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A COMPARATIVE STUDY ON THE ANTI-CANCER POTENTIAL OF *ENICOSTEMA AXILLARE* ON CANINE AND HUMAN BREAST CANCER BY TARGETING RUNX AS A PROGNOSTIC MARKER

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Keywords:

Anti-Proliferation, Accelrys discovery studio, MCF-7, Modeller, REM-134, RUNX

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ABSTRACT: The main objective of this study is to find a cure for Human Breast Cancer having Canine Mammary Tumor as a model using the ethyl acetate extract of *Enicostema axillare* targeting Runt-Related Transcription factor, which plays a vital role in Hippo Signaling Pathway. The preliminary phytochemical analysis and GC-MS analysis were used to determine the plant extract's bioactive compounds. The *in-vitro* Antioxidant and Anti-Proliferation assay was performed on MCF-7 and REM-134 cell lines by MTT assay. The Pharmacokinetics and Pharmacodynamics of Bioactive compounds were studied using Pre-ADMET and PASS SERVER software, respectively. Minimum Antioxidant potential Inhibition of extract was observed at 40µg/mL, and IC_{50} value calculated by MTT assay was found to be 31.2µg/mL for MCF-7 and 125µg/mL for REM-134. Bioactive compounds obtained from the GC-MS study were subjected to molecular docking on RUNX of Humans and Canine. The Dock score was found to be 96.7 for Humans and 102.13 for Canine. RUNX can be used as a prognostic marker for the treatment of mammary tumors for both Human and Dogs.

INTRODUCTION: The most frequent cancer in dogs is a mammary tumour; about half of these cancers are malignant [1]. Surgical resection is the preferred treatment for mammary gland neoplasia, and radiotherapy and chemotherapy are used as adjuvant therapies to surgery [2]. Major issues with cancer treatment that restrict the amount of chemotherapeutic drugs are drug toxicity and the emergence of drug resistance [3]. Thus, innovative chemotherapeutic medicines must be paired with new cytotoxic agents in new therapeutic strategies to minimize the cytotoxic agent dose while increasing the therapy's effectiveness.

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The injection of plant bioactive substances with cancer-preventive and growth-inhibitory properties into cancer cells is one potential strategy to cure cancer [4]. Breast cancer most frequently manifests in the cells that line milk ducts and the lobules that supply those ducts with milk. Ductal carcinomas originate from the ducts, whereas lobular carcinomas are cancers that originate from lobules [5]. Recent research has revealed that the gene RUNX1 is a novel altered gene in human luminal breast tumors. RUNX1 encodes a RUNX family transcription factor (TF) [6]. In this investigation, RUNX was put to the test against a number of the bioactive substances found in the plant extract.

Enicostema axillare is a perennial herb with sessile, lanceolate leaves that grows to a height of 15-20 inches and is widespread throughout India up to 1500 feet. In addition to being an *in-vivo* and *in-vitro* anti-inflammatory activity, the plant is used in traditional medicine to treat diabetes mellitus,

rheumatism, stomach ulcers, hernias, swelling, itching and insect poisoning [7]. The entire plant is used in medicine for its purported laxative, antipyretic, anti-inflammatory, liver tonic and digestive properties. *Enicostema axillare* was used to isolate steroids, alkaloids, saponins, flavonoids, triterpenoids, phenolic acids, and Xanthones. The primary objective of drug design is predicting how strongly a chemical will attach to a target. The most popular methods for modeling conformational changes that may take place in the biological target when the tiny molecule binds to it are molecular dynamics and molecular mechanics. The drug discovery uses *in-silico* interaction analysis to find drug-like compounds between therapeutic markers and plant bioactive principles. Using bioinformatics tools, *in-silico* approaches can aid in discovering pharmacological targets. Computational tools offer the advantage of delivering new drug candidates more quickly and cheaply.

MATERIALS AND METHODS:

Collection of Plants: *Enicostema axillare* was collected from Chennai district and was authenticated by Dr. Shankaranarayanan Head of Department Government Sidha Medical College.

Preliminary Phytochemical Analysis of *E. axillare:* Shade-dried plants (200 g) were pulverized separately and subjected to extraction by continuous hot extraction (Soxhlet). The extraction was done with Ethyl acetate solvent. Every time, the residue was dried in air at room temperature and later used for extraction. The extracts were evaporated using a rotary evaporator, and the percentage yield was thus recorded. Dried extracts were stored at 4°C in airtight containers for further studies. Concentrated extracts were subjected to various chemical tests to detect the presence of different phytoconstituents [8].

Extraction of Flavonoids: The extraction method used in this study was a modification of the method exercised by Zhao *et al.*,[9]. Dried and powdered plant material (10 g) was successively extracted in a flask using 50% Ethyl acetate. The extract was subjected to a maceration process in an incubator for 4 h at 150 rotations/min 50°C. The plant material was left to macerate for 20 h. Following this procedure, the extract was filtered using a blue band filter paper and a Buchner funnel. The hydro alcohol solution was evaporated to dryness under reduced pressure. Hydrolic extract was treated with petroleum ether (40°-60°) in a separation funnel and transferred in the aqueous phase to another separation funnel. After ethyl acetate was added, the funnel was gently mixed. It was found that ethyl acetate was a part of the separation funnel. The ethyl acetate was evaporated to dryness under reduced pressure. This extract was used for phytochemical and GC-MS analysis.

GC-MS Analysis: GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system. GC-MS operated using the following conditions: Column: Elite-5MS (30 x 0.25mm x 0.25 m df, composed of 5% Diphenyl / 95% Dimethyl poly siloxane), working in electron energy at 70 eV; helium was used as carrier gas at a constant flow of 1ml/min, and an injection volume of 2µl was employed (split ratio of 10:1). The injector temperature was -250°C; inlet and source temperature -200°C.

The oven temperature was programmed from upto 200 °C at the rate of 10 °C/min (no hold), to 5 °C/min - 9 min hold upto 280°C. Mass spectra were taken at 70 eV; a mass scan (m/z) fragments from 45 to 450 Da. Total GC and MS running time were 30min. Identification of components Interpretation on the mass spectrum of GC-MS was done using the National Institute of Standard and Technology (NIST) database, having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were determined.

***In-vitro* Antioxidant Activity:**

Inhibition of Lipid Peroxidation Activity: Lipid peroxidation induced by Fe^{2+-} ascarbate system in egg yolk by Bishayee and Balasubramaniyam 1971, was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al.* [10]. The reaction mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20mM, pH 7.0); KCl (30mM); $FeSO_4$ $(NH_4)2SO_4.7H_2O$ (0.06mM); and various concentrations of rich flavonoid extract of *E. axillare* in a final volume of 0.5mL. The reaction mixture was incubated at 37^{o}C for 1 h. After the incubation period, 0.4mL was

removed and treated with 0.2 mL sodium dodecyl sulphate (SDS) (1.1%); 1.5 mL thiobarbituric acid (TBA) (0.8%); and 1.5 mL acetic acid (20%, pH 3.5). The total volume was made up to 4.0 mL with distilled water and then kept in a water bath at 95 to 100^{o}C for 1 h. After cooling, 1.0 mL of distilled water and 5.0 mL of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously, and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed, and its absorbance at 532 nm Deep Vision (1371) UV–Vis Spectrophotometer) was measured to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of the test sample with the control. Ascorbic acid was used as standard.

Inhibition of lipid peroxidation (%) by the rich flavonoid extract of *E. axillare* was calculated according to

$$1- E / C \times 100,$$

Where C is the fully oxidized control's absorbance value and E is the test sample's absorbance (Abs 532+TBA – Abs532_TBA).

Cell Line and Culture: Cell lines MCF-7 and REM-134 were obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 µg /mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37 °C.

***In-vitro* Assay for Anti-Proliferation Activity: MTT Assay:** Cells (1 × 105/well) were plated in 24-well plates and incubated at 37°C with 5% CO2. After the cell reached the confluence, the various concentrations of the samples were added and incubated for 24 h.

After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µL/well (5mg/mL) of 0.5% 3-(4, 5-dimethyl – 2 - thiazolyl) - 2, 5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 h. After incubation, 1mL of DMSO was added in all the wells.

The absorbance at 570nm was measured with a UV- Spectrophotometer using DMSO as the blank [11]. Measurements were performed, and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

% Cell viability for Human Breast Cancer cells = MCF-7 of treated cells / MCF-7 of control cells × 100

% Cell viability Canine Mammary Cancer cells = REM-134 of treated cells / REM-134 of control cells × 100

Graphs are plotted using the percentage of Cell Viability at the Y-axis and the concentration of the sample in the X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

Pre-ADMET: Pre-ADMET 2.0 was used to study the Pharmacokinetics properties of the lead compound. Pre-ADMET is a web-based application for predicting ADME data and building a drug-like library using the *in-silico* method.

Homology Modeling: RUNX protein of Canine was designed using MODELLER using Human RUNX as a template. The structure with a low DOPE score was taken for further studies. The designed structure was evaluated using RAMPAGE by plotting the Ramachandran plot.

Docking: LIGANDFIT, which performs docking based on the cavity detection algorithm, was the docking method used to virtually screen compounds and predict the strongest binders based on various scoring functions.

For the molecular docking analysis of RUNX with ligands was carried out using the Dreiding parameter in which the partial charges of target protein and ligand in which the Gasteiger charging method was employed to calculate.

The energy grid extension was set to 5.0A° and ‘0’ was set as the conformation search number of Monte carlo trial. The number of poses for ligands in receptor cavity was limited to 10and other input parameters for docking were set as default options and docking was performed.

Broyden- Flecher Gold Farbshanno (BFGS) methods are employed on LIGANDFIT for the final energy refinement of the ligand pose optimization.

RESULTS:

Phytochemical Analysis of the *E. axillare* Extract: Phytochemical screening provides basic information about the medicinal importance of a plant extract. In this study, qualitative analysis of the chemical constituents of *E. axillare* showed the presence of various secondary metabolites, alkaloids, flavonoids, tannins, polyphenols, and terpenes. Phytochemical screening indicated the presence of flavonoids and tannin, which are phenolic compounds. Plant phenolics are known to be Antioxidants and free radical scavengers. The components present in the Ethyl acetate extract of *E. axillare* were identified by GC-MS analysis **Table 1** and **Fig. 1**. The active principles with their retention time, six compounds were identified in the Ethyl acetate extract of *E. axillare*.

TABLE 1: COMPOUNDS IDENTIFIED IN THE ETHYL ACETATE EXTRACT OF *E. AXILLARE* USING GC-MS CHROMATOGRAM

S. no.	Rt	Compound Name	Molecular Weight (G/Mol)	Chemical Formula
1	18.83	Phytol	296.539	$C_{20}H_{40}O$
2	22.82	Behenic acid, Methyl ester	354.619	$C_{23}H_{46}O_2$
3	21.87	Brassidic acid	338.576	$C_{22}H_{42}O_2$
4	20.87	Nonadecanoic acid, 18-oxo-, Methyl ester	326.565	$C_{21}H_{42}O_2$
5	17.17	Hexadecanoic acid, Methyl ester	270.457	$C_{17}H_{34}O_2$
6	14.07	Flavone	222.243	$C_{15}H_{10}O_2$

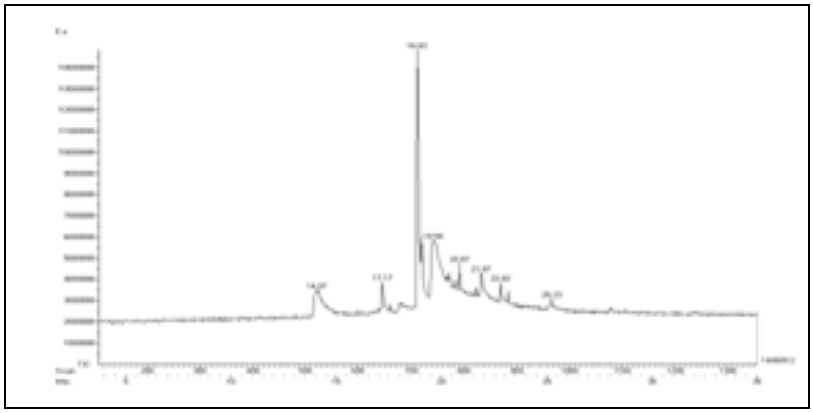

FIG. 1: GC-MS CHROMATOGRAM OF *ENICOSTEMA AXILLARE* ETHYL ACETATE EXTRACT

***In-vitro* Antioxidant Assay:**

Inhibition of Lipid Per-oxidation: In this assay, the per-oxidation of egg yolk was induced by ferrous sulphate and ascorbic acid as reducing agents. Hydroxyl radicals are generated by mixing Fe^{3+} and ascorbate, which attack the biological material.

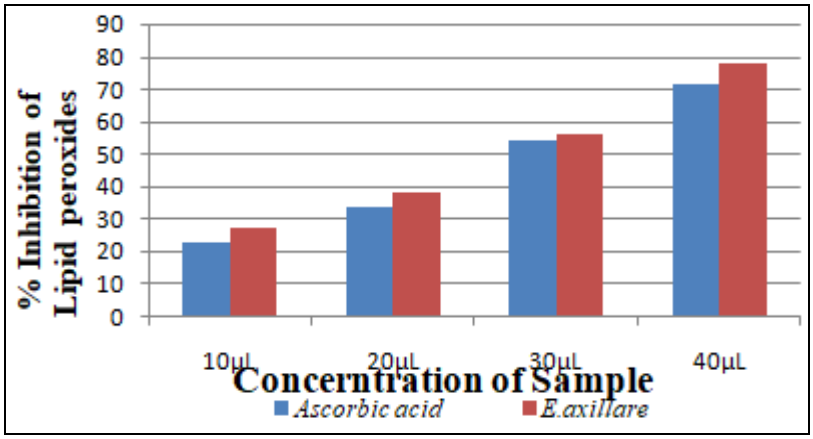

FIG. 2: INHIBITION OF LIPID PEROXIDATION BY THE VARIOUS CONCENTRATIONS OF *E. AXILLARE* EXTRACT

This leads to the formation of MDA and other aldehydes, which form a pink chromogen with TBA, absorbing at 532 nm. As shown in **Fig. 2**, the considerable amount of lipid peroxidation inhibitory effect by Gallic acid is 71%, while *E. axillare* significantly inhibited lipid peroxidation by 77.45%. The results were concentration-dependent and considered statistically significant ($P<0.05$).

***In-vitro* Assay for Anti-Proliferation Activity: (MTT Assay):** In the MTT assay, only cells that were viable after 24 hours exposed to the sample were capable of metabolizing a dye (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide) efficiently and the purple colored precipitate which is dissolved in a detergent was analyzed spectrophotometrically.

After 24 hours of post-treatment, MCF-7 and REM-134 cells showed IC_{50} of 31.25 µg/mL and 125µg/mL of *E. axillare,* respectively. The reduction of cell viability as the concentration of extract increases **Fig. 3.**

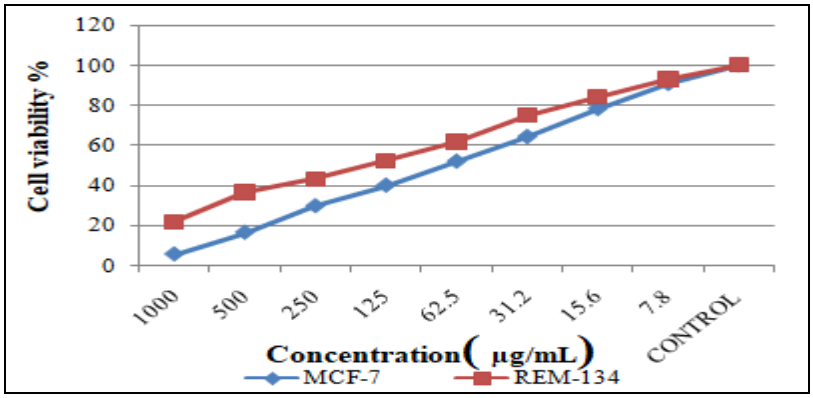

FIG. 3: CELL VIABILITY OF HUMAN BREAST CANCER CELLS AND CANINE MAMMARY TUMOR CELLS

Pre-ADME: The drug-likeliness property of the compounds identified from GC-MS was analyzed using the Pass server. The results showed that all 6 compounds obeyed Lipinski's rule 5, confirming the drug likeliness of the identified compounds. Following that, prediction of the Ames test and human ether-a-go-go related gene inhibition (hERG) supported that the compounds are non-mutagen and low risk of hERG inhibition **Table 2**. Thus the compounds present in the ethyl acetate extract of *E. axillare* are safe, and the same can be used as a lead molecule to design a drug.

TABLE 2: DRUG LIKELINESS AND TOXICITY PREDICTION OF COMPOUNDS OBTAINED FROM GC-MS

ID	Flavone	Behenic acid	Non Decanoic acid	Brassidic acid	Phytol	Hexadecanoic acid
Rule of Five	Suitable	Suitable	Suitable	Suitable	Suitable	Suitable
Ames test	Non mutagen	Non mutagen	Non mutagen	Suitable	Non mutagen	Non mutagen
hERG inhibition	Low Risk	Low Risk	Low Risk	Low Risk	Low Risk	Low Risk

Homology Modeling of RUNX-Canine using Modeller: RUNX protein of Canine was designed using MODELLER software using Human RUNX PDB id 3WTSas a template. The structure with low DOPE score was taken for further docking studies **Fig. 4**. The Ramachandran plot showed that most of the amino acid residues fall under the favorable region.

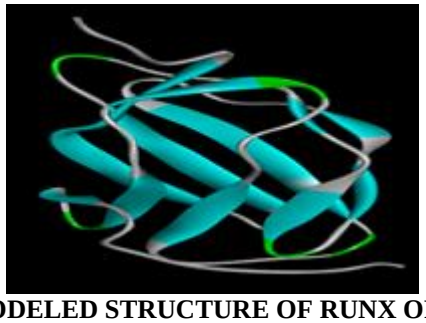

FIG. 4: MODELED STRUCTURE OF RUNX OF CANINE

Molecular Interaction: The 6 compounds possessing the drug-likeliness property were considered ligand molecules for molecular docking studies. Three and two possible binding sites were predicted in Humans and Canine using Discovery Studio software. The dock score is used to screen the top 10 poses which possess less ligand interaction energy. From the top 10poses, only the highest dock score poses were used for post-docking scoring. Among the 6 ligands, Brassidic acid has shown a higher Dock score with RUNX of Humans and Canine **Table 3** and **Fig. 5.**

TABLE 3: MOLECULAR DOCKING OF LIGAND WITH THE RUNX OF HUMAN AND CANINE

Compound Name	Relative Energy		Dockscore		Rmsd(A^o)	
	RUNX-Human	RUNX-Canine	RUNX-Human	RUNX-Canine	RUNX-Human	RUNX-Canine
Flavone	0	0	49.157	24.231	0	0
Behenic Acid	8.433	15.4147	88.0374	87.3376	0	0
Brassidic Acid	8.89	9.3082	96.8824	102.953	0	0
Nonadecanoic Acid	5.16	14.4038	86.0654	85.0778	0	0
Phytol	8.44	6.95469	82.1148	84.9331	0	0
Hexadecanoic Acid	7.16	14.1574	77.5848	92.0151	0	0

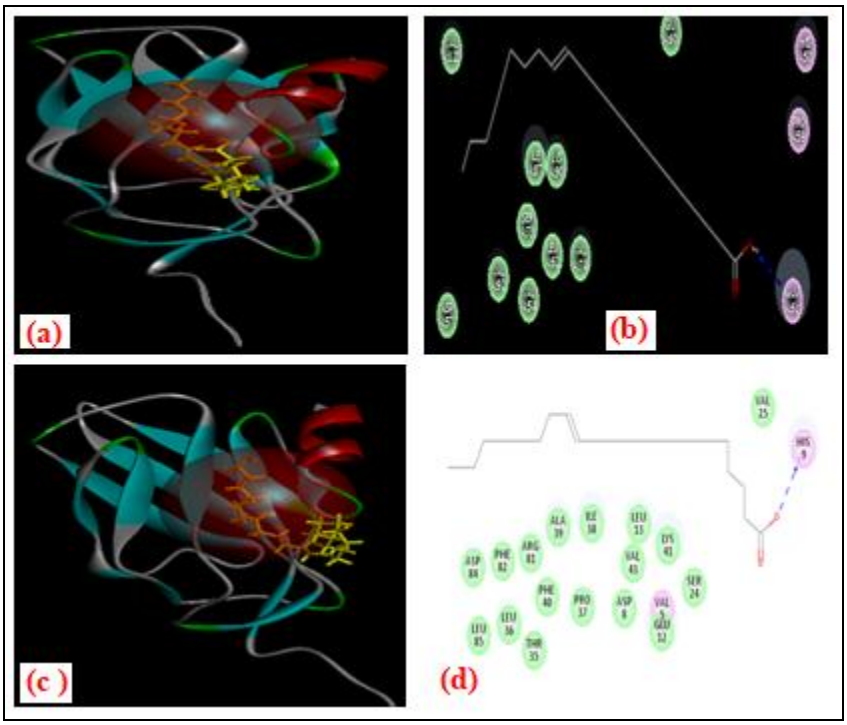

FIG. 5: DOCKING OF BRASSIDIC ACID WITH A) AND B) RUNX-HUMAN AT BINDING SITE 1 C) AND D) RUNX-CANINE AT BINDING SITE 1

DISCUSSION: Female breast cancer frequency is slowly increasing since the mid-2000s about 0.5% per year [12]. Synthetic Antioxidants have toxicological side effects, including carcinogenicity and neural damage. Due to this, natural antioxidants are used extensively for their strong capacity to scavenge reactive oxygen species. Many researchers have studied the free radical scavenging activity of plant extracts. *S. wallichii* free radical scavenging activity was studied *in-vitro* by Lalhminghlui *et al.*, 2018 [13]. The phytochemical constituents of Ethyl acetate extract showed the presence of tannins, flavonoids, alkaloids, and polyphenols. Flavonoids, phenolic acids, and tannins in the plant have grabbed much attention due to their antioxidant and free radical scavenging activities, which benefit mankind. The polyphenols and flavonoids contain the free-radical scavenging, and antioxidant activities of *S. wallichii* may be due to the presence of various polyphenols and flavonoids [13]. Lipid peroxidation assay inferred that the inhibition of peroxides drastically increases with an increase in concentration. From GC-MS analysis, the bioactive compounds are identified. The drug likeliness property of Brassidic acid was confirmed in Pre-ADMET study and the toxicity

report showed its non-mutagen from Ames test, Positive in carcinogenicity test and low risk of hERG inhibition. The IC_{50} value was found to be 31.25 ± 0.054 µg/Ml for MCF-7 and 125 ± 0.054 µg/mL for REM-134. Kaempferol-3-rhamnoside, a compound isolated from the leaves of *S. wallichii* inhibited MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway [14]. Phytochemicals extracted from *Ficus religiosa* and naringin have been reported to scavenge DPPH radicals in a concentration-dependent manner [15, 16]. From the docking studies, Brassidic acid has shown more hydrogen bonds and better Dock score and may be considered an inducer of RUNX protein in humans and Canine. In the future, it is important to isolate the Brassidic acid and correlate the structure of the compound with its biological effect, which will be valuable to propose new lead compounds with better cytotoxic potential against Breast cancer in Humans and Canine.

CONCLUSION: Mammary cancer is one of the major health problems in Adult Women in developed and developing countries. It's an emerging field in most of the current research. On continuous offer to explore new biocompatible agents, the present study was focused on a medicinal plant to find new and cost-effective Anti-Breast cancer agents. Like humans, Canine Mammary Tumor is one of the deadliest diseases in adult dogs. *In-vitro* Antioxidant study explains that on specific to RUNX a transcription protein, it plays a vital role in the development of Breast cancer. Further isolation and purifications of Brassidic acid and correlating the structure of the compound with their biological effect will be valuable to propose new lead compounds with better cytotoxic potential.

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