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EFFECTS OF COMBINED ETHANOLIC EXTRACTS OF CITRULLUS COLOCYNTHIS AND PLUMBAGO ZEYLANICA (L) ON MCF-7 HUMAN BREAST CANCER CELL LINES

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ABSTRACT

Background: Breast cancer is the most common disease in both developed and developing countries. According to the World Health Organization, breast cancer is the most prevalent cancer amongwomen worldwide and the second most common cause of cancer death in women. Annually, breast cancer causes over 1 million new cases, results in more than 400,000 deaths, and about 4.4 million women live with breast cancer.
Aim: The study aims to investigate the combined effect of the ethanolic extracts of ***Citrullus colocynthis*** and ***Plumbago zeylanica*** Linn on the MCF-7 human breast cancer cell line.
Methodology: The phytochemical screening was performed on the combined ethanolic extractof ***Citrullus colocynthis*** and ***Plumbago zeylanica*** Linn to identify the presence of alkaloids, terpenoids, flavonoids, tannins, and coumarins. GC-MS analysis was performed using thermoGC Trace Ultra Version 5.0 to determine the possible chemical compounds in the extract. Moleculardocking was performedforthetargetproteinestrogenreceptoralpha(ER-α),retrieved from the RCSB PDB (ID: 3ERT). MTT assay performed to determine the anti-proliferative activity of the combined ethanolic extract with different concentrations such as 6.25, 12.5, 25,50, and 100 µg/ml on MCF-7 human cancer cell line. The caspase3 and 9 inhibition assays were performed to demonstrate the cell death and apoptosis induced by the combined extract.
Result: GC-MS analysis revealed 16 chemical components present in the extracts. In that, 9-octadecenamide 50.43%, 1,2-benzenedicarboxylic acid, dioctyl ester 11.25% were predicted to be present in larger quantities. Molecular docking studies proved that the polyphenoliccompoundhasgoodbindingaffinitywithER-α,withaglidescorerangeof-9.85kcal/molto-5.98 kcal/mol, compared to tamoxifen, which has a glide score of -10.28 kcal/mol. Cytotoxicity measured as IC_{50} against the MCF-7 cell line was found to be 34.57 µg/ml. The extract was able toincreasetheactivityofcaspase3 and9, indicatingtheextract was ableto induceapoptosisand cell death.
Conclusion: According to the results, we concluded that the combined ethanolic extract of ***Citrullus colocynthis*** and ***Plumbago zeylanica*** linn act as potential chemotherapeutic agent for breast cancer.

Introduction

Breast cancer is the most prevalent cancer affecting women globally, resulting in roughly 570,000 fatalities in 2015. Worldwide, more than 1.5 million women are diagnosed with breast cancer annually, accounting for 25% of all cancer-affected women (1,2). In the United States, itis projected that breast cancer will account for 30% of all new cancer cases among women in 2017 (3). This type of cancer is metastatic, often spreading to distant organs such as the bones, liver, lungs, and brain, which significantly contributes to its incurability. However, early detection of the disease can result in a favourable prognosis and a high survival rate. In North America, the 5-year relative survival rate for breast cancer patients exceeds 80% due to the promptidentificationofthedisease (4). Mammographyisacommonlyutilizedscreenin gmethod fordetectingbreastcancerand hasbeenshownto effectivelyreducemortalityrates(1). ***Citrullus colocynthis (C. colocynthis)*** is a drought-tolerant, widely distributed, and geneticallydiversified desert plant that is a member of the Cucurbitaceae family. The Biomedical properties of C. colocynthis include antibacterial, anticancer, antioxidant, and antilipidemic properties (2).

Plumbago zeylanica L., often called white chitraka, is a member of the Plumbaginaceae family. It grows as a weed in tropical and subtropical countries worldwide. It is a perennial subscandian bush that thrives across India, but particularly in South India, Bengal, Uttar Pradesh, and Sri Lanka 1. There are 10 genera and 280 species in the Plumbaginaceae family. Three species, Plumbago indica L. (Plumbago rosea L.),Plumbago capensis L., and Plumbago zeylanica L., are found throughout India and belong to the genus Plumbago. Numerous chemical componentshave been identified, including steroids, flavonoids, terpenoids, and naphthoquinones (3). A naphthoquinone found in nature, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), is present in plants classified as Drosearaceae and Plumbaginaceae. Plumbago indica Linn., Plumbago zeylanica Linn., and Plumbago auriculata Linn. all have roots that contain high levels of plumbagin. Many pharmacological qualities are widely recognised about them (4). The anti- cancer properties of ***Plumbago zeylanica*** in relation to triple-negative breast cancer (TNBC) were demonstrated through both network pharmacology and in vitro studies. Network pharmacology has pinpointed significant therapeutic targets and pathways associated with apoptosis and cell cycle regulation, with zeylanone, a bioactive compound derived from P. zeylanica,exhibitingstronginteractionswithpr oteinssuchasPARP1,ESR1,andHSP90AA1 (5). Theproliferation and metastasis potential ofbreast cancercells can be inhibited bytreatment with ***C.colocynthis*** fruit extract. Nevertheless, to this there has been no documented research assessing the anticancer potential and cell cycle regulation of the methanolic extract of ***C.colocynthis*** leaves and its fraction in breast cancer cells (6). The current studywas undertaken to assess the anticancer potential of the ethanolic extract of ***C.colocynthis*** leaves and ***Plumbago zeylanica L.***, root, and its fractions on the MCF-7 breast cancer cell line. Additionally, the study also explored its effects on cell cycle regulation through various bioassays (7)

Material and methods

Collectionofplantmaterials

Fresh plants of ***Citrullus colocynthis and Plumbago zeylanica (L).*** were collected from nearby places. The leaves and roots

were separated and washed under running tap water. The washed leaves and roots were allowed to dry in the shade at room temperature in the laboratory. The dried leaves and roots were ground into fine powder using a blender. The powder was preserved in an air-tight bottle for further studies.

DetoxificationofPlumbagoZeylanica

A traditional method described in the API was applied to the roots of the ***Plumbago zeylanica*** species. One-kilogram sections of ***plumbago zeylanica*** roots were soaked in lime water containing CaCO3 for twenty-four hours. Then, the roots were rinsed with distilled water, dried, and kept as "***Plumbago zeylanica***," a purified variant of ***plumbago zeylanica***. Following adequate detoxification, the roots were dried in the shade, processed into a coarse powder with a grinder, and sifted through a mesh with a number 60 (8).

Samplespreparationofextracts

Five grams of each plant (***Citrullus colocynthis*** **and** ***Plumbago zeylanica*****)** were extracted in 250 mL of ethanol in a Soxhlet apparatus for 72 hours.

Removalof chlorophyll fromplant extract

Five volumes of ethyl alcohol were used to extract the plant extract, and two volumes of acetone were added to remove the chlorophyll. The extract was dried by a rotary evaporator under vacuum. The extract was stored at 4 °C in an air-tight container for further studies. (9)

GasChromatography-MassSpectroscopy

Gas chromatography and Mass spectrometry analysis were performed to identify the active compounds in the combined ethanolic extract of ***Citrullus colocynthis and Plumbago zeylanica.*** Extracts were diluted to 1 mg/ml, and 0.5 µL was separated on a nonpolar DB5-HT capillary column (20 m × 0.18 mm) with a 0.1-µm film fitted to an Auto System XL GC-MS. The injector temperature was 270°C, and the oven temperature was programmed at an initial temperature of 50°Cfor 1 min, then rising at 25°Cper minute to 160°C and maintained at that temperature for 1 min. The temperature was subsequently increased by 10°C per minute to 300°Cand maintained at that temperature for a further 4.6 min. The carrier gas was helium, which is kept at a constant pressure of 5 kPa. Interpretation of the mass spectrum was conducted using the database of the National Institute of Standard and Technology (NIST). The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library.

MolecularDocking

The crystal structure of the ligand-binding domain of ERα was downloaded from the Protein Data Bank (3ERT). The protein was prepared using Protein Prep Wizard using Schrödinger, Maestro 13.1. The missing atoms were added, and the protein is optimized for docking by adjusting the protonation state for a pH of 7.2 ± 0.2. The grid will define the binding site where theligandswouldbedocked.Thegridisesse ntialforaccuratelypredictingligandposes. Here, the grid is generated with cocrystal ligand 4-hydroxy Tamoxifen as the centroid of the binding grid.The phytochemicals, which were identified from the GC-MS, were used for dockingstudies. Here, 18 ligands are

taken for docking. The ligands were prepared using the LigPrep module in Schrodinger. The ligands were prepared for the target pH of 7.2 ± 0.2. The docking was carried out using Glide SP, Schrodinger.

Cytotoxicityassay

The MTT assay is used to measure the combined cytotoxicity of ***Citrullus colocynthis*** and ***Plumbago zeylanica*** extracts on MCF-7 cell lines. The principle of the assay involves reducing water-soluble yellow tetrazolium salt into water-insoluble blue/magenta formazan crystals (13). The MTT assay is a widely used testing method to measure the cytotoxic activity of plant extracts. The MCF-7 cells were cultured in DMEM medium supplemented with 10% FBS. Approximately5000 cells were seeded in each well in the 96-well plates. The cells were allowed to attach for 24 h. After that, various concentrations of the extract (6.25, 12.5, 25, 50, and 100 µg/ml) were added and incubated for 48 h.After the incubation period, the old medium was removed, and fresh phenol red-negative medium was added. Additionally, 10 µL of MTT (10mg/ml) was added. The plates were incubated for 4 h in the dark for the formation of formazon crystals. This was followed by the addition of 100 µL of solubilizing buffer DMSO. The96-wellplatewasreadforabsorbancedensityvaluestodeterminecellviabilityat570nm (14). The viable cells produced a dark blue formazan product. The percentage of the viable cells was calculated using the following formula, as shown below.

$$\%\text{Cytotoxicity} = \frac{(\text{Experimental OD - Control OD})}{(\text{Maximum OD - Control OD})} \times 100$$

Caspaseassay

The MCF-7 breast cancer cell line was used to perform an indirect ELISA for caspase-3 and caspase-9 as described in the previous study. The cells were treated with the extract at concentrations of 17.29 µg/mL and 34.57 µg/mL for 48 h. After treatment, the cells were lysed and the 96-well plate was coated with lysates containing caspase-3 and caspase-9 antigens in carbonate–bicarbonate buffer. The plate was then incubated overnight at 4 °C. To prevent nonspecificbinding,thewellswerewashed withPBScontainingTween-20andblockedwith5% bovineserumalbumin.Next,primaryantibodiesspecifictocaspase-3(rabbitanti-caspase-3 IgG) and caspase-9 (rabbit anti-caspase-9 IgG) were added separately and incubated for 4 h. This was followed by addition of HRP-conjugated anti-rabbit IgG secondary antibody. After extensive washing, 3,3′,5,5′-Tetramethylbenzidine substrate was added, producing a blue color, and the reaction was stopped with sulfuric acid. The absorbance was measured at 450 nm, and the increasein signal intensityreflected the expression levels ofcaspase-3 and caspase-9 in response to the extract treatment.

ROS Assay

Intracellular reactive oxygen species (ROS) levels were assessed using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA). When oxidized by ROS, $DCFH_2$-DA is converted to the fluorescent compound DCF, which emits green light. MCF7 cells were seeded into 96-well plates at a density of 3,000 cells per well and allowed to grow for 24 hours. Thecells were treated with the test extract and the standard substance for 24

hours. After the treatmentperiod,200µLof10µMDCFH$_2$-DAworkingsolutionwasaddedtoeachwell, and the plate was kept protected from light. Following a 30-minute incubation at 37°C, the probe solution was removed, and the cells were washed with PBS. The cells were then lysed using RIPA buffer according to standard procedures. After centrifugation, 100 µL of the resulting supernatantwastransferredtoablack 96-wellplate,andthefluorescenceintensitywa smeasured using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. (14)

Results

Phytochemicalanalysis

The GC-MS analysis of the combined ethanolic extract of **Citrullus colocynthis and Plumbago zeylanica** indicated the presence of key phytochemical active arenarol (*Table 1, Figure 1).*

Table:1:Phytochemicalspresentintheplantextractasper GC-MSAnalysis

S. NO	NAME OF THE COMPOUND	RETENTION TIME	AREAS %
01.	CYCLOHEXASILOXANE, DODECAMETHYL-	13.85	7.9
02.	1,3-DIPHENYL-1-((TRIMETHYLSILYL)OXY)-1(Z)-HEPTENE	17.458	4.53
03.	TRI-O-TRIMETHYLSILYL, N-PENTAFLUOROPROPIONYL DERIVATIVE OF TERBUTALINE	20.69	2
04.	1,2-BENZENEDICARBOXYLIC ACID, DIOCTYL ESTER	34.74	11.2 39.1525
05.	ARENAROL	37.492	7.62
06.	9-OCTADECENANIDE, (Z)-	37.784	50.43
07.	1H-FURO[3,4-C]PYRROLE-4-CARBOXYLIC ACID, 6-(2-FURANYL)HEXAHYDRO-1,3-DIOXO-4-PHENYL-, METHYL ESTER, (3A.ALPHA.,4.BETA.,6.BETA.,6	37.885	0.96
08.	ACETAMIDE, N-(ACETYLOXY)-N-[2-CHLORO-3-NITRO-5-(TRIFLUOROMETHYL)PHENYL]-	39.022	4.39
09.	6-[(TERT-BUTYLDIMETHYLSILYL)OXY]-3-METHYL-1H-NAPHTHO[1,2-C]PYRAN-1-ONE	39.152	1.35
10	DIMETHYL BIS(3-IODOPROPYL)MALONATE	39.968	1.64

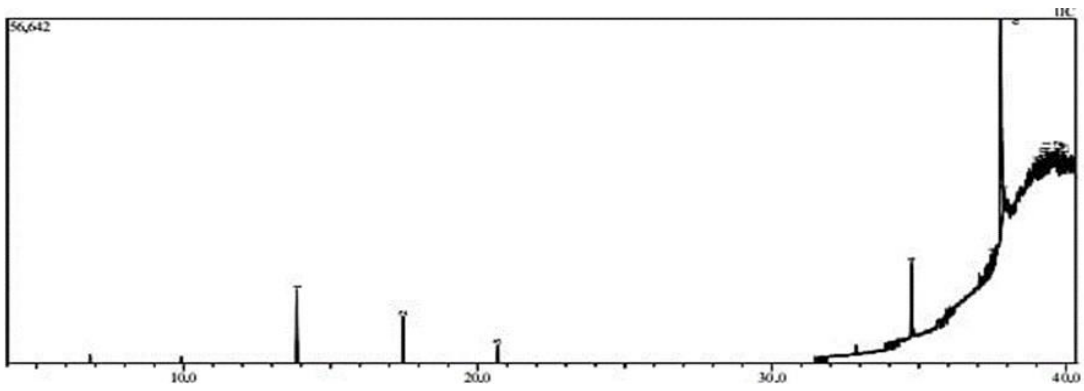

Figure 1. *Retention time of the phytochemical compounds in the combined plant extract as perGC-M*

Moleculardocking

The molecular docking revealed that the phytochemical Arenarol has a strong binding affinity withER-α.Arenarolisasesquiterpenoidhydroquinonewiththemolecularformula$C_{21}H_{30}O_2$. It features a rearranged drimane skeleton fused to a benzene-1,4-diol (hydroquinone) group. The structure includes a bicyclic sesquiterpene core with a methylidene group and specific stereocenters. Arenarol has a dockingscore of -9.85 kcal/mol, which isthebest amongthe tested phytochemicals. Its binding score is also comparable to that of the standard drug tamoxifen, which has a score of -10.29

kcal/mol (Table:2). Arenarol formed hydrogen bond interactionswith GLU353 and LEU387, while tamoxifen interacted with GLU353 and ARG394.

Table:2:Moleculardockingscoreoftest andstandard compounds

S. No	Name	GlideXPScore(kcal/mol)
1	Arenarol	-9.85336
2	9-Phenanthrylacetamide	-8.7718
3	2,4,6-trichlorophenylpentafluorophenylester	-7.29098
4	2-(bromomethyl)-2-tert-butyl-6-methyl-1,3-dioxan-4-one	-7.05363
5	Tris(trimethylsilyl)hydroxylamine	-6.37699
6	Oleamide	-6.30467
7	Tris(trimethylsilyl)hydroxylamine	-6.30193
8	(R)-adrenaline	-6.30682
9	Decamethylpentasiloxane-1,9-diol	-5.98933
10	(3-aminoisoindol-1-ylidene)azanium	-5.98197
11	Phthalate	-4.16102
12	Azinphos-ethyl	-3.69148
13	1,3-Diiminoisoindoline	-6.19887
14	3,4-Dihydroxyphenylglycol	-2.73888
15	4-Cyclopentene-1,3-dione	-2.27783
16	Succinatedianion	-2.16894
17	4-hydroxytamoxifen(Standard)	-10.2893

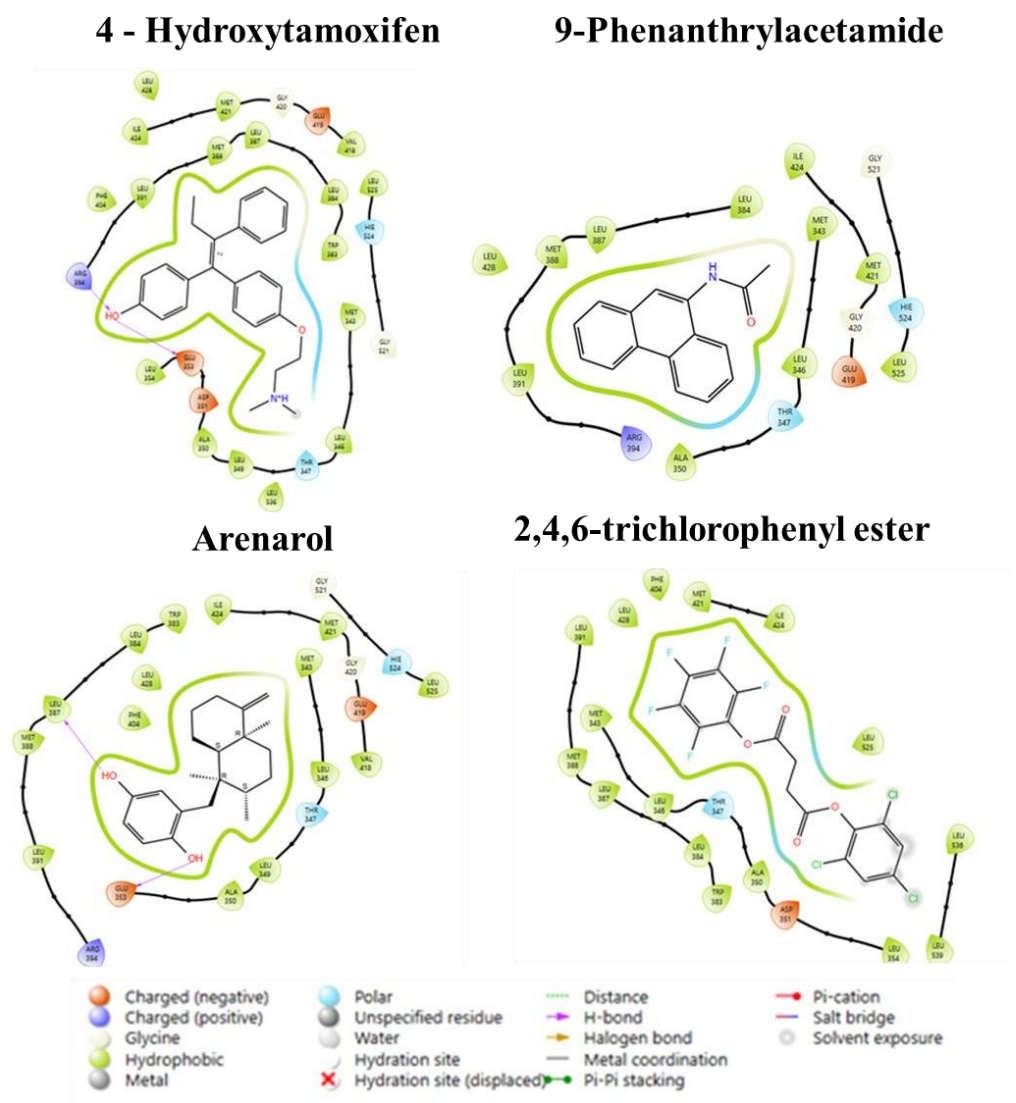

Figure2:2D interactionoftestand standard compoundswith ER-alpha

DeterminationofIC$_{50}$concentrationusingMTTassay

The combined ethanolic extract of ***Citrullus colocynthis and Plumbago zeylanica*** was tested for cytotoxic effects on breast cancer MCF-7 cells using a standard viability assay across concentrations from 3.125 to 100 µg/mL. Cells exhibited a dose-dependent decrease in viability, with cytotoxicity from 89.81 ± 3.4% at 100 µg/mL to 5.62 ± 1.8% at 3.125 µg/mL compared to the control (100 ± 3.1%) (Table 3, Figure 3). Nonlinear regression with a four-parameter logistic model estimated an IC$_{50}$ of 34.57 µg/mL, indicatingmoderate cytotoxicity. Treated cells showed reduced density and morphological changes, such as membrane blebbing, characteristic of apoptosis—programmed cell death involving cytoskeletal degradation, membrane bulging, and fragmentation into apoptotic bodies. These results demonstrate a concentration-dependent inhibitory effect on cell growth, highlighting its potential for further pharmacological investigations.

Table3:Cytotoxiceffectofdifferentconcentrationsofcombinedethanolicextractsof ***Citrulluscolocynthis* and*Plumbagozeylanica***onMCF-7breastcancer cells.

Concentration(µg/mL)	**Cellcytotoxicity(%)**
Control (0 µg/mL)	100.0 ± 3.1
100	89.81 ± 3.4
50	66.59 ± 4.3
25	46.74 ± 3.7
12.5	24.23 ± 4.2
6.25	7.51 ± 1.2
3.125	5.62 ± 1.8

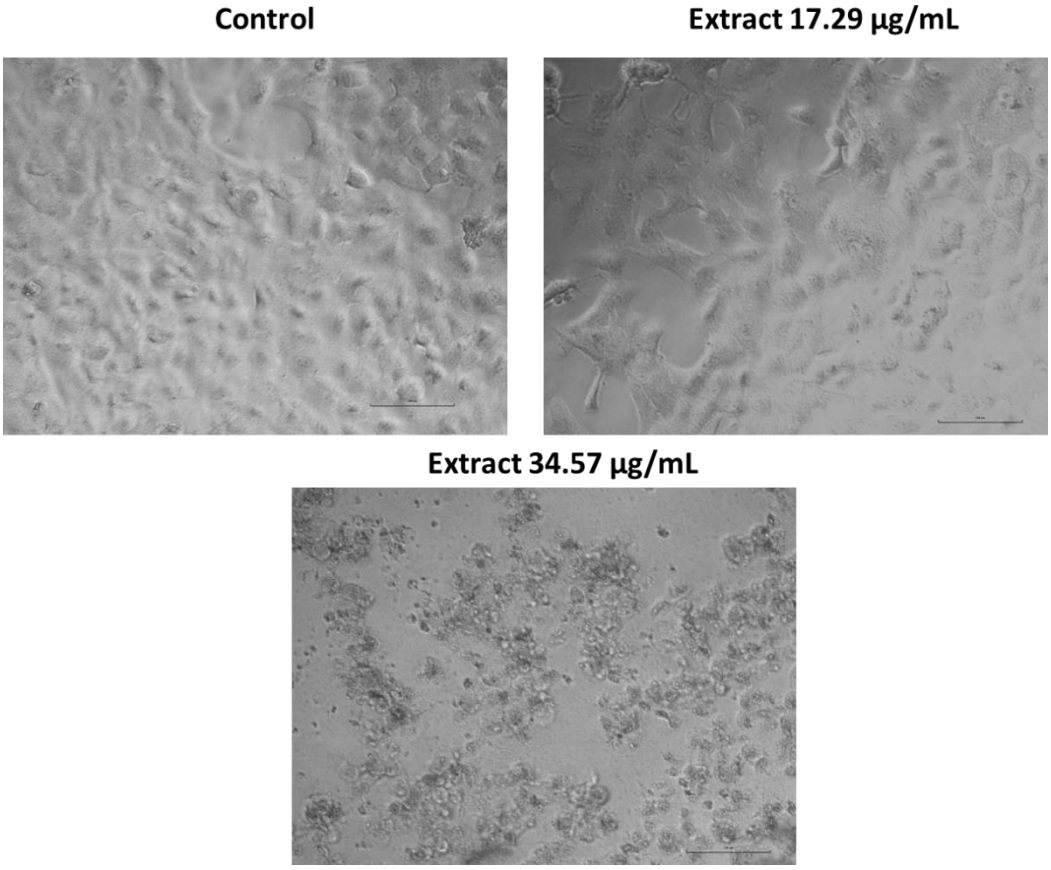

Figure 3: The comparison of cellular morphology of control and ***Citrullus colocynthis and Plumbago zeylanica*** treated MCF-7 cells. The phase contrast images of MCF-7 cells were treated with the extract's IC50 concentration.

CaspaseAssay

The apoptotic activity of the combined extract of ***Citrullus colocynthis and Plumbago zeylanica*** was confirmed by quantifying the caspase 3 and caspase 9 activity. An increase in thelevelofcaspases 3 and9indicatestheextract wasabletotriggertheapoptoticmechanism in MCF-7 cells. The standard drug paclitaxel significantly increased the caspase 3 and 9 activities compared to the control (Fig 4). The combined extract at 17.29 µg/mL did not significantly increase caspase 3 and 9 activities; however, the combined extract at 34.57 µg/mL was able to significantly increase caspase 3 and 9 activities in MCF-7 cells.

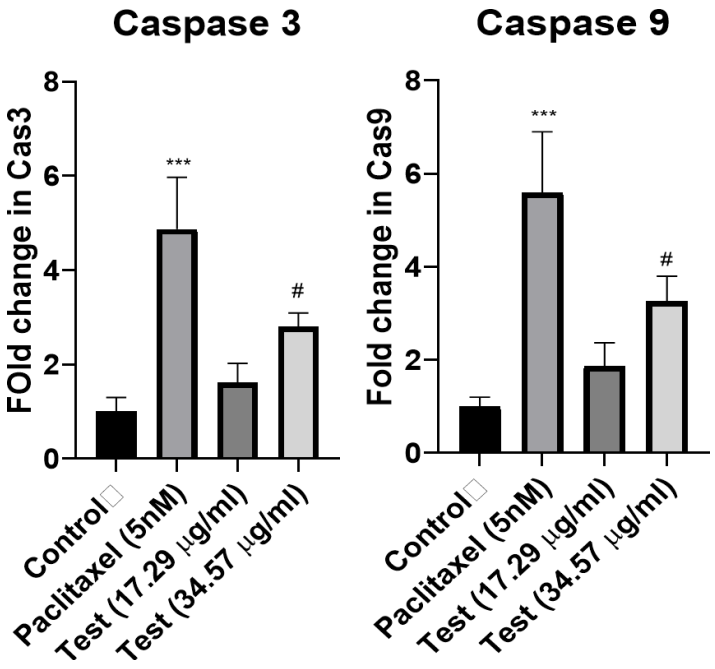

Figure:4. Represents the Caspase 3 and 9 expressions. A One-way ANOVA was performed to compare the means of each column. The results for all experiments are representative of at least three trials (n = 3). Data are expressed as Mean ± SEM. $p<0.05$ was considered significant. *Indicates comparison with vehicle treatment. #Indicates comparison with paclitaxel.

ROS Assay

ROS production was measured by the 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH2- DA) staining method. Similar to caspase activity, the combined extract did not increase the fluorescence intensity at 17.29 µg/mL (Figs 5 and 6). However, the fluorescence intensitywas remarkably increased in the 34.57µg/mL treated group.

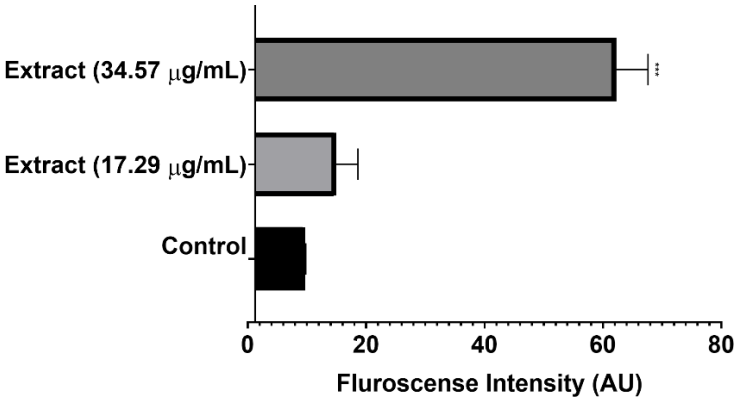

***Figure* 5**. Represents the ROS generation. A One-way ANOVA was performed to compare themeansofeachcolumn.Theresultsforallexperimentsarerepresentativeofatleastthree trials(n=3).DataareexpressedasMean±SEM.p<0.05wasconsideredsignificant.* Indicatescomparisonwithvehicle treatment.

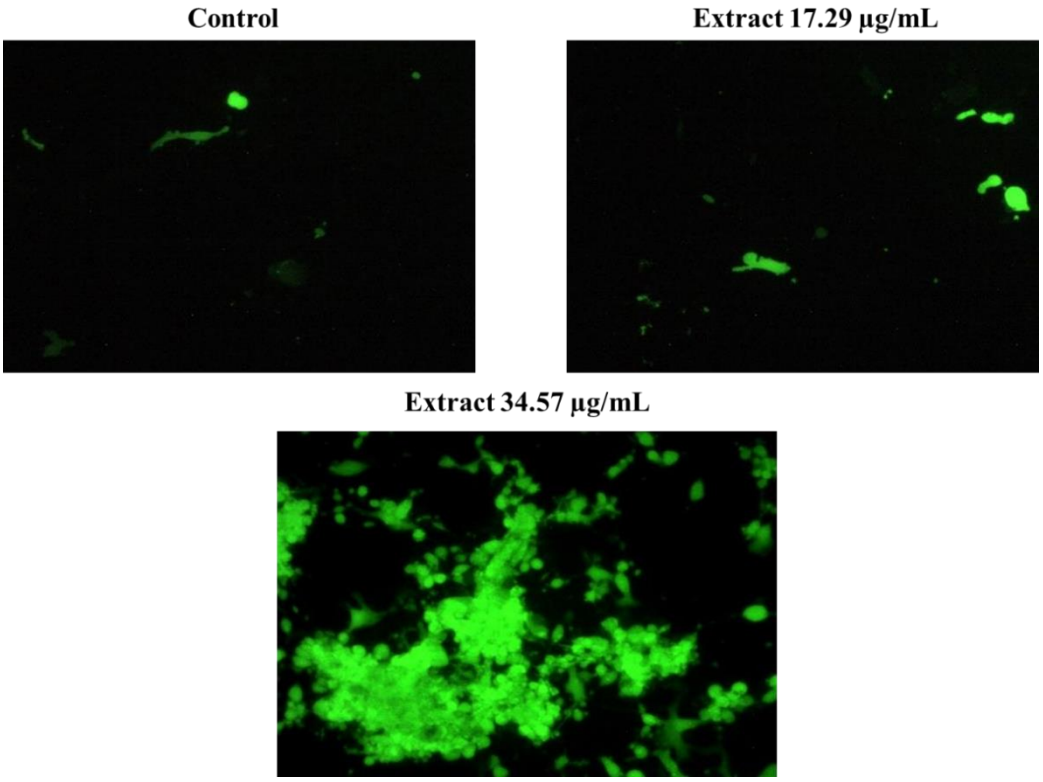

***Figure* 6**. Represents the ROS generation in various treatments as per DCFH2-DA staining methods

DISCUSSION

In this study, we analyzed a combined extract of ***Citrullus colocynthis and Plumbago zeylanica*** using GC-MS to identify bioactive compounds responsible for its anticancereffects. The mass spectra were matched with the NIST database, revealing severalcompounds with notable biological activity. Notably, arenarol, a sesquiterpenoid, was identified as having potential roles in both anticancer and anti-melanogenic processes. Cytotoxicity tests on MCF-7 breast cancer cells showed an IC_{50} of 34.57 µg/mL for the extract, indicatingmoderatepotency. At this dose, theextract significantlyincreased caspase- 9 and caspase-3 activity, indicating activation of the intrinsic apoptosis pathway. Caspase-9 triggers mitochondrial apoptosis following cytochrome c release, while caspase-3 serves as the executioner, cleaving key substrates to induce cell death. These findings suggest that the extract promotes apoptosis via mitochondrial signaling, a vital pathway for targeted cancer therapy.

Molecular docking studies targeting estrogen receptor alpha (ERα) were conducted to investigate possible receptor-mediated mechanisms behind the anticancer effects. Arenarol exhibited a high binding affinity with a Glide XP score of -9.85 kcal/mol and formed stable hydrogen bonds and hydrophobic interactions within the ligand-binding domain of ERα. Other compounds such as 9-Phenanthrylacetamide (-8.7 kcal/mol) and 2,4,6-Trichlorophenyl pentafluorophenylester(-7.29kcal/mol)alsoshowedpromisingdockingscores.Thestandard drug, 4-Hydroxytamoxifen, displayed the highest affinity at -10.29 kcal/mol, confirming the reliabilityofthedockingprotocol.Theseresultsimplythattheextractcouldactasapartial agonist or antagonist at ERα, potentially modulating estrogen signaling pathways to inhibit the growth of ER-positive breast cancer cells. This receptor-specific activity may contribute to the extract's cytotoxic effects (25, 26).

Beyond receptor modulation, the extract markedly increased intracellular reactive oxygen species (ROS) at the IC_{50} dose, suggesting that oxidative stress is a secondary mechanism of its cytotoxicity. Elevated ROS can harm cellular macromolecules, disrupt mitochondrial membrane potential, and enhance apoptotic pathways, working synergistically with caspase activation. The combined effects of receptor targeting, ROS production, and apoptosis highlight the extract's multi-faceted approach, which may lower resistance development risk compared to single-target treatments. Literature shows that sesquiterpenoids and phenanthryl compounds from medicinal plants often act through dual mechanisms—inducing apoptosis and engaging receptor pathways. This extract from ***C. colocynthis and P. zeylanica*** seems to sharethesefeatures,makingitapromisingcandidateforfurtherpharmacologicalresearch. Its multi-target actions, such as caspase activation, ERα binding, and oxidative stress induction, support advancing to in vivo studies and potential therapeutic applications (27).

CONCLUSION

This study shows that the phytochemicals in the combined extract have notable anticancer properties. Through various mechanisms—such as inducingmitochondrial apoptosis, causing oxidative stress, and modulating estrogen receptors—the extract offers a new natural strategy for treating ER-positive breast cancer. Future research

should focus on isolating specific bioactive compounds, exploring their combined effects, and performing preclinical in vivo tests to unlock its full therapeutic potential.

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