma longa* (CL), *Emblica officinalis*(EO), *Teriminalia bellerica*(TB), *Teriminalia chebula*(TC), *Withania somnifera*(WS), Vinblastine (VB).

The above data suggests that the alcoholic and the hydro alcoholic extracts of the selected plants show significant cytotoxicity as compared to the aqueous extracts of the plants. The alcoholic extract of *Withania somnifera and Curcuma longa*at 1000 µg/ml dose showed the most significant cytotoxicity, 76.91% and 74.98% respectively on the MCF7 cells. The hydro-alcoholic extracts of the selected plants shows moderate to mild cytotoxicty and the aqueous extract does not show significant cytotoxicity on the MCF7 cell line.

DISCUSSION

Breast cancer is the most commonly diagnosed cancer amongst the women in the major countries of the world in the present time.[22]Chemotherapy and radiotherapy is effective against breast cancer. The major problems in cancer chemotherapy are toxicity to normal cells and rapid clearance of the drug from the tumour tissues. Nature has been a source of medicinal agents since ancient times and an impressive number of modern drugs have been isolated from the natural sources.[23] In the present study the selected medicinal plants were tested for their cytotoxicity using brine shrimp lethality assay and MTT assay on the human breast carcinoma cell line, MCF7. From the observed lethality of the selected plant extracts to brine shrimps indicated the

presence of potent cytotoxic and probably anti-tumour components of these plants. According to Meyer *et. al*., crude plant extract is toxic (active) if it has an LD_{50} value of less than 1000µg/ml while non-toxic (inactive) if it is greater than 1000 µg/ml.[24]The LD_{50} values of the alcoholic extracts of *Curcuma longa, Terminalia bellerica, Terminalia chebula, Withania somnifera*were found to be less than 1000 µg/ml and hence supporting their use in the ethno medicine for its anti-cancer activity. Further all the prepared extracts were studied for their *in-vitro* cytotoxic activity using MCF7 breast cancer cell line via MTT assay. Viable cells with active metabolism convert MTT into a purple coloured formazan product which can be quantified using a spectrophotometric method.[25]The alcoholic extracts of all the selected ethnomedicinal plants showed cytotoxicity against MCF7 cell line out of which, *Withania somnifera and Curcuma longa*at 1000 µg/ml dose showed the most significant cytotoxicity of 76.91% and 74.98% respectively. These herbs are reported for their anti-cancer use in the literature along with their other reported uses, like immuno-modulators, anti-oxidants, etc. which not only helps in preventing cancer but also acts as chemo-protective, ultimately resulting in the improvement of the overall health of the patient. The antioxidant potentials of these plants, mainly contributed by their bioactive compounds, have been closely linked to their abilities to suppress growth of cancer cells, likely through reduced oxidative stress, which may play a role in the development and progression of cellular damages underlying cancerous growth. As such, it has been suggested that antioxidant supplementation may reduce breast cancer recurrence and mortalities and through bioassay systems and animal studies, there have been indications that numerous naturally-occurring antioxidant compounds possess anti-cancer properties.[26-28]Also the plants rich in polyphenols such as catechins, anthocyanines and flavones have been linked to lower occurrence of cancers.[29] Other phenolic compounds claimed to possess biological activities include coumarins, lignans, phenolic acids, flavonoids, quinones, stilbenes, tannins and curcuminoids.[30]By understanding the complex synergistic interaction of various constituents of anti-cancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body.

CONCLUSION

The selected ethno medicinal plants exhibited moderate to potent cytotoxicity on the human breast carcinoma cell line, MCF7. The ethno medicinal plants showing significant cytotoxic activity on the breast cancer cells can further be subjected to different *in-vitro* studies and *in-vivo* models and their mechanism of action can be studied. The reported *in- vitro* cytotoxicity of the ethno medicinal plants warrants further clinical investigation for their use in the treatment of breast cancer.

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REFERENCES

1. Angelopoulos N, Barbounis V, Livadas S, Kaltsas D, Tolis G, Effects of estrogen deprivation due to breast cancer treatment, EndocrRelat Cancer, 11, 2004, 523-535.
2. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ, Cancer Statistics, CA Cancer J Clin, 56, 2006, 106-130.
3. Ferguson PJ, Kurowska E, Freeman DJ, Chambers AF, Koropatnick DJ, A flavonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines, J Nutr, 134, 2004, 1529-1535**.**
4. Jo EH, Hong HD, Ahn NC, Jung JW, Yang SR, Park JS, Kim SH, Lee YS, Kang KS, Modulations of the Bcl-2/Bax family were involved in the chemo preventive effects of licorice root (Glycyrrhiza uralensis Fisch) in MCF-7 human breast cancer cell, J Agric Food Chem, 52, 2004,1715-1719.
5. Mukherjee AK, Basu S, Sarkar N, Ghosh AC, Advances in cancer therapy with plant based natural products, Curr Med Chem, 8, 2001, 1467-1486.
6. Michael AS, Thompson CG, Abramovitz M, Artemiasalina as a test organism for a bioassay, Science, 123, 1956, 464.
7. Vanhaecke P, Persoone G, Claus C, Sorgeloos P, Proposal for a short-term toxicity test with *Artemia nauplii*, Eco toxicol Env Safety, 5, 1981, 382-387.
8. Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD, A micro well cytotoxicity assay using *Artemia salina* , Plant Med, 59, 1993, 250-252.
9. Harwig J and Scott P, Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins, Appl. Microbiol, 21, 1971, 1011-1016.
10. Mc Lauglin JL, Chang CJ, Smith DL, Bench top" bioassay for the discovery of bioactive natural products: an update, Natural Products Chemistry, 1991, 383-409.
11. Jaki B, Orjala J, Burji HR, Sticher O, Biological screening of cyanobacteria for antimicrobial and molluscicidal activity, brine shrimp lethality, and cytotoxicity, Pharm Biol, 37, 1999, 138-143.
12. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, J. Immunol. Meth, 65, 1983, 55–63.
13. Asif M, A Review on Phytochemical and Ethnopharmacological Activities of *Curculigo orchioides*, Mahidol University Journal of Pharmaceutical Sciences, 39(3-4), 2012,1-10.
14. Labban L, Medicinal and pharmacological properties of Turmeric (Curcuma longa): A review, Int J Pharm Biomed Sci, 5(1), 2014, 17-23.
15. Panday CN, S.S.Raval, Sima Mali and Harshadsalvi, 'Medicinal plants of Gujarat'- species description and medicinal use*, Embillica officinale*, 179-80.
16. Panday CN, S.S.Raval, Sima Mali and Harshadsalvi, 'Medicinal plants of Gujarat'- species description and medicinal use *Terminalia bellerica*, 261.

17. Panday CN, S.S.Raval, Sima Mali and Harshadsalvi, 'Medicinal plants of Gujarat'- species description and medicinal use, *Terminalia chebulla*, 261.

18. Quality standard of Indian medicinal plants, medicinal plant unit, Indian council of medical research, newdelhi, *Withania somnifera*; 9,356.

19. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE , McLaughlin JL, Brine Shrimp: a convenient general bioassay for active plant constituents, Planta Med., 45, 1982, 31-34.

20. Lincoln RD, Strupinski K, Walker JM, The use of *Artemia naupli* (Brine shrimp larvae) to detect toxic compounds from micro algal cultures, Pharm. Biol., 34, 1996, 384-389.

21. Fotakis G, Timbrell JA, In vitro cytotoxicity assays: comparison of LDH, neutral red. MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride.,Toxicol. Lett, 160, 2006, 171–177.

22. Hulka BS, Moorman PG. Breast cancer: hormones and other risk factors, Maturitas, 38 (1), 2001, 103-116.

23. Dyamavvanahalli LS, Bioprospecting of selected medicinal plants for antibacterial activity against some pathogenic bacteria, J. Med. Plant. Res, 5(17), 2011, 4087-4093.

24. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL, Brine shrimp: A convenient general bioassay for active plant constituents, *Plant Med*, 45,1982, 31-34.

25. Marshall NJ, Goodwin CJ, Holt SJ, A critical assessment of the use of micro culture tetrazolium assays to measure cell growth and function, Growth Regul., 5(2), 1995, 69–84.

26. Fleischauer AT, Simonsen N, Arab L, Antioxidant supplements and risk of breast cancer recurrence and breast cancer-related mortality among postmenopausal women, Nutr Cancer, 46,2003, 15-22.

27. Aziz MH, Kumae R, Ahmad N, Cancer chemoprevention by resveratrol: In vitro and in vivo studies and the underlying mechanisms, Int J Onco.,23, 2003,17-28.

28. Primchanien M, Nuttavut K, Sineenart K, Omboon L, Narongchai P, Neelobol N, Antiproliferation, antioxidation and induction of apoptosis by Garciniamangostana (mangosteen) on SKBR3 human breast cancer cell line, J Ethnopharmacol,92, 2004,161-166.

29. Naasani I, Oh-Hashi F, Oh-Hara T, Feng WY, Johnston J, Chan K, Tsuruo T, Blocking telomerase by dietary polyphenols is a major mechanism for limiting the growth of human cancer cells in vitro and in vivo, Cancer Res,63, 2003, 824-830.

30. Cai Y, Luo Q, Sun M, Corke H, Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer, Life Sci.,74, 2004,2157-2184.

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