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In vitro and *Insilico* cytotoxicity activity of *Aconitum Heterophyllum* Phyto niosomes and its ethyl acetate root extract: A comparative study

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***Abstract*---**Niosomes as a novel drug delivery system is a nonionic surfactant-based vesicle, have attracted much attention in pharmaceutical fields due to their excellent behavior in encapsulating both hydrophilic and hydrophobic agents. In the present study a comparative free radical scavenging and anti-proliferative potential of ethyl acetate root extract of *Aconitum heterophyllum* (EAEAH) and *Aconitum heterophyllum* roots loaded phyto-niosomes (nEAEAH) were investigated. Initially the EAEAH and nEAEAH were prepared and their antioxidant potential were studied in terms of DPPH, Super oxide, Hydroxyl radical scavenging by employing the *In-vitro* method. Assessment of anti-proliferative activity of these extracts was done using MCF-7 cancer cell lines (human breast cancer) by employing MTT assay and trypan blue exclusion assay. *In-silico* docking was performed using Auto Dock Vina software to identify the suitable antagonistic ligand which can inhibit the proteins BRCA1, BRCA2 and HER2 to exert the cytotoxic effect. In the present study a concentration dependent antioxidant, and cytotoxic activity was observed for both the extracts. IC_{50} values were calculated for MTT and trypan blue assay and the nEAEAH showed 41.71µg/ml, 49.36µg/ml respectively, whereas for EAEAH it was found to be 54.89 and 67.13µg/ml. Among the 31 phyto components identified by the GCMS, molecular docking analysis revealed the antagonistic nature of

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phyto ligands namely 1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl) amino, Phenol,2,4-di-tert-butyl and isoindole-1,3 (2H)-dione-4,7-ethano-3a,4,7,7a-tetrahydro-2-phenyl**,** has the ability to inhibit the three tumour progressive proteins namely BRCA1, BRCA2, and HER-2 which can act as antagonistic ligand and their amino acid interactions were comparable to the positive drug Doxorubicin. It may be concluded that the increased antioxidant and cytotoxic activity observed for the *Aconitum heterophyllum* loaded phyto niosomes may be due to the increased bio availability of its phyto constituents.

***Keywords*---**Phyto noisome, ROS, RNS, Docking, Antitumor activity.

Introduction

Worldwide, bosom malignant growth keeps on being a most often analysed disease in females. The incidence of breast cancer varies worldwide; high incidence was reported in more developed countries than developing one [Ferley et al., 2010]. One in eight females' torments bosom disease during their lifetime [Tirona et al., 2010]. The extended number of cases for bosom malignant growth in India, in the year 2020 is 1,79,790 and this will comprise about 10% of all cancers [Mathur, Prashant 2020]. And recent research status says that exposure to pesticides is additionally displayed to have a positive relationship to existence of bosom cancer [Bassil 2007]. Developing bosom malignancy is related with non-modifiable risk factors like sex (female), age, conceptive factors like early menarche, late menopause, nulliparous and so forth. Modifiable risk factors include high fat and calorie diet, stoutness, inactive way of life, utilization of tobacco, utilization of liquor, preventative pills, endocrine disruptors and work shift [Weir et al., 2007]. Genetic based molecular modification, which includes transformation, uncontrolled proliferation, deregulation of apoptosis invasion, metastasis and angiogenesis are the characteristic features of cancer. During the biochemical reactions living cells continuously produces free radicals like superoxide, hydroxyl, hydrogen peroxide and nitric oxide radicals which are cumulatively called as Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) which gets scavenged with the help of endogenous antioxidant defence mechanisms. However, plant-derived phytochemicals and derivatives are intriguing options for improving therapeutic productivity in sick patients and reducing negative responses. Some of these phytochemicals are found in chemically dynamic combinations that have important anticancer properties. The examination of normal concentrates is the first step in improving successful and incidental effects free phytochemical-based anticancer treatment [Choudhari et al., 2020]. *Aconitum heterophyllum*, an individual from the *Ranunculaceae* family, they are used as expectorant, febrifuge, anthelmintic, anti-diarrheal, anti-emetic, and anti-inflammatory. It is a good source of diterpene alkaloids and flavonoids [Paramanick et al., 2017]. In recent decades, new anti-cancer drugs have been created with the goal of improving drug pharmacodynamics and bioavailability while also delivering targeted drug concentrations to cancer cells with little toxicity to healthy cells. Phytosomes are a type of advanced herbal medication technology that provides defined plant drug bioavailability over herbal extracts [Kumar et al., 2020]. The unclear bioavailability of lipid insoluble auxiliary

metabolites has been emarcated using phytosomes [Kumari et al.,2011]. The term 'Phyto' refers to a plant, while'somes' refers to a cell. Phytosome is a vesicular drug delivery system in which phytoconstituents are enclosed and confined by lipids (one phyto-constituent particle connected with no less than one phospholipid atom). Phytosomes protect a significant portion of natural concentrate from annihilation by stomach-related discharge and gut bacteria, resulting in improved ingestion, improved bioavailability, and improved pharmacological and pharmacokinetic bounds when compared to standard home-grown extract [Pawar et al.,2015]. The cycles in which the normalised concentrate of dynamic ingredients of herb is attached to phospholipids such as phosphatidylcholine (PC), phosphatidyl ethanolamine, or phosphatidyl serine via a polar end are known as phytosomes [Patel et al.,2013].

Molecular docking is a fundamental tool in structural biology and computer-aided drug design [John Dogulas Palleti et al.,2011]. It is the interface by which two molecules fit together in 3D space. The *BRCA1* and *BRCA2* genes protect from getting breast, ovarian, and other cancers. Women who have quality transformations in either BRCA1 or BRCA2 as well as HER2 genes due to mutation have a greater than 80% chance of developing breast cancer. Everyone has two copies of the *BRCA1* and *BRCA2* genes, each inherited from their parents. In the case of mutated *BRCA1* or *BRCA2* genes inherited from one parent, the normal copy of the BRCA*1* or *BRCA2* gene from the other parent has the ability to protect from cancer. Cancer occurs due to the second mutation occurring in the normal copy of the BRCA*1* or *BRCA2* gene, since the second mutation would not be present throughout our body, but would only be present in the cancer tissue [Thakur et al.,2005]. With the above scenario the present investigation is aimed to study, the comparative antioxidant and anti-proliferative activity of phyto niosomes loaded with root extract nEAEAH with that of the conventionally prepared ethyl acetate extract.

Materials and Method

Preparation of *Aconitum heterophyllum* ethyl acetate root extract (EAEAH)

Aconitum heterophyllum roots (1 kg) were dried and grinded into a coarse powder at room temperature. The powder was sieved through a 40-mesh sieve and extracted in a soxhlet unit at 60°C with ethyl acetate (CAS 141-78-6). The filtrate was evaporated under decreased pressure using a rotatory evaporator until all the solvent was removed and the EAEAH was then kept refrigerated for further studies, after ethyl acetate extraction.

Preparation of ethyl acetate extract of*Aconitum heterophyllum* loaded Phyto-Niosome (nEAEAH)

Aconitum heterophyllum roots stacked phyto-niosomes (nEAEAH) were incorporated by film hydration method. Initially, 10mM of Tween 60 (Polysorbate) 817076 and cholesterol (**C8667)** were broken up in chloroform with 1:1 molar proportion and 1.0 mg/ml of *Aconitum heterophyllum* was mixed and blended in a round base flask. The excess chloroform (CAS 67-66-3.102445) was taken out at 55℃ utilizing a rotational evaporator to acquire a thin film of nEAEAH on the

outer layer of the flask which was hydrated with PBS by unsettling in a water shower at 55°C for 2h. The subsequent arrangement was then exposed to shower sonication for 20 min to acquire better vesicles. Phyto-niosomes are isolated from the un caught materials by performing dialysis against distilled water for overnight.

In-Vitro Antioxidant Activity
DPPH radical scavenging activity

Cuendet et al., approach was used to test the ability of DPPH to scavenge radicals [Cuendet et al., 1997]. EAEAH and nEAEAH extracts (10 to 50g/ml) were added to 3.0 ml of DPPH methanolic solution at various concentrations (0.1mM). Methanol (179957) was used instead of root extract in the control group. The reaction mixture was incubated for 30 minutes at 37°C in a darkroom, and the absorbance was measured using a UV-visible spectrophotometer at 517nm. The percentage of inhibition was calculated using the formula A0-A1x100/A0, where A0 and A1 are the absorbance of the control and test samples, respectively. Ascorbic acid was used as a standard.

Super oxide scavenging activity

The scavenging effect of superoxide anion was calculated based on the technique described (Robak and Gryglewski, 1988). The oxidation of NADH created superoxide radicals in the nicotinamide adenine dinucleotide, phenazine methosulphate (PMS–NADH) (**S5037)** framework, which were measured by the decrease in nitro blue tetrazolium (NBT)(**N6495).** In 1ml of PMS solution, various concentrations of EAEAH and nEAEAH extracts were incubated at 25°C for 5 minutes, and the absorbance was evaluated at 590 nm in comparison to the clear arrangement. As a positive control, L-Ascorbic acid was used. The decrease of NBT reduction measured by the absorbance of the reaction mixture correlates with the superoxide radical scavenging activity of the study extracts.
The inhibition percentage of superoxide radical= [(A0 – A1/A0) x100],
Where A0 is the absorbance of the control, and A1 is the absorbance of test.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging action was determined according (Halliwell, et al., 1987) . The hydroxyl radical scavenging activity of EAEAH and nEAEAH extracts were measured by the competition between deoxyribose and the extracts for the hydroxyl radicals generated from the Fe3+/ascorbate/EDTA/H2O2 system (nonsite-specific assay). Briefly, the reaction mixture containing 2.8 mm deoxyribose, 0.1 mm $FeCl_3$(CAS NO 7705-05-0), 0.1 mm ascorbic acid, 0.1 mm EDTA, (226009) and 1 mm H_2O_2 were mixed with various concentrations of plant extracts in 1ml of final volume made with KH_2PO_4–KOH buffer (1551139) (20 mm pH 7.4), and was incubated in a water bath at 37 °C for 1 hr. The thiobarbituric corrosive (TBA)(T5500) method was used to determine the degree of deoxyribose breakdown. 1ml of 1% (w/v) TBA and 1ml of 2% (w/v) trichloro acidic corrosive (TCA)(116114) were added to the mixture and heated for 20 minutes at 100°c. The absorbance was determined at 532 nm against the clear reagent after cooling to room temperature. Mannitol(M3584), a classical hydroxyl radical scavenger was

used as positive control. The inhibition percentage of hydroxyl radical was calculated.

Anti Proliferative Activity
Maintenance of MCF-7 (human breast carcinoma) cell lines

The MCF-7 cell lines were obtained from National Centre for Cell Sciences, Pune, India was cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine (25030149), sodium bicarbonate (25080094), 10% FBS, streptomycin (3810-74-0) (100 µg/ml), and penicillin 15140122 (100 U/ml), nonessential amino acids and Hank's saltant appropriate conditions. Confluent monolayer cells were harvested by 0.25% trypsin (15050065) and cultured in 10% growth medium.

MTT (3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl- tetrazolium bromide) assay

The MTT method was used to assess the cytotoxicity and anticancer potential of *Aconitum heterophyllum* roots loaded Phyto-Niosomes (nEAEAH) and EAEAH on MCF-7 cell according to Horiuchi et al., 1988. 100 µl cell suspension (5×10^4 cells /well) was seeded in 96 well tissue culture plates with 0.2 ml of medium/well. To this different concentration of nEAEAH and EAEAH extracts were added and incubated at 37^0C with 5% CO_2 for 24hr, 48hr and 72hr in a humidified incubator to study the anti-proliferative activity. At the end of incubation of 24hr, 48hr and 72hr approximately 30 µl of MTT solution was added and further incubated for 4 hrs with same incubation conditions. MTT solubilisation solution in DMSO (D12345) 100µl was added to the resultant supernatant in each well and mixed with a micropipette for 45seconds to solubilize the formazan crystals. The suspension was transferred to the cuvette of a spectrophotometer and the optical density values were read at 595nm by using DMSO as a blank. Doxorubicin (DOX-14-002) was used as a positive control. The percentage of cell viability was calculated as follows:
Cell viability = optical density of Test drugs /optical density of control × 100

Trypan Blue Dye Exclusion Assay

A trypan blue staining assay was performed to further investigate the EAEAH cytotoxic effect in MCF-7 cells. In this method, viable cells with intact membranes do not absorb the trypan blue dye, whereas dead (non-viable) cells with intact membranes will absorb. MCF-7 cells were seeded in a 96-well plate and the plates were incubated in a 5% CO_2 incubator for 24 hours at 37^oC. The medium was then replaced with new medium containing different concentrations of the *Aconitum heterophyllum* roots loaded phyto-niosomes (nEAEAH) for MCF-7 (10,20,40,40,80,100 µg/ ml^{-1}) in DMSO and then incubated for 24 hours at 37^oC with 5% $C0_2$. Cells without any treatment was considered as negative control. The cells were placed on the Neubauer counting chamber at the end of the incubation period, stained with trypan blue dye(T10282), and the number of viable and nonviable cells counted under the microscope at 100x magnification (Halder et al., 2009) . The percentage of viable cells compared to the control was used to calculate cell viability.

GC–MS Analysis

A Shimadzu GC-2010 Plus gas chromatograph was used with a 15m Alltech EC-5 section (250 I.D.,0.25 movie thickness) and a straight deactivated 2 mm direct injector liner. For test organisation, a split infusion was used, with a 10:1 split percentage. The oven temperature programme was changed to start at 35°C and hold for 2 minutes, then slope at 20°C per minute to 450°C and hold for 5 minutes. The helium transporter gas stream rate was set to 2 ml/minute. All tests were conducted using a direct connection with a thin section metal quadrupole mass channel pre-pole mass spectrometer operating in electron ionisation (EI) mode and a programming GCMS configuration version 2.6. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 1000 at 0.3 seconds per scan with a 0.2-second inter-scan delay. High-resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 1000 at 1 second per scan.

***In silico* anticancer activities**
Preparation of the Receptor protein

The X-ray crystal structures of the breast cancer targeting protein HER2, BRCA1 and BRCA2 with PDB Code: 5JIK, 4IJK and 1N0W respectively were downloaded from the RCSB databank. The preparation of the crystal structures was performed by correcting the missing residues and missing hydrogens using the protein preparation wizard of Auto Dock Vina software. The bond orders were assigned. The non-essential water molecules were deleted and polar hydrogens were added. The crystal structures were optimized using the OPLS force field.

Preparation of the Ligand
The structures were drawn using ACD labs Chemsketch software and converted into MDL mol format, downloaded and imported. The LigPrep module in Auto Dock was used to geometrically refine the chemical structures. The ligands were possibly set to a pH of 7.0. Desalting and generation of tautomers with conformer generation were performed and at most 32 conformers per ligand were generated.

Receptor Grid Generation and Molecular Docking

The receptor grid indicates the area of interaction between the ligand and protein. The grid indicates an area of active site that is given coordinates of x, y and z. The centre of the active site residues is highlighted and the grid parameters were set to enable docking between the ligand and the prepared protein. The prepared and optimised protein structures were docked into ligands that were prepared using LigPrep. The docking analysis was performed using the Auto Dock on Maestro 11.5. The target proteins 5JIK, 4IJK and 1N0W were determined with their active site and the docking analysis was performed with the prepared ligands. The Glide standard precision (SP) algorithm was selected and flexible docking was performed. The docked results were viewed to determine the ligand interaction by viewing the interaction diagram at the residues of the active site of the protein.

Statistical analysis

All information was dissected measurably with Statistica/Macsoftware (Prism, USA). The exploratory outcomes were mean ± SD of three equal estimations. Successful focuses (EC50) esteem was determined by regression analysis. Mean differences were analysed statistically by running one-way analysis of variance test (ANOVA). $P < 0.05$ was considered statistically significant when compared to relevant controls.

Results

DPPH radical scavenging activity

The DPPH scavenging activity of EAEAH and nEAEAH extracts were shown in the Figure -1. A Concentration dependent DPPH scavenging activity was observed for both the extract in the present investigation. But at higher concentrations starting from 30µg/ml the phyto niosome extract of *Aconitum heterophyllum* exhibited similar inhibitory activity as that of positive control ascorbic acid.

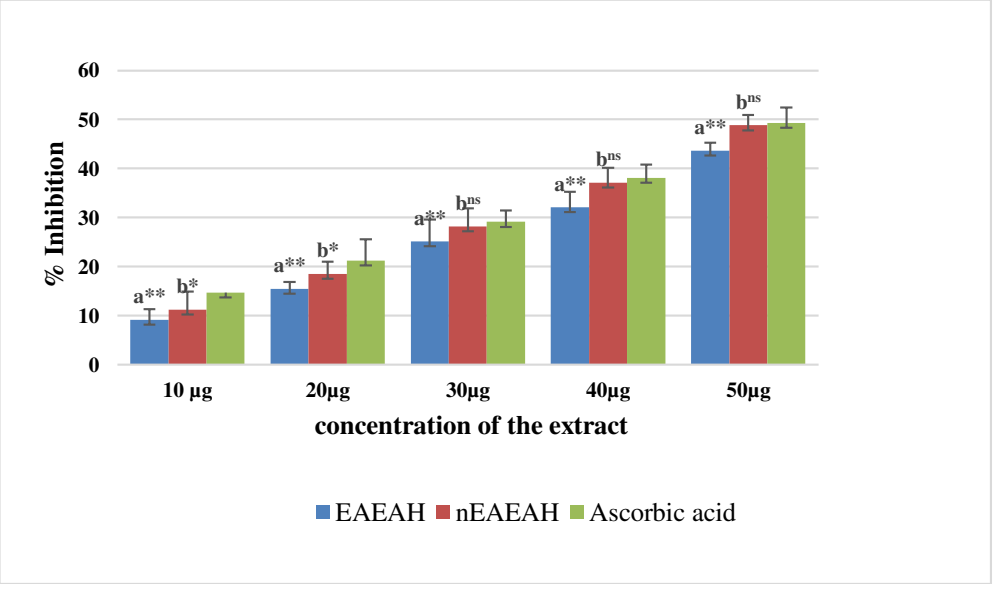


Figure -1 DPPH scavenging activity of EAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum)*, nEAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum* loaded Phyto-Niosomes) and Ascorbic acid

Values are stated in mean±SD (n=3), statistical substantial test for comparison was done by ANOVA (Analysis of Variance) followed by Dunnet's 't' test. Evaluation between a –Ascorbic acid vs EAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum*) b –Ascorbic acid vs nEAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum* loaded Phyto-Niosomes**)** *p<0.05, **p<0.01 and ns – non-Significant.

Super oxide scavenging activity

The potential of EAEAH and nEAEAH extracts in neutralizing the super oxide radicals were shown in the Figure-2. Both the extracts neutralized the generated super oxide radicals effectively. However, the phyto niosomes form of *Aconitum heterophyllum* neutralized the super oxide radicals effectively when comparable to the conventional ethanolic extract.

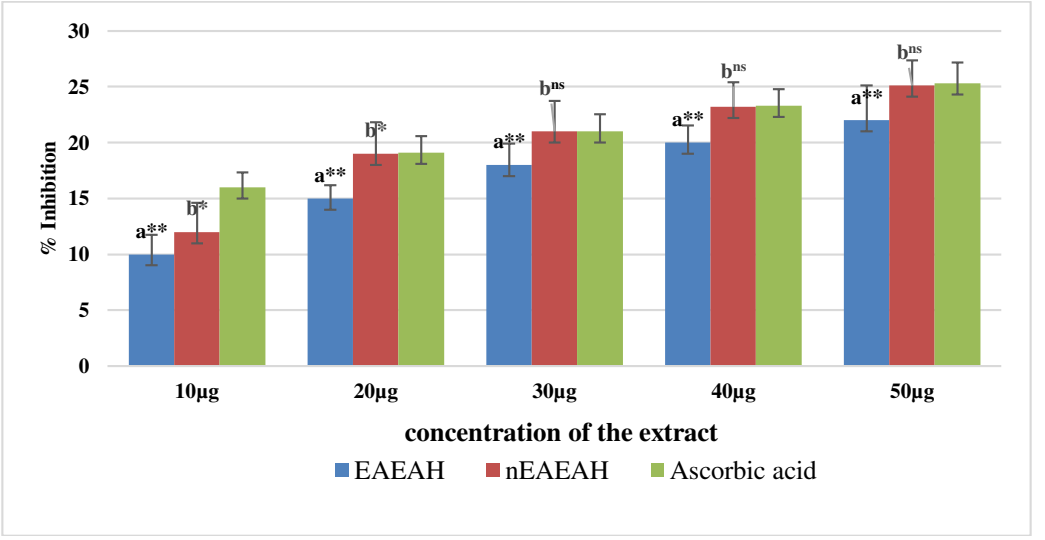


Figure -2 Super oxide scavenging activity of EAEAH (Ethyl Acetate Extract *Aconitum heterophyllum)*, nEAEAH (Ethyl Acetate Extract *Aconitum heterophyllum*loaded Phyto-Niosomes) and Ascorbic acid

Values are stated in mean±SD (n=3), statistical substantial test for comparison was done by ANOVA (Analysis of Variance) followed by Dunnet's 't' test.
Evaluation between a –Ascorbic acid vs EAEAH (Ethyl Acetate Extract *Aconitum heterophyllum)* b –Ascorbic acid vs nEAEAH (Ethyl Acetate Extract *Aconitum heterophyllum* loaded Phyto-Niosomes) *p<0.05, **p<0.01 and ns – non-Significant.
Experiments are performed in triplicates.and values mentioned are expressed in mean ±SD.

Hydroxyl radicals scavenging activity

Figure -3 depicts the hydroxyl radical scavenging activity of the three extracts namely EAEAH, nEAEAH and Ascorbic acid. Though all the three extracts scavenged the hydroxyl radicals in a dose dependent manner, we could not observe any increase in the scavenging potential nEAEAH extracts. In the present investigation ascorbic acid exhibits maximum scavenging potential when comparable with the two extracts.

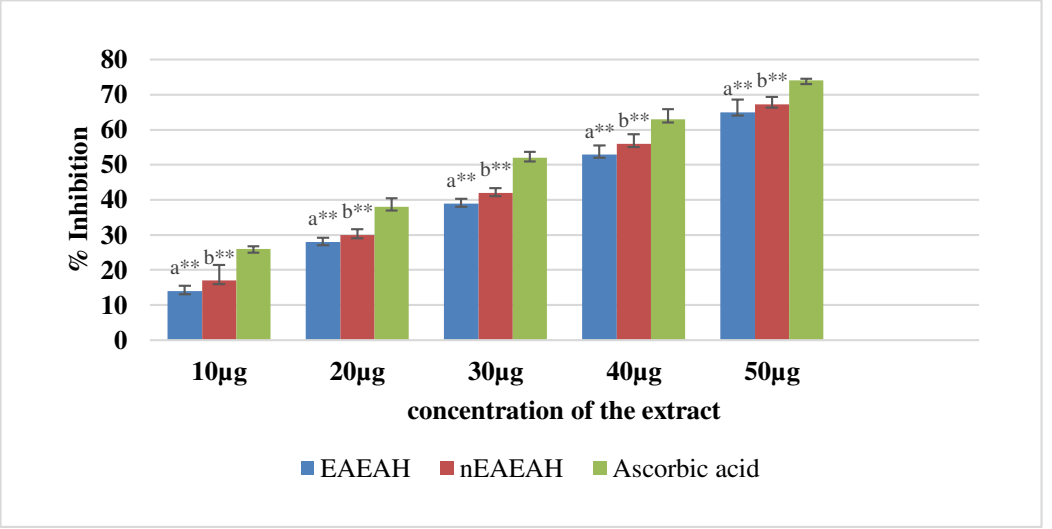


Figure -3 Hydroxyl scavenging activity of EAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum)*, nEAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum* loaded Phyto-Niosomes) and Ascorbic acid

Values are stated in mean±SD (n=3), statistical substantial test for comparison was done by ANOVA (Analysis of Variance) followed by Dunnet's 't' test. Evaluation between a –Ascorbic acid vs EAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum*) b –Ascorbic acid vs nEAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum* loaded Phyto-Niosomes) *p<0.05, **p<0.01 and ns – non-Significant.
Experiments are performed in triplicates and values mentioned are expressed in mean ±SD.

MTT (3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl- tetrazolium bromide) assay

MTT assay determines the cell viability due to the plant extract treatment. In the present investigation decrease in the cell viability is observed due to the treatment of EAEAH and nEAEAH (Table-1). The effect was found to be concentration dependent the cell viability of nEAEAH treated cells showed a deep decrease which was comparable with the doxorubicin the positive drug used in the present study. IC_{50} values were calculated and it was found to be 41.71µg/ml for nEAEAH extract.

Table-1 Cell viability of MCF-7 Cell lines after the treatment of different extracts

Concentration of extract(µg/ ml)	%Cell viability by EAEAH(EthylAcetate Extrac*t Aconitum heterophyllum*) extract	%Cell viability by nEAEAH(Ethyl Acetate Extrac*tAconitum heterophyllum* loaded Phyto-Niosomes) extract	%cell viability by Doxorubicin
10	84.21 ± 2.80a**	79.50 ± 1.55b*	79.20± 1.88
20	70.21 ± 2.00a**	69.18 ± 0.8b5*	61.93 ± 2.49
40	74.31 ± 1.85a**	64.44 ± 1.37b*	60.79 ± 1.30

80	62.26 ± 1.87a**	60.25 ± 1.32b*	44.41 ± 2.31
100	50.39 ± 2.12a**	46.51 ± 1.39b*	41.51 ± 2.40

Values are stated in mean±SD (n=3), statistical substantial test for comparison was done by ANOVA (Analysis of Variance) followed by Dunnet's 't' test. Evaluation between a –Doxorubicin vs EAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum)* b – Doxorubicin vs nEAEAH(Ethyl Acetate Extrac*t Aconitum heterophyllum* loaded Phyto-Niosomes) *p<0.05, **p<0.01 and ns – non-Significant. Experiments are performed in triplicates and values mentioned are expressed in mean ±SD.

Trypan Blue Dye Exclusion Assay

The trypan blue assay is used to find out the anti-proliferative activity of the plant extracts which measures the ratio of cell death due to the drug treatment. The ability of the EAEAH and nEAEAH extracts to induce the cell death in MCF-7 cells is analysed by trypan blue assay method and shown in Table-2. The nEAEAH extract suggestively inhibited the growth of MCF-7 cells and starting from the lower concentrations the anti-proliferative activity was quite similar to doxorubicin the positive drug with the IC_{50} concentration of 49.36µg/ml.

Table-2 Cell death of MCF-7 Cell lines after the treatment of different extracts

Concentratin of extract(µg/ ml)	%Cell death by EAEAH((EthylAcetate Extrac*t Aconitum heterophyllum*) extract	% cell death by nEAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum* loaded Phyto-Niosomes) extract	% Cell death by Doxorubicin
10	5.80 ± 0.14a**	7.70 ± 1.25b*	12.97 ± 0.58
20	12.50 ± 0.44a**	14.39 ± 1.56b*	19.13 ± 1.60
40	19.40 ± 0.69a**	21.39 ± 0.76b*	29.16 ± 0.47
80	25.07 ± 1.17a**	26.56 ± 2.21b*	30.01 ± 1.41
100	30.88 ± 3.04a**	32.66 ± 2.60b*	31.39 ± 1.81

Values are stated in mean±SD (n=3), statistical substantial test for comparison was done by ANOVA (Analysis of Variance) followed by Dunnet's 't' test. Evaluation between a –Doxorubicin vs EAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum)* b – Doxorubicin vs nEAEAH (Ethyl Acetate Extrac*t Aconitum heterophyllum* loaded Phyto-Niosomes)*p<0.05, **p<0.01 and ns – non-Significant

Experiments are performed in triplicates and values mentioned are expressed in mean ±SD.
The nEEAH extract significantly inhibited the growth of MCF-7 cells and starting from the lower concentrations the anti-proliferative activity was quite similar to doxorubicin the positive drug

GC MS Analysis

In the present investigation GC-MS was performed to find out the components responsible for the antioxidant and anti-proliferative activity. Figure4 and Table3 depicts the chromatogram and list of phytochemicals with their molecular weight and formula. Totally 31 phyto constituents were identified in the ethyl acetate root extract of *Aconitum heterophyllum*. These compounds were further subjected to *In-silico* docking studies to find suitable antagonistic ligand for the tumour progressing proteins.

Figure -4 Chromatogram of Ethyl acetate root extract of *Aconitum heterophyllum*

Table -3 Phyto-components identified in *Aconitum heterophyllum* Ethyl acetate root extract

Peak	R.time	Name of compound	Molecular Formula	Molecular weight	Area%
1	8.649	Phenol, 2,4-di-tert-butyl	$C_{14}H_{22}O$	206	0.82
2	8.889	2,4-diethyl-6-methyl-1,3,5-trioxane	$C_8H_{16}O_3$	160	0.09
3	8.992	4-Allyl-2-t-butyl-4-methyl-1,3-oxathiolan-5-one	$C_{11}H_{18}O_2S$	214	0.07
4	9.595	Z,Z-2,5Pentadecadien-1-ol	$C_{15}H_{28}O$	224	0.10
5	9.667	Isoindole-1,3(2H)-dione-4,7-ethano-3a,4,7,7a-tetrahydro-2-phenyl-	$C_{16}H_{15}NO_2$	253	0.01
6	10.655	Pyridine, 3-(phenylmethyl)-	$C_{12}H_{11}N$	169	0.04
7	12.262	Methyl ricinoleate	$C_{19}H_{36}O_3$	312	0.99
8	13.317	N-(1-Anilino-2,2,2-trichloroethyl)decanamide	$C_{18}H_{27}C_{l3}N_2O$	392	0.01
9	15.834	2,3-O-Benzal-d-mannosan	$C_{13}H_{14}O_5$	250	0.11
10	17.568	Thiazolidin-4-one, 5-ethyl-2-imino-	$C_5H_8N_2OS$	144	3.60
11	18.042	2H-Pyran,2-(2 heptadecynyoxy)tetrahydro-	$C_{22}H_{40}O_2$	336	0.84
12	18.658	3-Propylnorleucine	$C_9H_{19}NO_2$	173	0.83
13	20.230	1,2,3-Thiadiazole-4-carboxylic acid, (1-amino-2-chloroethenylideneamino) ester	$C_5H_5ClN_4O_2S$	220	0.73
14	21.291	1,5-Cyclooctadiene,1-t-butyl-	$C_{12}H_{20}$	164	1.11
15	23.667	2H-Pyran, tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-	$C_{10}H_{18}O$	154	0.03
16	24.000	2H-Pyrimido[1,2-a] pyrimidine, 1,3,4,6,7,8-hexahydro-1-methyl	$C_8H_{15}N_3$	153	0.23
17	26.714	CitronellylTiglate	$C_{15}H_{26}O_2$	238	1.14
18	28.865	4,4-Dimethyl-2-allylcyclohexanone	$C_{11}H_{18}O$	166	1.17
19	28.984	Bis(cic-13-docosenamido) methane	$C_{45}H_{86}N_2O_2$	686	3.46
20	29.179	2-(p-Methoxyphenyl) ethyl beta.-d-glucopyranoside	$C_{15}H_{22}O_7$	314	2.35
21	35.025	Azacyclononan-2-one,5,5,8,8-tetramethyl-	$C_{12}H_{23}NO$	197	0.03
22	35.218	dl-alanyl-l –leucine	$C_9H_{18}N_2O_3$	202	0.07
23	37.125	5-Bromovaleric acid, oct-3-en-2-yl ester	$C_{13}H_{23}BrO_2$	290	0.01
24	38.153	N-maleic acid 2,2,6,6-Tetramethyl,-4-piperidylmonoamide	$C_{13}H_{22}N_2O_3$	254	0.02
25	38.551	4-(3-Hydroxy-2,2,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-but-3-en-2-one	$C_{13}H_{20}O_3$	224	0.06
26	41.921	Fumaric acid,2-decyl tridecyl ester	$C_{27}H_{50}O_4$	438	0.97
27	42.483	Batilol	$C_{21}H_{44}O_3$	344	2.91
28	42.625	13-docosenamide,(z)	$C_{22}H_{43}NO$	337	1.20
29	43.238	13,14-epoxyursan-3-ol,acetate	$C_{31}H_{50}O_3$	470	5.19
30	45.131	1,2,4-Triazol-3-amine, 5-(1,3,5-trimethyl-4-pyrazolyl)amino-	$C_8H_{13}N_7$	207	46.93
31	45.486	Triaraachine	$C_{63}H_{122}O_6$	974	16.50

Depicts the chromatogram and list of phytochemicals with their molecular weight and formula

***In silico* Anticancer Activities**

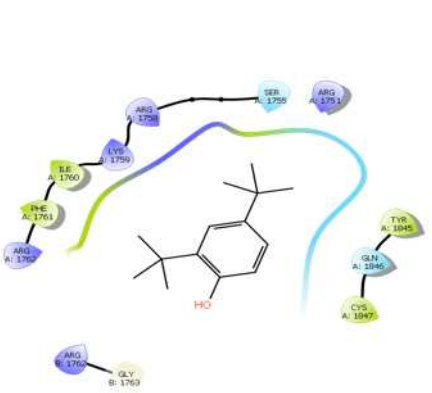
The *in-silico* docking analysis was performed by a molecular docking method using Auto Dock Vina software to find a suitable antagonistic ligand from the plant compound for the tumor progressive proteins BRCA1, BRCA2 and HER2. All the compounds identified from GC-MS analysis were docked against the proteins BRCA1, BRCA2 and HER2 and compounds showing lowest binding energy were selected as a suitable antagonistic ligand and the mode of inhibition is analysed in terms of their interaction with specific amino acids. Table 4 shows the docking score in terms of binding energies of the selected ligand and the protein. Based on the binding energies three ligands namely Phenol,2,4-di-tert-butyl, 1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl) amino and isoindole-1,3(2H)-dione-4,7-ethano-3a,4,7,7a-tetrahydro-2-phenyl showed the lowest binding energy. So, these three ligands were further investigated for their inhibition of the BRCA1 protein. The Protein BRCA1 interaction with their phyto chemical ligand in terms of amino acid binding is shown in the Table-5 and Figure 5. It is observed that phyto constituents inhibited certain amino acids like valine and tyrosine which were inhibited by the positive drug Doxorubicin. Table and Figure 6 shows the amino acid interactions observed between the protein BRCA1 protein and the phyto ligands namely which were selected based on their binding energies. Here also the same ligands**,** 1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl) amino, exhibited lowest binding energy for BRCA1 also. Additionally, 5-cyclooctadiene,1-t-butyl ,2-(p-methoxyphenyl) ethyl beta-d-glucopyronoside ligand also showed better binding. So, these three ligands were further investigated for their amino acid binding with the protein BRCA1 to show their antogonisic nature. Here only few amino acid interactions were observed unlike the interactions seen in BRCA1.
The Phytoligands such as 1,2,4-triazol-3-amine, 5- (1,3,5-trimethyl-4-pyrazolyl) amino, Phenol, 2, 4-di-tert-butyl and 2- (p-methoxyphenyl) ethyl beta –d -glucopyronoside from *Aconitun heterophyllum* exhibited antagonistic action in terms of amino acids binding with protein HER2.

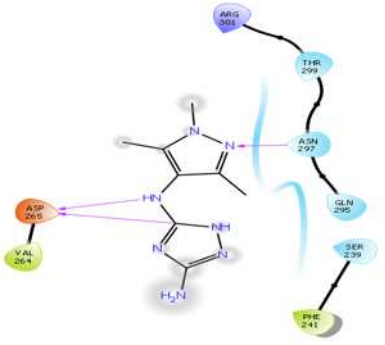
Table: 5 Amino acid Interaction with the BRCA1(Breast Cancer Gene) protein phyto ligands of EAEAH (Ethyl Acetate Extract *Aconitum heterophylum*.)

S.No	Compound	BRCA1(Breast Cancer Gene) Protein amino acid binding site	
		H bonding sites	Hydrophobic contact sites
1	Phenol,2,4-di-tert-butyl	1	5
		Val 240	Pro 238, Ser 239, Phe 241, Ile 332, Lys 334
2	1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl)amino	2	6
		Asp265, Asn 297	Ser 239, Phe 241, Gln 295 Thr 299, Arg 301, Val 264
3	isoindole-1,3(2H)-dione-4,7-ethano-3a,4,7,7a-tetrahydro-	1	9
		Cys 1847	Gln 1846,Tyr 1845,Arg 1751,Ser 1755,Arg 1762,Arg 1758,Lys 1759,Ile 1760,Arg

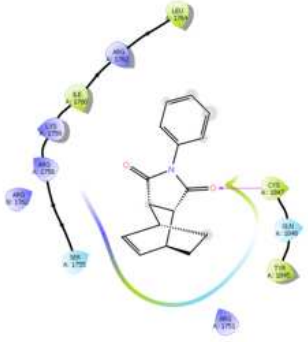
	2-phenyl		1762,Leu 1764
4	Doxorubicin	2	19
		Tyr 836 Val 851	Val850,Trp780,Pro778,Ser774,Ile800,Met772,Lys 802,Ser773,Gln859,Thr856,Ser919,His917, Asn920, Asp933, Ile848,Phe930,Glu849, Met922

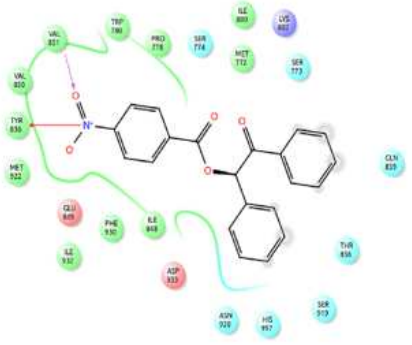
Shows the amino acid interactions observed between the protein **BRCA1(Breast Cancer Gene)** protein and the phyto ligands namely which were selected based on their binding energies.





(A)1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl) amino C) isoindole-1,3(2H)-dione-4,7- ethano3a,4,7,7a- tetrahydro-2-phenyl





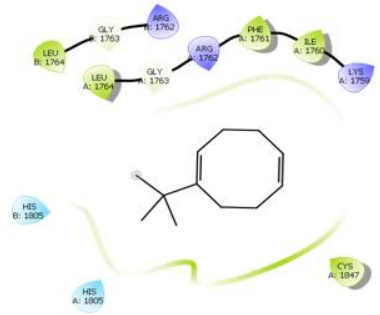
(B)Phenol,2,4-di-tert-butyl phenyl (D)Doxorubicin

Figure 5: BRCA1(Breast Cancer Gene) protein binding interactions with the phyto chemicals of *Aconitum heterophyllum*

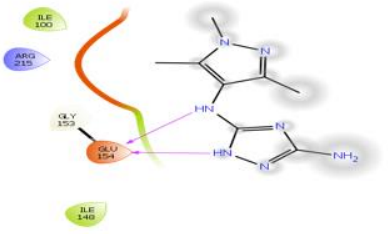
Table: 6 Amino acid Interaction with the BARC2(Breast Cancer Gene) protein phyto ligands of EAEAH (Ethyl Acetate Extract *Aconitum heterophylum)*

S.No	Compound	BARC2 (Breast Cancer Gene)Protein amino acid binding site	
		H bonding sites	Hydrophobic contact sites
1	1,5-cyclooctadiene,1-t-butyl		12
		-	Cys 1847,His 1805 ,His 1805 ,Leu 1805 Ile 1760,Phe 1761,Arg 1762,Gly 1763,Leu 1764,Leu 1764,Gly 1763,Arg 1762
2	1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl)amino	1	4
		Glu 154	Ile 100, Arg 215, Gly 153, Ile 148
3	2-(p-methoxyphenyl)ethyl beta-d-glucopyronoside	3	10
		Ser 1755,Arg 1758,Tyr 1845	Arg 1751,Ile 1760,Phe 1761,Arg 1762,Leu 1764,Gly 1763,Arg 1762,Gln 1846,Cys 1847,His 1805
4	Doxorubicin	3	15
		His47, Gly29 Phe5	His27,Cys44,Gly22,Phe98,Tyr21,Ile9,Ala 94,Ala17,His6,Ala18,Leu2,Val3,Cys28,Val30,Asp48

Shows the amino acid interactions observed between the protein BRCA 2(**Breast Cancer Gene)** protein and the phyto ligands namely which were selected based on their binding energies.



(A)1,5-cyclooctadiene,1-t-butyl amino



(B)1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl)

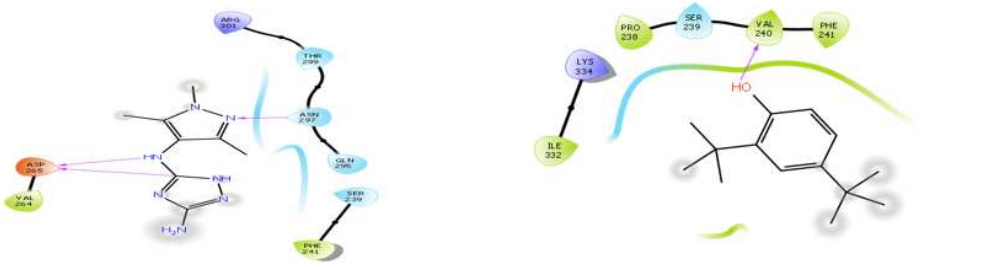
(C) 2-(p-methoxyphenyl) ethyl beta-d-glucopyronoside (D) Doxorubicin

Figure 6: BRCA2 (Breast Cancer Gene) protein binding interactions with the phyto chemicals of *Aconitum heterophyllum*

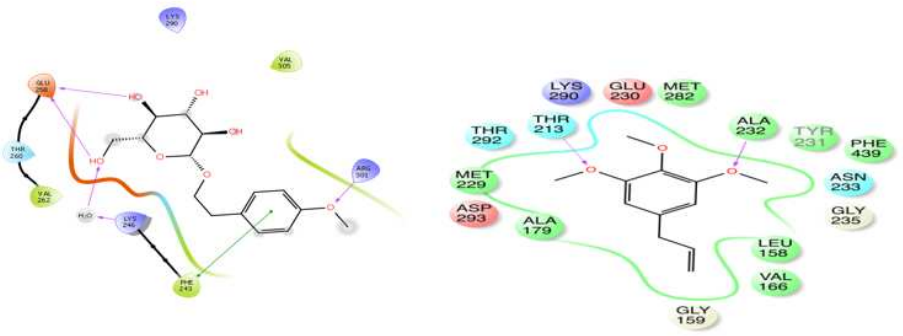
Table: 6 Amino acid Interaction with the HER2 (Human Epidermal growth factor Receptor) protein phyto ligands of EAEAH (Ethyl Acetate Extract *Aconitum heterophylum)*

S.No	Compound	BRCA2(Breast Cancer Gene) Protein amino acid binding site	
		H bonding sites	Hydrophobic contact sites
1	Phenol,2,4-di-tert-butyl	1	5
		Val 240	Pro 238, Ser 239, Phe 241, Ile 332, Lys 334
2	1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl)amino	2	6
		Asp265, Asn 297	Ser 239, Phe 241, Gln 295 Thr 299, Arg 301, Val 264
3	2-(p-methoxyphenyl)ethyl beta-d-glucopyronoside	3	5
		Glu 258,Lys 246,Arg 301	Val 305,Lys 290,Thr 260,Val 262,Phe 243
4	Doxorubicin	2	14
		Ala232,Thr213	Lys290, Glu230, Met282, Tyr231,Phe439,Asn233, Gly235, Leu158, Val166, Gly159,Ala179,Asp293, Met229,Thr292.

Shows the amino acid interactions observed between the protein **HER2(Human Epidermal growth factor Receptor))** protein and the phyto ligands namely which were selected based on their binding energies.



(A) 1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl) amino (B) Phenol,2,4-di-tert-butyl



(C) 2-(p-methoxyphenyl) ethyl beta-d-glucopyronoside (D) Doxorubicin

Figure 6: HER 2(Human Epidermal growth factor Receptor) protein binding interactions with the phyto chemicals of *Aconitum heterophyllum.*

Discussion

Living cells during metabolic reactions produces free radicals like hydroxyl, nitric oxide and super oxide radicals which are collectively called as Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) according to Rajeswary Hari et.al., 2019. These are recognized as the major cause of oxidative stress and also is responsible for uncontrolled cell proliferation and various number of disorders. Antioxidants play important role in maintaining balance between the formation of reactive oxygen species (ROS) and their removal from the system. All human cells prevent themselves from free radical by some antioxidant mechanism, sometimes these are not adequate to protect cells from the ROS damage. The plant bioactive compounds due to their antioxidant potential can inhibit the development of cancer by reducing oxidative stress caused by free radicals. Phytosomes are herbal formulations which offer a greater bioavailability when compared to ordinary plant extracts. They are obtained by adding conventional plant extracts or phytochemicals with phospholipids, generally Phosphatidylcholine (PC) to produce lipid-compatible molecular structures. Phosphatidylcholine possesses medicinal properties in addition to acting as a carrier, offering it a dual benefit when it is used in phytosome preparations [Nilesh Jain et al., 2010].

In the present investigation we have demonstrated the comparative antioxidant and anti-proliferative activity of Ethyl acetate root extract and phytosomes of *Aconitum heterophyllum.* The antioxidant potential of the root extracts to scavenge the DPPH free radical by donating hydrogen proton was indicated by stage of discolouration. According to Shyaula et al., 2012 the roots of *Aconitum heterophyllum* contains many phenolic and flavonoids which may contribute towards the DPPH scavenging activity observed in the present study. Superoxide anion radical plays a key role in the production of other ROS such as hydrogen peroxide or singlet oxygen. So, deactivation of superoxide by the plant extracts is considered as an important activity since most of the chain reactions initiated by the super oxide radicals can be halted. The role of hydroxyl radicals as mutagenic agent is well documented since it attacks the deoxy ribose sugar present in the DNA molecule leading to its breakdown leading to the carcinogenicity and cytotoxicity. Phaniendra et.al., and Sarkar et.al., 2015 2012] have done the phyto chemical analysis of the roots of *Aconitum heterophyllum* and reported the presence of carbohydrates, alkaloids, amino acids, proteins, cardiac glycosides, tannins, quinones, steroids, saponins tannins, terpenoids and flavonoids. The presence of diverse group of phytochemicals with high therapeutic nature makes the plant extract in scavenging the free radicals in an efficient manner and contributes towards its antioxidant activity. In the present study the phyto niosomes of *Aconitum heterophyllum* extract exhibited higher free radical scavenging potential than its conventional ethyl acetate extract which may attributed to their increased bioavailability in the form of niosomes.

MCF-7 breast cancer cells used as an ideal model for understanding the cancer pathway occurring in the human breast cancer and the MTT (4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay to assess the cellular metabolic activity. It is a widely used method for screening the cytotoxic activity of crude extracts, isolated compounds and synthetic drugs proposed for anticancer activity in different cell lines [Morshed et al., 2011]. Moreover, the trypan blue assay is usually performed to find out the viability of the tumor cells as a result of drug treatment. In the present investigation significant cytotoxicity was observed for the *Aconitum heterophyllum* loaded phyto niosomes in the MTT assay and trypan blue assay. Babazadeh et.al.,2016 have developed food grade nano delivery systems in the form of phyto niosomes using the phytochemicals such as rutin, anthocyanidins, catechins, and silymarin to treat several types of cancer. These lipids based nano carriers facilitate the entry of large molecular sized and poor lipid soluble phyto molecules through the biological membranes to exert its effect [Bhattaram et al., 2002]. The increased cytotoxic activity observed for the *Aconitum heterophyllum* loaded phyto niosomes may be due to the increased bio availability of its phyto constituents to the cancer cells leading to their death.

It is also important to gain a deeper understanding of breast cancer molecular processes in order to find new therapeutic targets. The mutation in the BRCA1, BRCA2, and HER-2 genes plays a significant role in breast cancer. BRCA and HER2 proteins, which play a critical role in developing breast and ovarian cancer in women, are functionally disrupted by mutations in the BRCA1 and BRCA2 genes. So, in the present investigation we made an attempt to identify a suitable antagonistic ligand which can bind with the BRCA and HER2 proteins to down

regulate the actions of these proteins. Based on the docking studies three phyto ligands 1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl) amino, Phenol,2,4-di-tert-butyl and isoindole-1,3(2H)-dione-4,7-ethano-3a,4,7,7a-tetrahydro-2-phenyl has the ability to inhibit these three tumour progressive proteins. Reshma et al., 2018 have isolated 2,4-di-tert-butylphenol a chloroform derivative from the *Humboldtia unijuga* and showed it possess anticancer activity. The presence of Phenol,2,4-di-tert-butyl functional group present in our root extract may be responsible for the anti-proliferative activity of MCF-7 cell lines in the present study. Moreover, in our docking studies 1,2,4-triazol-3-amine,5-(1,3,5-trimethyl-4-pyrazolyl) served as a best antagonistic ligand for the tumour progressive proteins BRCA1 and BRCA2 and the synthetic derivative of same compound was shown to possess anticancer activity by Brzozowski, et al., 2002. The authors Dominika Szkatuła et al., 2021 and Umit. Kocyigit et al., 2019 in their studies have reported the cyclooxygenase inhibitory as well as anticancer activity of the compound 1H-Isoindole-1,3(2H)-Dione. Among the 31 compounds identified in the GCMS analysis the presence of 1H-Isoindole-1,3(2H)-Dione compound in our plant extract.

Conclusion

It can be concluded that phyto niosomes produced from the root extract of *Aconitum heterophyllum* (nEEAH) exhibited enhanced anti-carcinogenic activity and bioavailability compared to its EEAH. Due to the encapsulation of *Aconitum heterophyllum* in the form of niosomes the release rate of the phyto constituents were slow to prolong the therapeutic activity. Moreover, bio active compounds present in *Aconitum heterophyllum* could act as an antagonistic ligand for the proteins namely BRCA1, BRCA2, and HER-2, thereby used as a potential drug for reducing the cytotoxic condition observed in Breast cancer.

Abbreviations

BRCA1 Breast Cancer gene 1, BRCA2 Breast Cancer gene 2, HER2 Human Epidermal growth factor Receptor2, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide.

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