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Activities of Cinnamaldehyde from *Boswellia Serrata* on MCF-7 Breast Cancer Cell Line

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Abstract- *Boswellia serrata* also known as Indian frankincense is known for its medical practice for thousands of years. B. serrata is recognized for its anti-tumor and anti-inflammatory actions. Cinnamaldehyde is one of the common compounds derived from B. serrata from Methanolic extract (ME), Dichloromethanol extract (DME) and Hydrodistillate extracts. MCF-7 cell lines were tested to understand the effects of cinnamaldehyde on hemolysis, its cytotoxic effect, and antioxidant activity (Hydroxyl radical scavenging assay and Clonogenic Survival Assays). Cinnamaldehyde is a natural substance derived from *B. serrata* that is known for its anti-proliferative activity. The anti-proliferative effect of cinnamaldehyde was investigated to work out its mechanisms of inhibition of growth proliferation at the morphological level. 100µM of ME was found to be more efficient to deliver the expected results while deriving less inhibition coefficient when compared with 100µM of DME. The study reveals the mechanisms of the anti-proliferative activities of cinnamaldehyde in MCF-7 breast cell lines, and further emphasizes that cinnamaldehyde could be a safe and effective natural agent for the treatment of breast cancer. These studies indicate that Cinnamaldehyde could be a potential candidate for further studies related to chemo therapy for breast carcinoma.

Keywords- Breast Carcinoma, MCF-7 cell line, Boswellia serrata, Cinnamaldehyde, Catechin.

I. INTRODUCTION

Cancer is one of the major causes of mortality all over the world. Various chemotherapeutic drugs are established to treat and manage this devastating disease through systemic drug discovery and development [1]. According to the studies, Breast Cancer (BC) accounts for 22% and one of the most prevalent diseases worldwide [2]. Male breast cancer rates are less than 1 in one hundred thousand men [3]. BC hits the urban population three times greater than the rural populations [4].

Treatment of breast carcinoma

The major treatments for breast cancer include therapies, such as, radiation, chemo, hormonal therapy, targeted therapy etc. Chemotherapy targets to remove tumor or cancerous cells with the aid of medications. These investigations are aimed at evaluating the anti-proliferative property of cinnamaldehyde of Boswellic acids from *Boswellia serrata* through Complementary Alternative Medicine (CAM) with the standard chemotherapeutic drug, Catechin on MCF-7 cell line. This cell line originated from a breast adeno-carcinomal tissue of a 69-year-old female. MCF-7 cell lines actively express the estrogen alpha along with progesterone, androgen, and glucocorticoid receptors hence it is helpful in understanding drug resistance which aids in development of chemotherapeutic drugs [5]. This research tries to address the usage of CAM as a supplement with the chemotherapeutic drug to minimize side effects. CAM includes herbal remedies, aromatherapy, biofeedback, massages, and so on. Here we have involved herbal or bioactive compound from B. serrata which has shown anticancerous property, previously [6].

Studies suggest that various bioactive compounds from B. serrata exhibit anti-proliferative properties and are known to initiate Apoptosis. According to Shashi Bhushan et al., (2007), an isomeric mixture of B. serrata triterpenediol (TPD) has shown apoptosis (organized cell death) in tumors [7]. Various reports suggest that the resins derived from Boswellia species have medicinal properties. Researches on Boswellia have not been done vastly and the scientific data has many gaps which need to be filled to understand the prime importance of the species [8]. According to Sankpal UT et al., (2011), a gum resin derivative of B. serrata is found to be useful in cancer therapeutics (in traditional medicine) and its analogs restrain the growth of cancer associated biomarkers along with the metastasis of human CRC in vivo conditions [9]. According to Lin HK et al., (2013) essential oils derived from Boswellia species have found to be biologically active. They suppressed the proliferation along with cytotoxicity of various cancer cell lines. Acetyl-11-keto-β-boswellic acid (AKBA)

was found to be effective and induced cytotoxic activities in vitro and vivo human models [10]. This study aims to study the activity of cinnamaldehyde extracted from resins of *B. serrata* on MCF-7 cell line.

Cinnamaldehyde is present in all the extracts in large percentage compared to the other terpenes. According to Lea-YeaChuang. et.al, 2012, Cinnamaldehyde caused apoptosis in HepG2 and Hep3B (hepatoma cancer cell lines) and blocked cell proliferation, suggesting its potential as an anti-neoplastic substance that may be useful in treatment of various cancers including breast, colon and prostate cancers [11, 12]. Hence its activity was evaluated on MCF-7 breast cancer cell line. This cell line is useful in expressing Estrogen receptor (ER) alpha in invasive human breast cancer [13]. MCF-7 has been effectively utilized in studies related to functional genomics. This cell line is also known to produce copious quantity of RNA/DNA that allows for carrying out further investigations [14].

II. MATERIALS AND METHODS

All the experimentations were conducted at Dextrose Technologies, Kengeri, Bangalore, India under the guidance of Dr. Farah Deeba.

Essential extraction methods:

- Methanolic extract (ME) (Table I)
- Dichloromethanol extract (DME) (Table II)
- Hydrodistillate (Table III)

Gas chromatography mass spectrometry (GC–MS) was utilized for characterization of the extracted essential oils. GCMS results (I, II and III respectively) are given in the table section. It was analyzed that Cinnamaldehyde is found to be common among all the three extracts.

GC-MS ANALYSIS:

It was performed in Agilent Technologies connected with MS. The chemical constituents were separated on a 30 m × 0.32 mm non-polar capillary column with phase thickness of 1.0 µm. This was and interfaced with a quadrupole mass spectrometer. The injector temperature was270 °C and the interface were kept at 320 °C. The temperature range was kept between 60 °C to 260 °C @ of 2 °C /min. Helium was the carrier gas (linear velocity 74.6 cm/s, total flow rate 39.0 ml/min). The ionization voltage was 70 eV and the scan rate was 500 amu/sec.

THIN LAYER CHROMATOGRAPHY (TLC):

The samples were chromatographed with Thin Layer Chromatography (TLC) on silica gel (60F-TLC plates). These were developed using ternary-solvent system (hexane-chloroform-methanol, 5:5:0.5, v/v) and scanned at 260 nm [15].

MCF-7 CELL CULTURING:

The cell culture utilized EMEM (EBSS) media supplemented with 1% non-essential amino acids (NEAA), 10% foetal bovine serum (FBS) and 2mM glutamine. For our hormone-related studies, the medium is grown on low serum and phenol-red-free media was used [16].

CATALOGUE NO:

Cell Line	Catalogue No.	Characteristics
MCF-7/S0.5	16022501	Human Breast Cancer Estrogen receptor, MCF-7 Adapted to grow in low-serum media.

DOCKING STUDIES:

The molecular docking studies are carried out to simulate the interaction between a small molecule and a protein in order to characterize the binding site of the target proteins. Docking efficiency can be increased by knowing the binding sites before docking process. If docking is done without any postulation of the binding site, it is known as blind docking [18]. Docking studies were carried out against Cinnamaldehyde and 5 different receptors to investigate and to access the binding efficiency of Cinnamaldehyde with 5 different anticancer receptors or proteins, a molecular docking imitation studies was undertaken. Estrogen receptors are the main receptor molecules for the breast cancer which mutates at various segments. Cinnamaldehyde acts upon various receptors but the below mentioned receptors were cautiously chosen on the basis of lately found mutation among the population. 5 protein ligands/receptors which were taken for docking studies are:

- 3ert: Human Estrogen Receptor Alpha Ligand-Binding Domain in Complex With 4hydroxytamoxifen
- 3hb5 Binary and Ternary Crystal Structures of a Novel Inhibitor Of 17 Beta-Hsd Type 1: A Lead Compound for Breast Cancer Therapy
- 3ols Crystal Structure of Estrogen Receptor Beta Ligand Binding Domain.
- 3s7s Crystal Structure of Human Placental Aromatase Complexed with Breast Cancer Drug Exemestane
- 5nqr Potent Inhibitors of Nudt5 Silence Hormone Signaling In Breast Cancer

CYTOTOXIC ASSAY:

Toxicity of unknown compounds is determined by counting viable cells after staining with a dye in vitro. The activity of living cells is measured via mitochondrial dehydrogenases. This MTT method is simple, accurate and yields high reproducible results. MTT (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is a water soluble tetrazolium salt that yields a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. Formazan can be solubilized using acidified isopropanol or other solvents. The purple solution which is obtained is measured spectrophotometrically. The associated change in the amount of formazan formed is due to increase or decrease in cell number, indicating the degree of cytotoxicity caused by the test compound [19].

PROTOCOL:

104 – 106 cells were plated in 200 ml PBS in 96-well (flat bottom).

↓

20 ml of MTT solution is added and mixed well.

↓

Plate is incubated at 37˚C in dark for 4hours.

↓

An aliquot is removed for the analysis; 200 ml of acidic isopropanol is added and mixed well.

↓

The above solution is incubated for an hour at 37˚C in dark

↓

The plate is read in Elisa Reader at 570nm and measured OD (background wavelength is 630nm) [20]

HEMOLYSIS ASSAY:

Hemolytic effect of a compound is determined through this assay; it utilizes a suspension of red blood cells

- PBS as negative control
- 1% SDS as positive control
- Test: DME and ME compound on MCF-7 cell lines.

Ten Separate test tubes containing MCF-7 cell lines were dosed with PBS, 1% SDS, DME and ME (4 different concentrations) compounds respectively.

- Water bath incubation at 37°C, with gentle agitation
- Each sample was centrifuged for released hemoglobin is quantified using a UV/Vis spectrometer at 405nm.
- % hemolysis is calculated at each sampling time point
- % hemolysis is calculated using the following formula:
  % Hemolysis = 100 x[(Absorbance of sample – Absorbance of negative control) ÷ (Absorbance of positive control – Absorbance of negative control)] [21]

ANTIOXIDANT (HYDROXYL RADICAL SCAVENGING) ASSAY:

Free radicals are produced in response to various stimulants (UV radiation, smoke, chemicals etc.) The Reactive oxygen species (ROS) includes: O2 - , OH, H2O2, ferric ion, NO etc. these are known to cause oxidative stress. This oxidative stress associated with diseases and conditions, such as, cancer, inflammation, diabetes, liver cirrhosis, cardio vascular disease, Alzheimer's, aging and acquired immunodeficiency syndrome. The amplified production of ROS affects enzymes and causes cell damage. Organisms need to evolve mechanisms to overcome these stressful situations by using various antioxidant molecules and enzymes [22].

CLONOGENIC ASSAY:

This is an assay to analyze survival of tumor cells and to determine cell reproductive death after treatment with ionizing radiation as well as the action of other cytotoxic agents [23, 24].

Cancer cells were plated at $5x10^3$ cells/dish containing DMEM complete media, incubated at 37^0C, 5% CO_2 for 24 h. After 24 h, cells are treated with 50 and 100 mg/ml of both *DME* and *ME* compound for 24 h. Cells are grown for 14 days and add the fresh media on fourth day. On 14^{th} day to produce colonies of >50 cells/ colony, remove the media from the dishes and washed with 1X PBS, cells were fixed with 3.7% formaldehyde for 5 min, followed by colonies were stained with 1 ml of 1% crystal violet in 1X PBS for 20-30 minutes on a platform.

Rinse the dishes three times with 1X PBS and air-dried, and then count the colonies. Finally take the picture at 4X by using inverted microscope).

III. RESULTS & DISCUSSION

Boswellic acids are terpenes found in small portions.

1. Compound has cinnamaldehyde which is active in the all the compounds and common in all the compounds according to the GCMS analysis. Table I, II & III depict the compounds present in Methanolic extract (ME) , Dichloromethanol extract (DME) & Hydrodistillate respectively.

Table 1: Compounds Found In Sample 1 of Methanolic Extract (ME)

Compound label	Name	Hits
Cpd 6: Acetophenone	Acetophenone	10
Cpd 12: Cinnamaldehyde, (E)-	**Cinnamaldehyde, (E)-**	**10**
Cpd 14: 2-Methoxy-4-vinylphenol	2-Methoxy-4-vinylphenol	10
Cpd 15: Cyclohexanemethanol, 4-hydroxy-.alpha.,.alpha.,4-trimethyl-	Cyclohexanemethanol, 4-hydroxy-.alpha.,.alpha.,4-trimethyl-	10
Cpd 17: Cyclohexanemethanol, 4-hydroxy-.alpha.,.alpha.,4-trimethyl-	Cyclohexanemethanol, 4-hydroxy-.alpha.,.alpha.,4-trimethyl-	6
Cpd 21: Vanillin	Vanillin	9
Cpd 27: Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	10
Cpd 29: Benzene, (1-methoxyethyl)-	Benzene, (1-methoxyethyl)-	8
Cpd 39: n-Pentadecanol	n-Pentadecanol	10

Table 2: Compounds Found In Sample 2 of Dichloromethane Extract (DME)

Compound label	Name	Hits
Cpd 1: Cyclohexene, 4- methylene-1-(1-methylethyl)	Cyclohexene, 4- methylene-1-(1-methylethyl)	10
Cpd 2: (+)-4-Carene	(+)-4-Carene	10
Cpd 3: endo-Borneol	: endo-Borneol	10
Cpd 4: 3-Cyclohexen-1-ol, 4- methyl-1-(1-methylethyl)-, (R)-	Cyclohexen-1-ol, 4- methyl-1-(1-methylethyl)-, (R)-	6
Cpd 5: L-.alpha.-Terpineol	L-.alpha.-Terpineol	10
Cpd 6: Cinnamaldehyde, (E)-	**Cinnamaldehyde, (E)-**	**10**
Cpd 7: 3,6-Dimethyl-2- nitrobenzaldehyde	3,6-Dimethyl-2- nitrobenzaldehyde	7
Cpd 9: Bicyclo[7.2.0]undec-4- ene, 4,11,11-trimethyl-8-methylene	Bicyclo[7.2.0]undec-4- ene, 4,11,11-trimethyl-8- methylene	10
Cpd 10: Bicyclo[3.1.1]hept-2- ene, 2,6-dimethyl-6-(4- methyl-3-pentenyl)	Bicyclo[3.1.1]hept-2- ene, 2,6-dimethyl-6-(4- methyl-3-pentenyl	10
Cpd 11: 2-Hydroxy-1,8-naphthyridine	2-Hydroxy-1,8-naphthyridine	10
Cpd 12: 1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	10
Cpd 13: Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	10
Cpd 14: Benzoic acid, 2-(1-oxopropyl)-	Benzoic acid, 2-(1-oxopropyl)-	7
Cpd 15: (R)-(-)-14-Methyl-8-hexadecyn-1-ol	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	10
Cpd 16: N-Benzyloxy-2,2-bis(trifluoromethyl)aziridine	N-Benzyloxy-2,2-bis(trifluoromethyl)aziridine	8
Cpd 17: Tetradecanoic acid	Tetradecanoic acid	7
Cpd 22: 1-Nonylcycloheptane	1-Nonylcycloheptane	10
Cpd 24: Andrographolide	Andrographolide	10
Cpd 29: 1-Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-1,4a,7-trimethyl-, methyl ester,[1R-(1.alpha.,4a.beta.,4b.alpha.,7.alpha.,10a.alpha.)]-	1-Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-1,4a,7-trimethyl-, methyl ester, [1R-(1.alpha.,4a.beta.,4b.alpha.,7.alpha.,10a.alpha.)]-	7
Cpd 36: Methyl abietate	Methyl abietate	7
Cpd 32: 4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	8

Table 3: Compounds Found In Sample 3 of Hydrodistillate

Compound label	Name	Hits
Cpd 13: N-Benzyloxy-2,2-bis(trifluoromethyl)aziridie	N-Benzyloxy-2,2-bis(trifluoromethyl)aziridie	10
Cpd 35: 4-Hydroxy-2-methylacetophenone	4-Hydroxy-2-methylacetophenone	10
Cpd 73: (S)-6,6-Dimethyl-2-azaspiro[4.4]non-1-ene	(S)-6,6-Dimethyl-2-azaspiro[4.4]non-1-ene	10
Cpd 74: Tricyclo[4.3.1.0(2,5)]decane	Tricyclo[4.3.1.0(2,5)]decane	10
Cpd 76: Cis-8-methyl-exo-tricyclo[5.2.1.0(2.6)]decane	Cis-8-methyl-exo-tricyclo[5.2.1.0(2.6)]decane	10

TLC is working (Fig 1: Run 1 and 2).

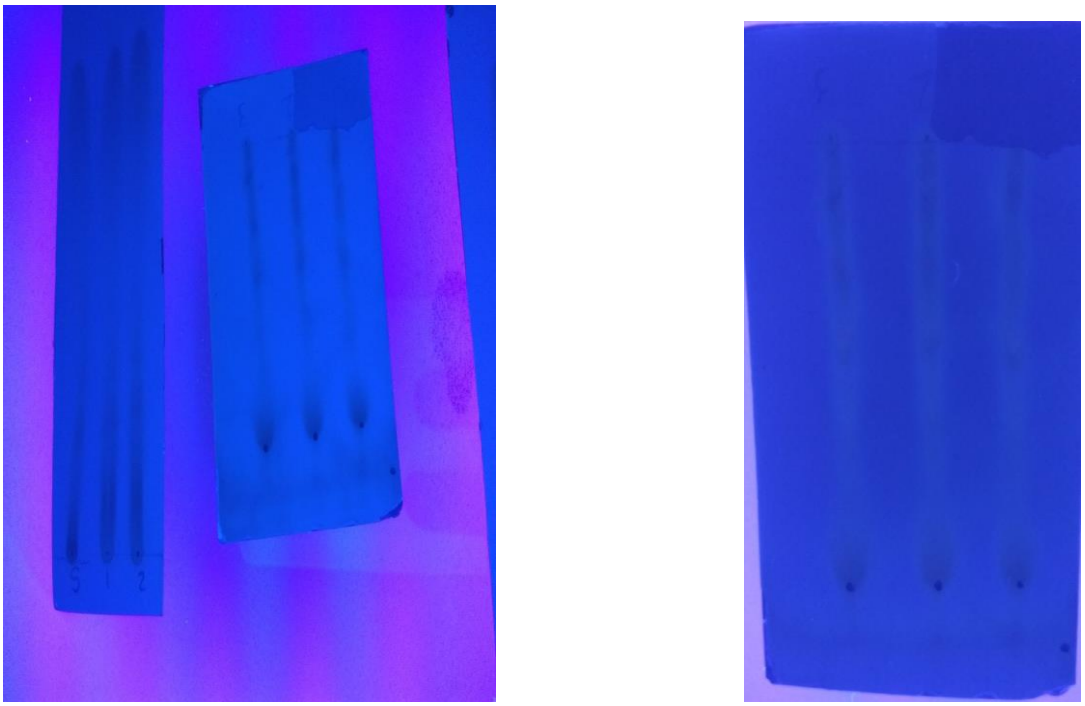

Fig. 1: Run 1 & 2: Thin Layer chromatography of Standard (Catechin) , DCM extract, Methanolic extract.

2. Bioinformatics work: Breast Cancer (BC) denotes mutations. Estrogen receptors are the main receptor molecules for the breast cancer which mutates at various segments. Cinnamaldehyde is the common bio-active compound to be found in all the 5 receptors docking studies. So the compound cinnamaldehyde acts upon various receptors chosen in bioinformatics work. This docking study shows the mechanism of action of the cinnamaldehyde by how it binds to BC receptors. Mutating enzymes are many; but 5 different proteins ligands were chosen which were commonly found among the population.

3. Structure of breast cancer receptors were retrieved from the Protein Data Bank and the structures of flavonoids compounds have been collected from PubChem databases. Molecular docking and drug likeness studies were performed for those natural compounds to evaluate and analyze the anti-proliferative breast cancer activity. Binding affinity is observed. Lower the scores, higher the binding affinity (BA). Accordingly, 3OLS Crystal Structure of Estrogen Receptor Beta Ligand Binding Domain has showing least binding affinity (-3) and hence is considered to be best among the 5 receptors. The results of this study can be implemented in the drug designing pipeline.

4. Catechin is a natural phenol and antioxidant, belonging to the group flavanols. It has anticancer properties and it's widely used as chemical formulations hence Catechin is used as standard control in this study with our extracted test samples.
5. According to Cytotoxic assay (MTT Assay) Fig.2 & 3 and Table IV, it can be interpreted that 100µM has lesser IC50 value and is more potent than the 50 and 100 µM of DME.

Fig. 2: Cytotoxic assay (MTT Assay; ELISA PLATE)

Formula:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Sample})}{(\text{Control})} \times 100$$

Table 4: Formula & Summary of Percentage Inhibition of DME & ME Compounds on MCF-7 Cell Lines

	Samples		Conc.(µl)		OD 590 nm	% Inhibition	IC 50
			Control		0.6451	0.00	
	DME		0.7		0.6353	1.52	35.7
			1.5		0.6145	4.74	
			3.1		0.5921	8.22	
			6.2		0.5529	14.29	
			12.5		0.5005	22.42	
			25		0.2697	58.19	
			50		0.1062	83.54	
			100		0.0551	91.46	
	ME		0.7		0.6186	4.11	11.44
			1.5		0.5762	10.68	
			3.1		0.4974	22.90	
			6.2		0.4227	34.48	
			12.5		0.2877	55.40	
			25		0.1883	70.81	
			50		0.0926	85.65	
			100		0.0583	90.96	

OD: Optical Density; IC50: Half Maximal Inhibitory Concentration

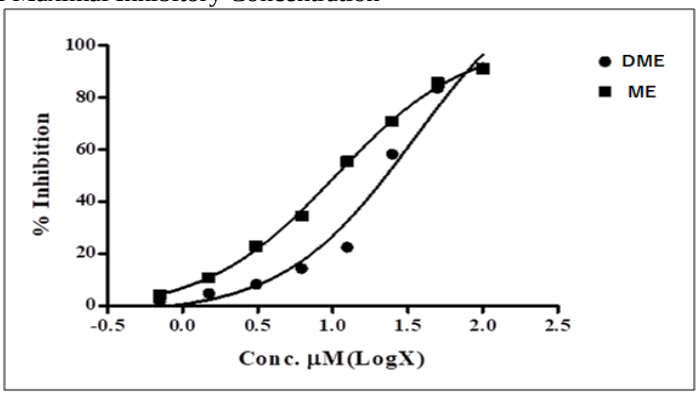

Fig. 3: Determination of Percent Inhibition (IC50) of DME and ME on MCF-7 cell lines

6. From hemolysis studies (Table V, VI & Fig .4), we are able to conclude that 320 µM of DME and ME are equally potent. 100µg/mL of DME is efficient to exhibit its Antioxidant activity according to the Antioxidant assay/ Hydroxyl radical scavenging assay.

Table 5: Summary of Hemolytic Activity Absorbance of 1% SDS (+VE CONTROL) and PBS (-VE CONTROL), DME and ME Compound on MCF-7 Cell Lines.

Sample	Conc. µg/ml	Absorbance	% Inhibition
PBS		0.8964	0.00
1% SDS		0.1987	78.65
DME	40.00	0.89	0.00
	80.00	0.8706	2.18
	160.00	0.8111	8.87
	320.00	0.7283	18.17
ME	40.00	0.89	0.00
	80.00	0.8526	4.20
	160.00	0.8196	7.91
	320.00	0.7421	16.62

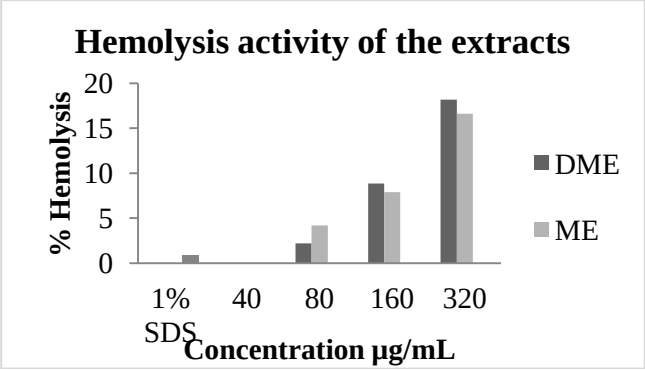

Fig 4. Determination of Percent hemolysis of DME and ME compounds in MCF-7cell lines

Table 6: Summary of Absorbance and Percent Inhibition of Hydroxyl Radical Scavenging Activity in DME and ME Compounds on MCF-7 Cell Line.

Name	Concentration (µg/ml)	Absorbance 590nm	% Inhibition	IC_{50}
Control (1% SDS)	0.0	0.585	0.00	8.130 µg/ml
+Standard (Catechin)	0.3	0.562	3.93	
	0.6	0.548	6.32	
	1.2	0.506	13.50	
	2.5	0.429	26.67	
	5	0.254	56.58	
	10	0.151	74.19	
DME	0.0	0.585	0.00	
	3.1	0.575	1.63	86.21 µg/ml
	6.3	0.566	3.18	
	12.5	0.543	7.26	
	25.0	0.486	16.98	
	50.0	0.354	39.53	
	100.0	0.282	51.71	
ME	0.0	0.585	0.00	
	3.1	0.576	1.60	Not active
	6.3	0.572	2.27	
	12.5	0.555	5.13	
	25.0	0.509	12.92	
	50.0	0.470	19.67	
	100.0	0.393	32.82	

Catechin is used as Positive standard drug.

7. From Clonogenic Survival Assay (Fig 5 & 6), MCF-7 cells treated with 50 and 100 µM/ml of DME compound for 24 h showed around 50% of inhibition at 50 µM/ml and around 20 % inhibition of colonies forming ability at 100 µM/ml. However, the compound ME has shows inhibition of 15% at 50 µM/ml and 100% inhibition at 100 µM/ml of colony forming capability. The summation of the various assays result suggests that ME has more significant colony forming inhibition abilities compare to DME, which also shows inhibition at higher concentration.
8.

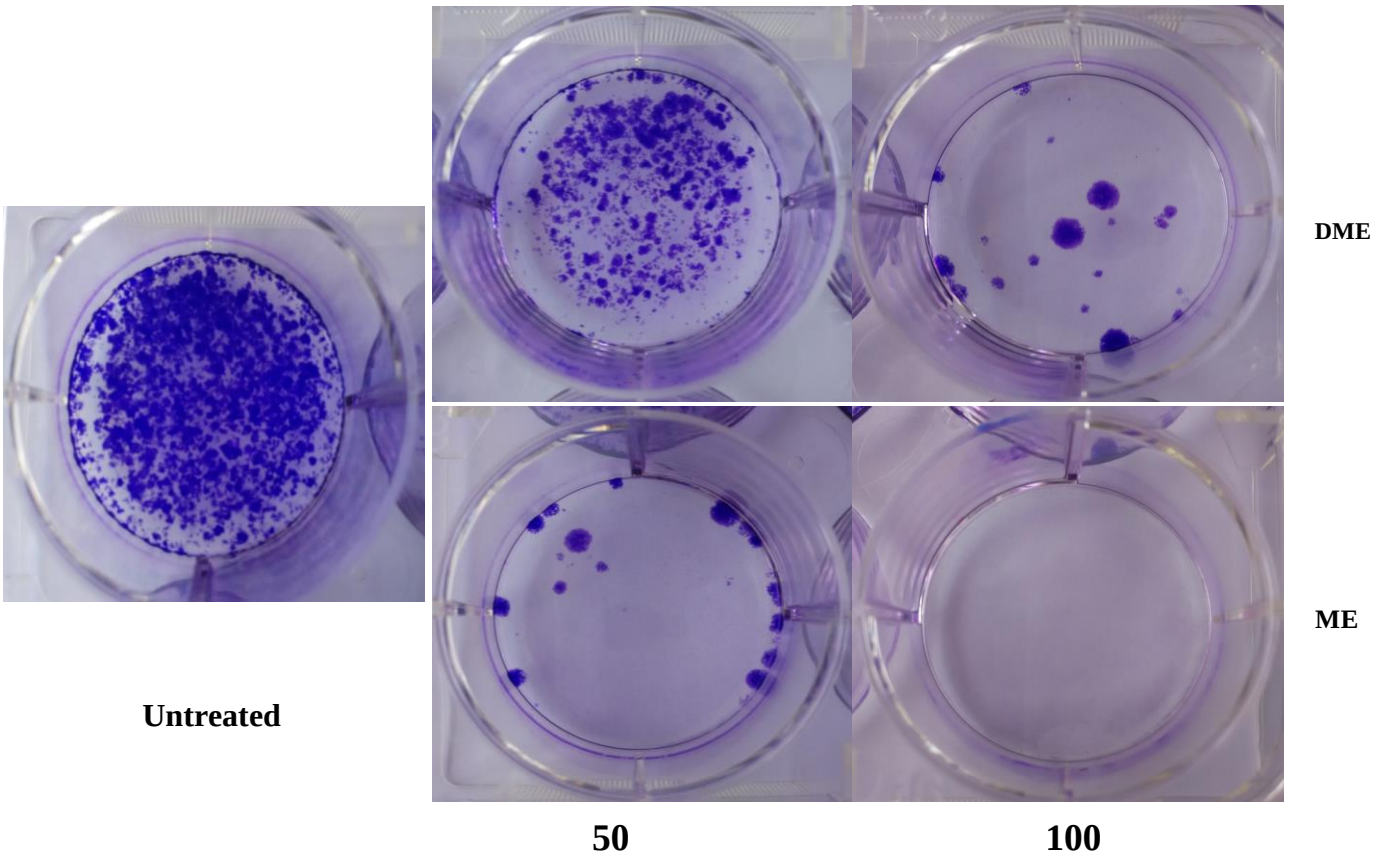

Fig. 5: Determination of Clonogenic activity of ME and DME compounds on MCF-7 culture cells

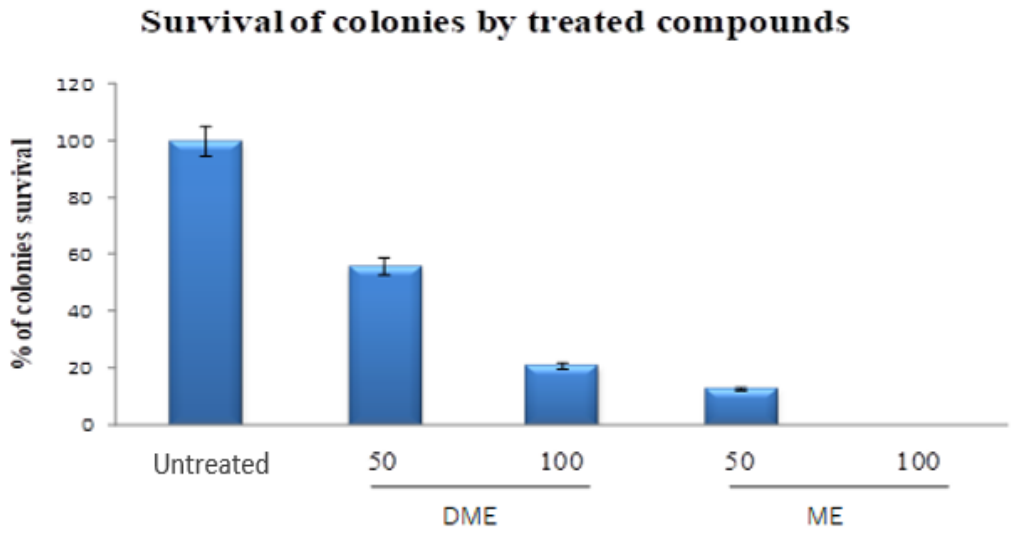

Fig. 6: Graphical representation of Clonogenic activity of ME and DME compounds on MCF-7 culture cells

Overall, these results show that both ME and DME have showed inhibition of the growth of MCF-7cells by preventing the formation of colony and thereby inducing apoptosis.

IV. CONCLUSION

Alternative medicine is becoming popular in recent times. In this brief report, we described that Cinnamaldehyde obtained from methanolic extracts of the resins of *Boswellia serrata* has shown the anti-cancer activity on MCF-7 breast carcinoma cell lines, effectively. Based on preliminary observations with various assays, it is evident that Cinnamaldehyde may represent an alternative medicine which can be supplemented with chemotherapy in breast cancer patients. It is desirable to conduct further experiments using this compound to test various cancer cell lines, animal models and in combination with anti-cancer therapies to reaffirm its efficacy and therapeutic properties.

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