ABSTRACT

Objective: The present study was aimed to evaluate the anticancer property of eremanthin isolated from Costus speciosus against breast cancer using in vitro and in silico approaches and thereby to develop eremanthin as a typical phytotherapeutic drug against cancer.

Methods: The presence of specific biologically active extract was confirmed under GC–MS/MS (gas chromatography–mass spectrometry) analysis. The cell proliferation inhibitory effect of the eremanthin was confirmed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and LDH (lactate dehydrogenase) assay. In silico studies were performed to predict the targeted interaction of eremanthin with cancer proteins.

Results: The GC–MS/MS analysis confirmed the presence of eremanthin with peak value of RA: 20.03. The MTT and LDH assays revealed the antiproliferative activity of eremanthin on MCF-7 and MDA-MB-231 breast cancer cell lines. The results provide stable interaction between eremanthin and cancer target proteins.

Conclusion: Thus, the compound can be used as an effective herbal therapeutic molecule to treat cancer with further explorations.

Keywords: Costus speciosus, Eremanthin, Cancer, Molecular docking.

INTRODUCTION

Cancer is a complex genetic disease that is caused by gene mutations, dysregulation of cellular pathways [1] or through environmental factors, the carcinogens [2]. Surgery combined with chemotherapy and radiotherapy has become the local therapy for cancer, which has been often found to result in significant side effects and related toxicities [3]. Alternative and traditional medicines, mostly herbal, are now regarded as important source of therapy but underutilized tools against disease which have better compatibility and lesser side effects [4]. Natural product-based cancer therapy is flourishing with substantial utilization of anticancer drugs in clinics being either natural or derived from natural products [5]. The therapeutic potential of Costus speciosus [6] is referred in Ayurveda and can be emphasized for identifying an array of biologically active compounds in different parts of the plant [7,8]. Thus, the present study aspires to estimate the anticancer property of a biologically active constituent isolated from C. speciosus, eremanthin against breast cancer. Eremanthin is a common secondary metabolite present in medicinally valuable plants and a bioactive compound with various pharmacological properties [9]. In recent times, search for new cancer drugs has moved toward a more mechanistic approach on identifying molecular targets for cell transformation rather than developing drugs that kill tumors cells [10]. We evaluated the potentials of eremanthin isolated from n-hexane extract of C. speciosus rhizome on breast cancer cells and cancer targeted protein.

METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemicals Pvt. Ltd. (USA). Solvents were obtained from Fisher Scientific Ltd., India. All the chemicals used were extra pure and were of culture grade.

Plant collection

The rhizome of C. speciosus was collected from the local market of Tiruchirappalli District, Tamil Nadu, India. The species was authenticated by the Department of Botany, Holy Cross College (Autonomous), Tiruchirappalli, India. The voucher specimen is preserved in the herbarium of the department. The rhizomes were dried under shade and mechanically reduced to moderate coarse powder and sieved.

Preparation of plant extract

The rhizome powder was collected and soaked in organic solvents such as hexane, ethyl acetate, and methanol and used for the preparation of extract. Five hundred grams powder was extracted with 1.5 l of hexane (1:3 w/v) for 72 h with frequent mixing and filtered. The filtrate was evaporated to dry under reduced pressure using rotary evaporator at 40°C. The remaining plant material are used for further extraction with ethyl acetate and methanol sequentially in a similar manner and obtained for the present study.

Phytochemical screening

Chemical tests were carried out on the rhizome extracts of C. speciosus using standard procedures to identify the constituents as described by Harborne (1973) for alkaloids, flavonoids, terpenoids, quinines, phenols, volatile oil, glycosides, tannins, and saponins [11].

Isolation and identification of the active compound

One kilogram powder was soaked in 3 l of hexane for 72 h with intermittent shaking and after filtering through Buchner funnel, it was concentrated using vacuum rotary evaporator at 40°C. Twenty-five gram of active crude hexane extract was chromatographed on a silica gel column (Merck 10–200 mesh, 750 g 3.5 i.d.×60 cm) and successively eluted with stepwise gradient of petroleum ether and hexane solvent system (5%, 10%, 20%, 30%, 50%, 70%, and 100%). A total of 116 fractions were collected and each fraction was spotted on a pre-coated silica gel (Merck-60 F254, 0.25 mm thick) plate and eluted in hexanemethyl acetate (3:1). An oil substance was obtained in subfraction. The oil substance was checked on TLC (thin-layer chromatography). It showed single spot on TLC. It was subjected to GC–MS/MS analysis.
GC–MS/MS analysis
The oil (subfraction) was quantified by an ITQ900-GC–MS/MS instrument of Thermo Fisher make, Holy Cross College, Trichy. One microliter of the sample was run in a DB-1 fused silica capillary column with helium (1 ml/min) as carrier gas, 250°C injector temperature, 280°C ion source temperature, and isothermal temperature 110°C (2 min), with an increase of 10°C/min–200°C then 5°C/min–280°C and 9 min–280°C.

Cell lines
MCF-10A, MCF-7, and MDA-MB-231 breast cell line of human were obtained from National Centre Cell Sciences, Pune, and were cultured in Dulbecco’s Modified Eagle Medium culture medium with 10% FBS (Fetal Bovine Serum) at 5% CO₂ and 37°C. Cells were passaged using trypsin-EDTA at 70–80% confluence.

Cell viability assay
The effect of the extracts and eremanthin on MCF-7, MDA-MB-231, and MCF-10A cells was seeded in 96-well plate at 1×10⁵ cells/well in medium containing 10% FBS and incubated for 24 h under 5% CO₂ at 37°C for attachment. The cells were washed with phosphate buffer solution (PBS), 100 µl of the prepared samples were added to the wells, and 100 µl of SFM (Serum-Free medium) was added to the control well and incubated for 24 h. The medium was then removed and washed with PBS and 100 µl of 0.5 mg/ml MTT solution was added to each well and incubated for 2–3 h. Five hundred microliters of dimethyl sulfoxide were added for solubilization of cells and kept in dark for 1 h. The intensity of the color developed was read at 570 nm in an ELISA reader.

Cell cytotoxicity assay
After 24 h incubation following the treatment with compounds (10, 20, 40, 80, and 100 µM), the cells were washed with 100 µl of 1× PBS. One hundred microliter of SFM with working reagent (10:1000) was mixed and incubated for 1 min at 37°C. The change in absorbance per minute (Δ OD/min) during 3 min observed at 340 nm. The amount of LDH leakage was calculated as follows:

LDH activity $= \left( \frac{\Delta \text{OD}}{\text{min}} \right) \times 16030$

Cytotoxic effect of the extracts, the compound (eremanthin) on MCF-10A, MCF-7, and MDA-MB-231 was assessed by LDH assay and the most active extract and compound were identified.

In silico studies
The exact interaction of eremanthin with the cancer targets was analyzed using in silico molecular docking analysis.

PDB (Protein Data Bank)
PDB is the single worldwide archive of structural data of biological macromolecules [12]. It serves as a repository for the 3D structural data of large biological molecules such as the proteins. The data typically obtained by X-ray crystallography submitted by biologists and biochemists from around the world are released into the public domain and can be accessed at no charge on the internet. The PDB is overseen by an organization called the worldwide PDB.
Receptor-ligand interaction was subjected to energy minimization using the CHARMM force field. The 3D structures of eremanthin, which was drawn using ChemSketch, were prepared for docking with an energy minimization process carried out using CHARMM (Chemistry at HARvard Macromolecular Mechanics) force field for eremanthin.

**ChemSketch**

The three-dimensional (3D) structure of eremanthin was developed using ACD/ChemSketch software (Fig.1). ChemSketch is a chemical intelligence in a comprehensive drawing package and provides a portal to an entire range of analytical tools and facilitates the transformation of structural or analytical data into professional, easy-to-decipher reports, or presentations.

**Ligand preparation**

The 3D structures of eremanthin which was drawn using ChemSketch were prepared for docking with an energy minimization process carried out using CHARMM (Chemistry at HARvard Macromolecular Mechanics) force field for eremanthin.

**Preparation of protein structures**

The ligand, crystallographic water molecules, heteroatom, and unwanted amino acid chains were removed from the PDB downloaded protein structures and the chemistry of the protein was corrected for missing hydrogen. Crystallographic disorder and unfilled valence atoms were corrected using alternate conformations and valence monitor options. Following the above steps of preparation, the protein was subjected to energy minimization using the CHARMM force field.

**Receptor-ligand interaction**

After the preparation of eremanthin and cancer targets for docking, the receptor and ligand were docked using the receptor-ligand interaction protocol of LibDock module in the Discovery Studio 2.1 version software. LibDock score and energy values were analyzed to screen and evaluate for the best interaction. The interacted molecules were explored for the presence of hydrogen bonds and their bond length. The specific amino acid residues to which eremanthin fitted were identified.

**Statistical analysis**

The data were analyzed using the SPSS 17.0 Version. For all quantitative measurement, data were expressed as mean±SEM for triplicates. The data were analyzed using one-way ANOVA and the group means were compared by Duncan’s multiple range tests. The result was considered statistically significant if p<0.05.

**RESULTS**

**Identification of active compound**

Eremanthin isolated from the hexane extract of *C. speciosus* rhizome was studied for anticancer activity. The n-hexane extract was fractionated and was rechromatographed and an oil substance obtained was confirmed as eremanthin using GC–MS/MS analysis (Fig.2).

**Effect of MTT assay on human breast cancer cell lines**

Eremanthin significantly less variation in the viability of normal breast cells (MCF-10A). On the other hand, a decrease in breast cancer cells (MCF-7 and MDA-MB-231) viability was found to be induced by eremanthin (Fig.3).

**Effect of LDH assay on human breast cancer cell lines**

In contrast, when treated on breast cancer cell lines (MCF-7 and MDA-MB-231), eremanthin-induced cytotoxicity was identified with an appreciable increase in lactate dehydrogenase leakage in a dose-dependent manner compared to control (Fig.4).

**In silico receptor-ligand interaction analysis**

The respective 2D and 3D structures of eremanthin sketched using ChemSketch. The X-ray crystallographic structures of NF-κB (nuclear factor kappa B) subunit proteins, cell cycle proteins, and apoptotic proteins of the study were retrieved from PDB. The retrieved proteins were prepared and docked with eremanthin using Discovery Studio 2.1.

A number of docked poses were obtained for each docking analysis made and the best poses out of them were chosen based on the LibDock score and absolute energy (Table 1). The pose which had highest score value and energy value was chosen as the best pose. The docked complex was then explored for the presence of H-bonds, their bond length and labeled with the amino acid in the active site with which eremanthin interacted (Figs 5–7).

**DISCUSSION**

The naturally derived products such as the plant extracts or their pure compounds are the major possessions that stand ahead in drug discovery, with their property of novel identity with that of the available chemical diversity [13]. In the present study, the biological activity of *C. speciosus* extracts against breast cancer cell proliferation may be due to the action of the phytochemicals and the level of antiproliferative activity in extract may be either due to the presence or absence of specific bioactive phytochemicals. The n-hexane extract of *C. speciosus* rhizome was confirmed to contain costunolide and eremanthin as the two major phytochemicals, which is the derivation based on comparison of the mass spectrum obtained in GC–MS/MS analysis of n-hexane extract. This analysis indicated that the anticancer potentials of this extract may be attributed to these major phytochemicals, and hence, the individual compound was isolated and studied for anticancer activity. The n-hexane extract may be either due to the presence or absence of specific bioactive phytochemicals. The n-hexane extract of *C. speciosus* rhizome was confirmed to contain costunolide and eremanthin as the two major phytochemicals, which is the derivation based on comparison of the mass spectrum obtained in GC–MS/MS analysis of n-hexane extract. This analysis indicated that the anticancer potentials of this extract may be attributed to these major phytochemicals, and hence, the individual compound was isolated and studied for anticancer activity. There are several studies reporting the ability of plant extracts and plant-derived compounds to inhibit cell proliferation and induce apoptosis in breast cancer cells [14-17]. Plant extracts and plant-derived bioactive molecules have been found to regulate and control the enhanced or uncontrolled cancer cell proliferation by specific modulation frequencies, targeting expression of genes associated with...
regulation of cell cycle and thereby promoting apoptosis [18]. In these concepts, the cell viability analysis of the present study revealed the efficacy of *C. speciosus* extracts of the compound (eremanthin) isolated from the active n-hexane extract to inhibit MCF-7 and MDA-MB-231 cell proliferation. The significant antiproliferative effects of n-hexane extract may be due the presence of eremanthin at high intensity of RA: 20.03 in the extract. The potential action of eremanthin to inhibit MCF-7 and MDA-MB-231 cell proliferation with reduction in cell viability may be due

---

**Fig. 5**: Eremanthin interacted with (a) cyclin D1, (b) cyclin D3, (c) CDK-4, (d) CDK-6, (e) p18 INK4c, (f) p21 CIP1, (g) p27 KIP1 with hydrogen bond interactions (dotted lines)

**Fig. 6**: Eremanthin interacted with (a) Bax, (b) Bak, (c) Bcl-2, (d) Bcl-xL, (e) caspase 3, and (f) caspase 9 with hydrogen bond interactions (dotted lines)
to the ability of eremanthin to regulate and control cell proliferation by altering the expression of various signaling molecules involved. Cancer cells have been reported to possess high levels of intracellular LDH [19]. These cancer cells undergo anticancer agent triggered apoptosis by the activation of cell death receptors superfamily, where alteration in cell membrane is noted. Such alteration includes loss of membrane integrity due to which the intracellular contents are released [20]. Hence, release of LDH mediated by external agents indicates the occurrence of cell death by apoptosis. Similarly, eremanthin was also found to induce MCF-7 and MDA-MB-231 cell death with an increase in LDH leakage in a dose-dependent manner. Hence, the release of LDH may be due to the action of the compound in stimulating the permeabilization of MCF-7 and MDA-MB-231 cell membrane and the corresponding induction of cell death. The study analyzed the exact fit of eremanthin with cancer targets by in silico molecular docking approach. Molecular docking is a widely used computational tool to access the exact fit of a small molecule within the active site of a protein and their modes of binding, which may support the prediction of the basic biochemical process that a small molecule may trigger [21]. Docking is reliable in recognizing the binding affinity and choosing the best drug-protein complex based on the energy value and LibDock score. A high LibDock score indicates a stronger drug-protein binding affinity [22]. Hence, drug-protein complex with high score was used in the present study. The presence of hydrogen bonds in drug-protein complex is a vital criterion to identify the binding affinity. A better interaction between a drug and a protein is achieved when they interact with hydrogen bond(s) and their bond lengths being <3Å root-mean-square deviation (RMSD) [23]. Less RMSD found in the interactions of eremanthin with the target proteins in the present study indicates their interaction with good affinity. Substantiating the in silico analysis in the present study provided the exact fit of eremanthin with cancer targets and these interactions may inhibit or stimulate protein function. Interaction of eremanthin

![Fig. 7: Eremanthin interacted with (a) nuclear factor kappa B (NF-κB)/p100, (b) NF-κB/p52, and (c) NF-κB/p65 with hydrogen bond interactions (green lines)](image)

| Table 1: Interaction of eremanthin with cancer targets |
|-----------------------------------------------|
| Cell cycle proteins                          |
| Cyclin D1                                      | 2W99_A | 3  | 36.993 | 71.818 | 1 | 1.58392 | SERQ58 | O16 |
| Cyclin D3                                      | 3G33_B  | 6  | 36.993 | 57.492 | 1 | 2.18171 | ARG174 | O13 |
| CDK4                                           | 3G33_A  | 2  | 36.993 | 55.184 | 2 | 2.475   | THR173 | O13 |
| CDK6                                           | IBLX_A  | 2  | 36.993 | 31.524 | 2 | 41.011  | LYS147 | O11 |
| p18 INK4c                                      | IMX2_A  | 5  | 36.993 | 58.734 | 1 | 1.92031 | LYS136 | O13 |
| p21 CIP1                                      | 3A99_A  | 28 | 36.993 | 64.582 | 1 | 2.1107  | VAL52  | O15 |
| p27 KIP1                                      | IJSU_C  | 22 | 36.993 | 51.899 | 2 | 2.36962 | GLN55  | 15 |
| Apoptotic proteins                            |
| Bax                                            | 2G55_A  | 4  | 36.993 | 76.25  | 1 | 2.18622 | GLN65  | 15 |
| Bak                                            | 4U2V_A  | 22 | 36.993 | 69.552 | 1 | 2.40459 | ARG13  | O15 |
| Bcl-2                                          | 1G5M_A  | 25 | 36.993 | 86.699 | 2 | 1.69174 | ASN11  | O15 |
| Bcl-xL                                         | 1G5J_A  | 8  | 36.993 | 68.533 | 1 | 2.14208 | SER47  | O18 |
| Caspase 3                                      | 3PD1_A  | 5  | 36.993 | 76.059 | 1 | 2.11355 | ARG207 | O15 |
| Caspase 9                                      | 1NW9_B  | 20 | 36.993 | 66.837 | 1 | 2.38411 | HIS237 | O15 |
| NF-κB subunit proteins                        |
| NF-κB/p100                                     | 3D07_B  | 7  | 36.993 | 64.011 | 1 | 2.34953 | HIS105 | O15 |
| NF-κB/p52                                      | 1A3Q_A  | 7  | 36.993 | 65.691 | 2 | 2.30811 | ARG155 | 16 |
| NF-κB/p65                                      | INFL_A  | 13 | 36.993 | 81.637 | 1 | 1.815556| ASN115 | O15 |
with positive Bax, Bak, Bcl-2, Bcl-xL, and caspase3 and 9 active sites docked with eremanthin might be responsible for their upregulation and downregulation. Binding of eremanthin with NF-κB/p100, NF-κB/p52, and NF-κB/p65 may be responsible for their downregulation or might inhibit the formation of mature NF-κB/p50 from NF-κB/p100 or the formation of their heterodimers. Thus, the outcome of in silico analysis in the present study stands as a supportive evidence to predict the mechanism of eremanthin action to induce apoptosis.

CONCLUSION

Thus, from the present study, it is found that the bioactive compound of n-hexane extract of the C. speciosus has a cytotoxic effect against human breast cancer cells. A potential role of the plant extract as a powerful chemotherapeutic agent was found to be the best cell proliferation inhibitor. However, further experiments are required to assess the cell survival pathways and other signaling pathways of the extract in the breast cancer cells to define the mechanism at the molecular level. The study could extend in advance by the isolation and testing for the activity of individual active principles present in the extract and analyzing its anticancer activity. This may help in the discovery of a new herbal drug against breast cancer, making herbs a strong therapeutic agent.

AUTHORS’ CONTRIBUTIONS

Dr. Anita Roy, experimental data analysis and interpretation and drafting the article. Ms. Angel Mary, data collection and compiling. Ms. Indu Sabapathy, manuscript correction. Dr. Rajalakshmi Manikkam, research guidance, critical revision, and final approval.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Haber DH. Roads leading to breast cancer. N Engl J Med 2001;343:1566-8.
2. Alison MR. Hand Book on Cancer. London, UK: Imperial College School of Medicine; 2001.
3. Dechant KL, Brogden RN, Pilkington T, Faulds D. Ifosfamide/mesna: A review of its antineoplastic activity, pharmacokinetic properties and therapeutic efficacy in cancer. Drugs 1991;42:428-67.
4. Kamboj VP. Herbal medicine. Curr Sci 2000;78:35-51.
5. Cragg GM, Kingston DG, Newman DJ. Anticancer Agents from Natural Products. Boca Raton, FL: Brunner-Routledge Psychology Press, Taylor and Francis Group; 2005.
6. Sari IP, Nurrochmad A. Sub-acute toxicity study of ethanolic extract of pacing (Costus speciosus) in male mice. Int J Pharm Pharm Sci 2016;8:97-101.
7. Upadhyay HC, Saini DC, Srivastava SK. Phytochemical analysis of Ammannia multiflora. Res J Phytochem 2011;5:170-6.
8. Kala C, Ali SS, Chauhdary S. Comparative phamacognostical evaluation of Costus speciosus (wild ginger) and Zingiber officinale (ginger) rhizome. Int J Curr Pharm Res 2016;8:19-23.
9. Eliza J, Daisy P, Ignacimuthu S. Antioxidant activity of eremanthin and eremanthin isolated from Costus speciosus (Koen ex. Retz) Sm. Chem Biol Interact 2010;88:467-72.
10. Richter M, Zhang H. Receptor-targeted cancer therapy. DNA Cell Biol 2005;24:271-82.
11. Ajay PM, Okaka AN, Onu PN, Ibiarn U, Urako AJ. Phytochemical composition of Talinum triangulare (water leaf) leaves. Pak J Nutr 2010;9:527-30.
12. Berman H, Henrick K, Nakamura H, Markley JL. The worldwide protein data bank (wwPDB): Ensuring a single, uniform archive of PDB data. Nucleic Acids Res 2006;35:D301-3.
13. Cosa P, Vlieutinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger in vitro “proof-of-concept”. J Ethnopharmacol 2006;106:290-302.
14. Khoja KK, Shaf G, Hasan TN, Syed N, Al-Khalifa SA, Al-Assaf AH, et al. Fenugreek, a naturally occurring edible spice, kills MCF-7 human breast cancer cells via an apoptotic pathway. Asian Pac J Cancer Prev 2011;12:3299-304.
15. Jang M, Kim SS, Lee J. Cancer cell metabolism: Implications for therapeutic targets. Exp Mol Med 2013;45:e45.
16. Ludas A, Indu S, Hinduja S, Nirmala AK, Rajalakshmi M. Antioxidant potential of polysaccharide isolated from methanolic extract of Tinospora cordifolia stem bark. Asian J Pharm Clin Res 2018;11:447-51.
17. Reddy MN, Reddy RN, Jamil K. Spicy anti-cancer spices: A review. Int J Pharm Pharm Sci 2015;7:1-6.
18. Nerurkar P, Ray RB. Bitter melon: Antagonist to cancer. Pharm Res 2010;27:1049-53.
19. Decker T, Lohmann-Mathies ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods 1998;115:61-9.
20. Compeau D, Higgins CA, Huff S. Social cognitive theory and individual reactions to computing technology: A longitudinal study. MIS Q 1999;23:145-58.
21. Mcconkey K, Drake D, Meehan H, Parsons N. Husking stations provide evidence of seed predation by introduced rodents in Tongan rain forests. Biol Conserv 2003;109:221-5.
22. Al-Nadaf AH, Taha MO, Aldal’in HK. Haloperidol inhibits Memapsin 2: Innovation by docking simulation and in vitro assay. Pak J Pharm Sci 2015;28:139-46.
23. Pitchai D, Roy A, Banu S. In vitro and in silico evaluation of NFκB targeted costunolide action on estrogen receptor-negative breast cancer cells a comparison with normal breast cells. Phytother Res 2014;28:1499-505.