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Pharmaceutical characterization and exploration of *Arkeshwara rasa* in MDA-MB-231 cells

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ABSTRACT

Background: The diverse specificity mode of cancer treatment targets and chemo resistance demands the necessity of drug entities which can address the devastating dynamicity of the disease.
Objectives: To check the anti-tumour potential of traditional medicine rich in polyherbal components and metal nanoparticle namely *Arkeshwara rasa (*AR).
Material methods: The AR was prepared in a modified version with reference from *Rasaratna Samuchaya* and characterized using sophisticated instrumental analysis including XRD, SEM-EDAX, TEM, TGA-DSC, and LC-MS and tested against the MDA-MB-231 cell line to screen cell viability and the cytotoxicity with MTT, SRB and the AO assay.
Results: XRD pattern shows cubic tetrahedrite structure with Sb, Cu, S peaks and trace elements like Fe, Mg, etc. The particle size of AR ranges between 20 and 30 nm. The TGA points thermal decomposition at 210 °C and the metal sulphide peaks in DSC. LC-MS analysis reveals the components of the formulation more on the flavonoid portion. The IC_{50} value of MTT and SRB are 25.28 μg/mL and 31.7 μg/mL respectively. The AO colorimeter substantiated the cell viability and the apoptosis figures of the same cell line. The AR exhibits cytotoxicity and reaffirms the apoptosis fraction with SRB assay.
Conclusions: The Hesperidine, Neohesperidin, Rutin components in the phytochemical pool can synergize the anti-tumour potential with either influencing cellular pathways or decreasing chemo resistance to conventional treatment. AR need to be further experimented with reverse transcription, flow cytometry, western blotting, etc.

1. Introduction

Cancer dominates the host mechanism with multiple accessibility demanding the management with diverse specificity and breast cancer stands as the prevalent cancer with the most DALYs (Disability Adjusted Life Years) [1]. The variants such as Triple negative breast cancer (TNBC) with less molecular targets are challenging the scientific community and are responding with minimal attributes to conventional therapy [2]. The TNBC lacks the receptors to estrogen, progesterone and HER2, representing the basal type carcinoma among the seven category. Literatures support the role of traditional medicine in the aspects of prevention, treatment and palliative purpose extending with the diversified target activity on the encroachment of the oncogenes [3]. The target accuracy [4], economical assurance [5], marked absence of side effects [6], supportive part to conventional management [7], epigenetic prophecies [8], metabolomics suggestion [9], lowered drug resistance

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[10], etc repeatedly demand the beneficial phytochemical and metal nanoparticle addition to be analyzed in the anticancer pathway.

Arkeshwara rasa, has been mentioned in treatises for different ailments like *Raktha pitta, Mandala Kushta* [11,12]*, Daruna Raktha Pitta* [13,14]*and Supthivatha.* [15] The progressive cancer pathology can be halted with anti-inflammatory compounds and such combinations like celecoxib are already in practice with conventional medicine. The selected *Arkeshwara rasa* formulation [15] includes those constituents with anti-inflammatory and anti-tumor potential and so was checked against the breast cancer cell line. It constitutes *Dwiguna Kajjali*(Mercury: Sulphur = 1:2), *Tamra bhasma* (Incinerated Copper ash) levigated with *Calotropis procera* leaf juice, *Plumbago zeylanica* root decoction and *Triphala* decoction (*Terminalia chebula, Terminalia bellerica, Emblica Officinalis*). The formed compound was subjected to in-vitro cytotoxicity assays like MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl -2H-tetrazolium bromide) and SRB (Sulforhodamine B) Assay on MDA-MB-231 cell line. The colorimetric evaluation with Ethidium Bromide/Acridine Orange Assay and the molecular quantification analysis with LC/MS were carried out along with other structural characterization techniques. The compounds such as Hesperidine, Neohesperidin, Rutin, Maleic acid, Thiabendazole, Kaempeferol, Petunidin, Vitexin, Resveratrol, and Luteolin dominated the hit score ratio among the derived molecules. The compounds are already known in their previous studies for the anti-inflammatory, Immunomodulatory and anti-tumor activities. The copper metal nano particles are known DNA cleavage agents [16] and traditionally prepared mercurial compounds are not causing any toxic ill effects when analyzed with OECD guidelines in the acute, sub-acute and the sub-chronic periodicity in many literatures. The AR was also subjected to acute and sub-acute toxicity experimentation with OECD 420 and 407 Test Guide line. The act of apoptosis by Doxorubicin can be cancelled by the activation of P13k/Akt/NfkB pathway but the presence of the suppressor of the same pathway with Hesperidin like phytochemicals, synergizes the tumor suppression [17]. Likewise the drugs like Cisplatin endures the resistance in ovarian cancers and there the stimulation to FOXO3a protein is linked with tumor suppression actions which is sourced from Quercetin like fraction [18]. The alteration to gut microbiota benefits colon cancer and the ability of Neohespiridin, the flavonoid from citrus fruits can be seen experimented in both in-vitro and in-vivo ventures [19]. So the formulation AR with the cytotoxicity, growth inhibition quotient and analytical scrutinizing will be explicitly evaluated with respect to anti-tumor potential.

2. Materials and methods

2.1. Synthesis of Arkeshwara rasa (AR)

The Mercury (CAS No:7438-87-6), Sulphur (S094112) and Copper (CAS No:7440-50-8) of analytical grade were purchased from the Advance Quality Traders, Varanasi, India. The raw materials for different procedures were procured from the local market of Varanasi, India. The dairy products were from the Institution *Goshala,* inside the campus of Banaras Hindu University, Varanasi, India. The *Plumbago zeylanica* and *Triphala* are from the Herbal drug market of Varanasi, *Gola Dinanath*, whereas *Calotropis procera* for the fresh leaf juice was available in the premises of the University. The each of the herbal inclusions were identified before the procedure at NISCAIR (National Institute of Science Communication and Information Resources, Council of Scientific and Industrial Research, Ministry of Science and Technology, Government of India) with catalog numbers as *Calotropis procera* (Ait) Ait.f. - NISCAIR/RHMD/Consult/2021/3812-13-2 *Plumbago eylanica*L.- NISCAIR/RHMD/Consult/2021/3812-13-1, *Terminalia chebula* Retz. - NISCAIR/RHMD/Consult/2021/3812-13-4, *Terminalia bellirica* (Gaertn.) Roxb.- NISCAIR/RHMD/Consult/2021/3812-13-5, *Phyllanthus emblica* L. NISCAIR/RHMD/Consult/2021/3812-13-3, respectively.

The *Tamra* was subjected to detoxification procedure *(Samanya Shodhana),* by the method of heating and quenching in different medias - Sesame oil, Butter milk, Cow's urine, *Kanji*/Fermented liquid*, Dolichos biflorus Linn.* decoction, 7 times in each liquid media [20]. It was then subjected to subsequent detoxification procedure (*Vishesha Shodhana*) by boiling in cow's urine containing rock salt [21]. The *Shodhana* (detoxification) of Mercury was done with lime powder, *Allium sativum* and the rock salt [22] For Sulphur, *bhudharputa methodology* was adopted with cow's milk and *ghrita* to remove the toxins [23]. For Incineration (*Marana*), *Shodhita Tamra* was treated with *Samaguna Kajjali* (Hg:S = 1:1) and levigated (*Bhavana)* in the juice of *Citrus lemon,* and subjected to Traditional *Puta*. The same process was repeated for 3 times. Further, for incineration*, kajjali* was replaced with *Shuddha Gandhaka* and levigated in the juice of *Citrus lemon,* [24] till the attainment of desired criteria for *Bhasma* preparation(*Bhasma pareeksha)i.e.*19 *puta*. Followed by *marana*, the process of *Amrithikarana*, was done by treating the prepared *bhasma* with *Panchamrita, (*Milk, ghee*,* curd, honey and sugar) and subjected to *Puta*, to enhance the qualities of *bhasma* [25].

For preparation of AR, the *amrithikrita Tamra bhasma* was mixed with *Dwiguna Kajjali* and levigated in the juice of *Calotropis procera* for 12 times. The same procedure was repeated with the decoction of *Chitraka* and *Triphala,* 12 times in each liquid media. The pictorial description of pharmaceutical processing is detailed in Fig. 1.

2.2. Material characterization of AR

XRD/X-ray Diffraction was carried on the prepared sample with Bruker AXS D8 Advance- Department of Chemistry, IIT, BHU (see Fig. 2). The particle size and elemental analysis with Scanning Electron Microscopy- Energy Dispersive X-ray Analysis/SEM- EDAX were carried out in the Department of Geology, with Zeiss EVO| 18 Research with EDAX-AMETEK Materials Analysis Division, B.H.U. The TEM/Transmission Electron Microscopy with Jeol/JEM 2100 were send for analysis in the SAIF, CUSAT, Cochin. Whereas, the DSC/TGA – Differential Scanning Calorimeter/Thermogravimetric Analysis were implied with the DSC-60 Plus, Shimadzu and TGA-50 Shimadzu in, Department of Chemical Engineering and Technology, B.H.U.

2.2.1. LC-MS derivation of AR formulation

Sample preparation: The powdered raw sample was dissolved in water and 50 % methanol to prepare the stock solution in a concentration of 50 mg/ml. The solution was filtered through syringe filter of 0.2 μm, 4 mm diameter and stored at 4 °C for further use. Metabolites were identified from the aqueous and methanol extracts using reverse phase**. Procedure** -UPLC-ESI-MS (UHPLC, Exion LC Sciex, USA) using a Kinetex C18, 2.1 × 50 mm column (Phenomenex, USA) with a particle size of 1.7 μm column oven kept at 40 °C, coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (4500 Q-TRAP, SCIEX, USA), in Amity Institute of Biotechnology, Gurugram. Aqueous extract preparation- Solvent A: Formic acid 0.1 % in water and Solvent B: 50 % Methanol, 50 % Acetonitrile and later added with 0.1 % Formic acid for positive ion mode; Alcoholic extract preparation- Solvent A: Formic acid 0.1 % in water add 5 mM ammonium acetate Solvent B: 50 % Methanol, 50 % Acetonitrile, further added with 0.1 % Formic acid. Lastly mixed with 5 mM ammonium acetate for negative ion mode. A linear gradient elution was performed at the flow rate of 0.250 mL (time 5–8 min); 70–95 % B for 8–23 min; 95-30 % B for 23–25 min; and 30 % B up to 30 min. The ion Spray heater was maintained at 500 C°, with ion Spray needle voltage set to 5500 V, curtain gas was set to 30 psi, and GS1 & GS2 were set to 50 psi, declustering potential (DP): 200 V and entrance potential (EP) 10 V. The sample was introduced using an auto sampler with 10 μL and 5 μL of sample injection volume for aqueous and alcoholic extracts respectively. The data were recorded in the mass range, *m/z* 100–2000 Da in ESI ± mode and processing was performed with the Analyst software, where each chromatogram was smoothened and the background was subtracted. Detected compounds were validated on the basis of molecular formula, molecular mass, retention time and *m/z*

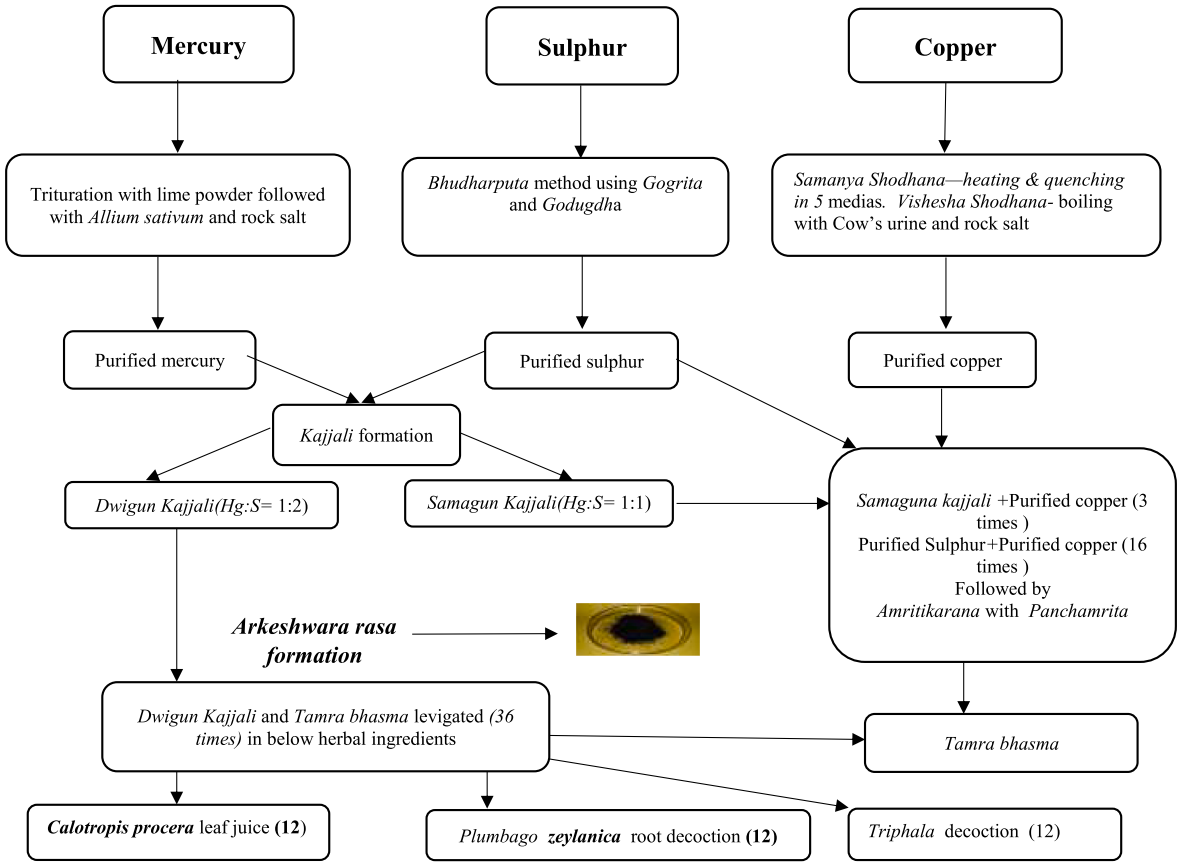


Fig. 1. Pharmaceutical processing of AR.

ratio. For the authentication of compounds, all the details were compared with published database and respective metabolite database.

2.3. MTT assay with MDA-MB-231 cell line

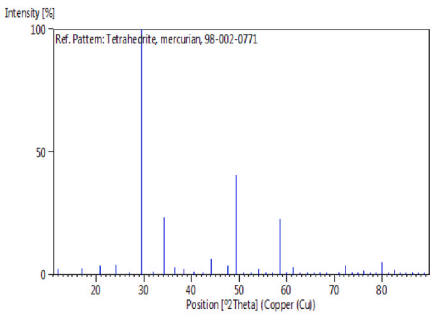
MDA-MB-231 cells were purchased from N.C.C.S, Pune, India and the experimentation was carried at PBRI, Bhopal, India. The cells were treated with DMEM-high glucose media –(Cat no: AL149, Himedia) supplemented with 10 % FBS(#RM10432, Himedia) along with the 1 % antibiotic-antimycotic solution in the atmosphere of 5 % CO_2, 18–20 % O_2 at 37 °C temperature in the CO_2 incubator. The cytotoxicity of the preparation AR was taken with 200 µl cell suspension in 96-well plate for incubation and later added up with teat agent to be incubated for 24 h at 37 °C in a 5 % CO_2 atmosphere. The MTT dye (0.5mg/mL- #4060, Himedia) on introduction (the plates were then incubated for 3 h at 37 °C in CO_2 incubator) get used up by the viable cells after the treatment transforming the yellow tetrazolium salt into purple blue formazan crystals. The formazan crystals after removing the medium were dissolved in dimethyl sulfoxide (DMSO-#PHR1309, Sigma) with incubation for 10 min and OD (Optical Density) at 540 nm measured with synergy H1 hybrid microplate reader. Accordingly, the dose–response graph was plotted between concentration of the drug and the inhibition rate. Cell viability percentage was calculated using the formula which forms the ratio between the absorbance of sample and absorbance of untreated cells × 100 [26].

2.4. SRB assay with MDA-MB-231 cell line

The cells (MDA-MB-231, NCCS, Pune) were grown in appropriate medium containing 10 % fetal bovine serum (Cat#092916854, M.P. Biochemicals) and 2 mM L-glutamine (Cat #12800-017, GIBCO) These were incubated at 37 °C, 5 % CO_2, with 5000 cells in each well of the 96 well plate. The test drug was diluted and different concentrations were prepared with further incubation for 48 h. The addition of TCA (Cat #2844 M, Fisher Scientific) in 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) for assay termination. The well washed and air dried sample is further treated with the Sulforhodamine B (SRB) (Cat# S9012 CAS- 3620, SIGMA) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid kept for 20 min at room temperature. Unbound dye recovered and residual dye washed off and treated with trizma base (Cat# T1378, SIGMA) before OD measured at 540 nm (Model# ELx808, BIOTEK instruments Inc). Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells × 100 [27].

2.5. Acridine orange assay with MDA-MB-231 cell line

The ethidium bromide/acridine orange assay is the colorimetric assay to define the apoptosis based on the reaction to ethidium bromide (50 µg/mL solution, Thermo Fischer, USA) and the acridine orange (20 µg/mL solution, Thermo Fischer, USA) on MDA-MB-231 cell procured from the NCCS, Pune, India. The cells were cultured in the 12 well plates (Biolite-Thermo) in the concentration of the 2 × 105 cells/2 ml and used the cover slips with Poly L-ornithine solution (0.01 % -#A-004-M, Sigma), followed by incubation for 24 h. The cell culture medium used was DMEM- High Glucose media-(Cat No: 2120785, Gibco). The cells were incubated for another 24 h with test and standard drug (Doxorubicin-#D1515, Sigma) and followed with D-PBS (#TL006, Himedia) wash. The well plates were again washed with D-PBS after removing the cover slips and stained with 200 µl solution. These were again washed, mounted on the fluorescence microscope and emission checked with filter cube of Excitation 560/40 nm, emission with 645/75 nm for EtBr. The excitation of 470/40 nm and emission of 525/50 nm for acridine



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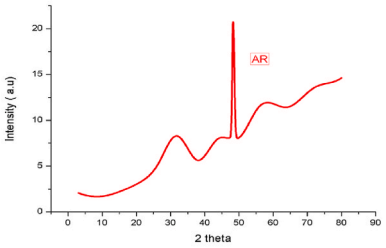

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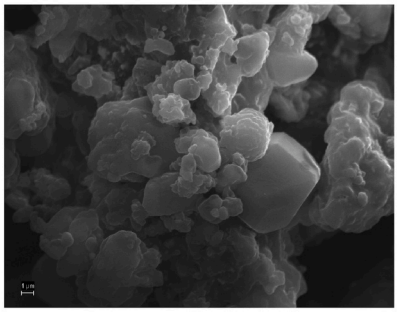

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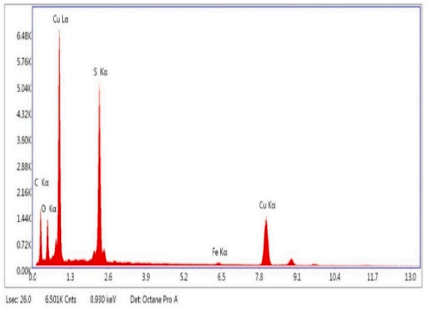

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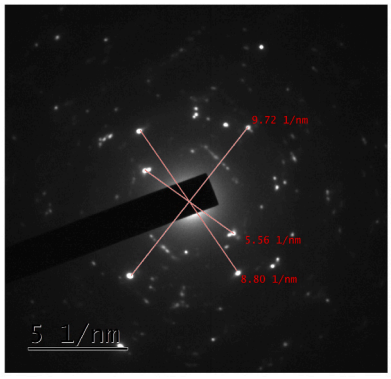

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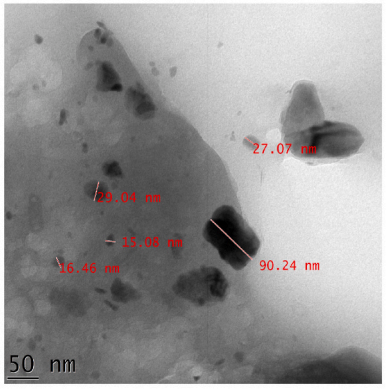

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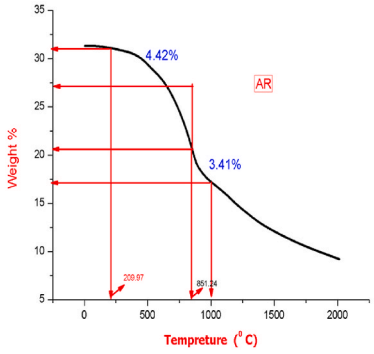

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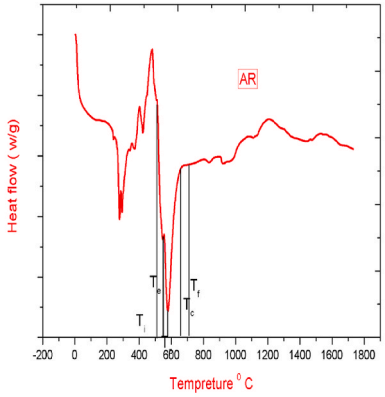


H

(caption on next page)

Fig. 2. Material Characterization of AR- Stick pattern X-ray Diffraction **and B-**X-ray Diffraction peaks**, C-**SEM image showing the particles spherical to irregular shapes indicates the tetrahedrite morphology of AR. **D-** EDAX exhibiting the peaks of Cu, Fe, S, O, C **E-**Diffraction pattern in HR-TEM **F-** TEM image showing particle range (20–30 nm). **G**-TGA of AR showing the decomposition temperature at 210 °C. **H**-DSC peaks showing the thermal decomposition of metal sulphides at 600 °C. Figure 2[112]

orange were used for evaluation results [28].

2.6. Statistical analysis

The MTT and the SRB assay with different drug concentrations were converted to mean and Standard deviation. The p value significance was checked with the unpaired T test, were p value less than .05, 95 % confidence interval using the Graph pad online statistical provision was considered as significant. The results of the LC/MS analysis are presented as mean ± standard error (SE) of at least three independent replicates. Statistical analysis was performed using Analysis of Variance (ANOVA) and the significances of the differences between sample mean were determined using duncan's multiple range test (DMRT) using SPSS software ver. 16. Statistical significance was set at a level of $p < 0.05$. Principal component analysis (PCA) was performed using Minitab ver. 18 software.

3. Results

3.1. AR preparation

The detoxified mercury (*Shodhita Parada*) and sulphur (*Shodhita Gandaka)* were mixed in a proportion of 1:2. This mixture designated as *Dwigun Kajjali* was taken in a w/w ratio of 60 gm with Incinerated copper ash (*Tamra bhasma*). The particular combination was carried over to 36 levigations with *Calotropis procera* leaf juice*, Plumbago zeylanica* root decoction and *Triphala* decoction to attain the AR formulation. The weight of AR with each herbal levigation increased in the manner 80 gm, 95 gm, and 130 gm respectively. The weight gain of extractive principles were more with *Triphala* decoction representing 26.923 % in quantity with the levigation (mentioned in the Table-1)

3.2. Characterization of AR

AR on XRD analysis exhibited cubic tetrahedrite structure. The three diffraction peaks of (222), (044) and (226) Bragg planes of the tetrahedrite structure are of Sulphur, Copper and Antimony, respectively (Fig no:2 (A)). The rod, oval, spherical, or irregular shaped particles in SEM analysis expresses the intensified peaks of sulphur along with many other trace elements like Copper, Iron, etc in the EDAX outcomes (Fig no:2 (C) & (D)). The particle size range as revealed with TEM analysis borders the 20–30 nm (Fig no:2(E) & (F)). TGA analysis expresses the decomposition from *Arkeswara rasa* occur at 210 °C (Fig no:2(G)). DSC experimental data suggest that studied complex decompose in two-step process with sulphide inclusion. (Fig no:2(H)).

3.2.1. LC/MS derived molecules and their target identification

The LC-MS analysis of the test drug AR revealed more than 100 compounds and the data with peaks in hit score were scrutinized for therapeutic target specifying any potential to tumour alleviation or synergising the existing conventional management. The hikes included different flavonoids, flavones, combined glycosides, polyphenols, etc represented in the images of Fig. 5 (in supplementary file also). These compounds are elaborately quoted with their evidenced activities in the Table-5.

Table −1
Details of different levigations in AR during the preparation.

Bhavana drug	Initial weight	Final weight (after 12 bhavna in each drug)	% gain
Arka patra swarasa bavana/Calotropis procera	60 gm	80 gm	**25 %**
Chitraka kwatha/ Plumbago zeylanica	80 gm	95 gm	**15.78 %**
Triphala Kwatha	95 gm	130 gm	**26.923 %**

3.3. Inhibition constant, cytotoxicity and colorimetric evaluation of AR with MTT, SRB and EtBr/AO assay

The MTT assay on MDA-MB-231 revealed the IC_{50} value to be 25.28 μg/ml of the AR and the SRB assay with the drug revealed the GI_{50} as 31.7 μg/ml. The AO/EtBr assay represented the colorimetric image of the apoptosis and viable cells in the IC_{50} range of 25.28 μg/ml, marked in the Fig. 4. The MTT assay with AR treatment are figured in the supplementary file-*Fig. 3,* whereas the SRB assay charted in the *Fig. 3* (images-supplementary file-*Fig. 4* and graph-figure section-*Fig. 3)* with the dose and related response points. The graphical representation of MTT and SRB assays denotes the decline with increase in drug concentration of constituted formulation.

4. Discussions

The TNBC tumours apart from lacking the three main receptors, their heterogeneous composition, and the aggressiveness of metastasis are seen to be responsive to the phytochemical targeted therapies in many clinical [29], in-vivo [30] and in-vitro [31] experiments. Arkeshwara rasa (AR), prior to the anti tumour testing was subjected to Toxicity analysis with OECD guideline 420 and 407, on Charles Foster Female Rats. The one time (acute toxicity with single dosage and the 14 days observation period-Test guideline 420) and repeated dosage pattern (sub acute toxicity with repeated dosage for 28 days-Test guideline 407) didn't create any toxicity signs in the therapeutic dose level. The higher dosage group with minor histological changes (vacuolation, glycogen deposition, of liver parenchyma) were normalised when the medication was stopped in the retrieval group kept for 14 days.

The tetrahedrite modified DNA are used as targeted precision medicine, in the conventional practise. The uptake of a compound depends specifically on the shape, size, and many other factors including the diverse cell particularity too [32].The cubic tetrahedrite AR with the hundreds of molecules as assessed from the LC-MS quantification, prominently bear the anti-tumour (related pathway actions including the anti-oxidant or anti inflammatory potential, summarized in the Table 5) effects and might be promptly able to enter the cellular uptake criteria with the peculiar shape. The formulation is also synergised with

Table-2
Cell viability and Drug concentration of AR in the MTT Assay against MDA-MB-231 cell line.

Drug concentration (μg/ml)	Cell viability (Mean ± SD)	Control	Positive Control	Negative Control
6.25	91.91 ± .005	Medium	Doxorubicin in	Medium and cells
12.5	73.966 ± .004	without	5 μg/ml	with no Test drug
25	50.243 ± .01	cells		or Standard drug
50	28.406 ± .006	achieved as 1 %		as100 %
100	5.9 ± .005			

Table-3
Results of SRB Assay with different concentrations of AR and percentage of cell viability.

Drug concentration (µg/ml)	Cell viability (Mean ± SD)	Control	Positive Control/ Doxorubicin	Negative Control
10	77.46 ± 1.46	Medium without cells	−54.5 ± 4.87	Medium and cells with no Test drug or Standard drug and cell viability as 100 %
20	70.96 ± 5.91		−55.3 ± 12.32	
40	37.2 ± 2.26		−68.8 ± 20.54	
80	−18.1 ± 6.81		−60.3 ± 4.2	

Table-4
Mean and Standard deviation of the MTT and SRB assay for AR for different concentrations.

Results	MTT Assay	SRB Assay	p Value
Mean ± SD	50.09± 34.43	41.9 ± 43.70	..7784

Confidence interval of 95 % and significance to be noted at $p < 0.05$. The p value = .7784 of Unpaired t Test and df=6.

the copper nano-medicine and the modern parameters substantiate the use of Copper oxide nano particles, which aids the apoptosis signals with the MMP (Mitochondria Membrane Potential) regulation and the ROS generation in TNBC cell line [33]. Whereas, the TIC (Tumour Initiating Cells) aided Copper nano medicine creates apoptosis in the Pancreatic cancer cell line such as PANC1 in the in-vitro and in-vivo model [34]. The presence of Sulphur peaks in the formulation can aid the caspase 3, p53, Bax activation and the Bcl2 down-regulation as with the Keratinocytes and oral cancer cells using highly purified sulphur [35]. The intense resistance developed by anticancer agents -Anthracyclin or Alkylating agents -doxorubicin, cisplatin, etc, has forced the expedition diverge to new domains. The antimony compounds also possess the antitumor activity with the inhibition of the phosphatase [36]. The Cu, Fe, etc in the trace forms along with the Sb constitute a polynuclear metal type compound that can bind cancer cell DNA at different sites for un-repairable damages. The TEM analysis confirms the nano range of the AR formulation with in the 20–30 nm and the nano particle size enhances the concentration of the drug at the tumour site. The cancer disease is also associated with the retention of lymphatic circulation which further synergizes nano-drug action with the liquid pool. Thus the convergence of Tetrahedrite structure, nano size, presence of Sulphur, Copper, and Antimony fabricates an anticancer mechanism to pave for.

The mitochondrial membrane mediated apoptosis were reverberated in the MTT, SRB and EtBr/AO assays on MDA-MB-231 cell line. These experiments provide the anti-neoplastic potential in comparison to the standard drug with cyto-toxicity potential, inhibition quotient(IC_{50}), etc. The MTT assay is considered a relevant approach to derive primary level cytotoxicity potential of a compound [37,38]. AR represented 50 % inhibition of cancer cells at the dose of 25.28 µg/ml whereas, the Doxorubicin (Positive Control) achieved the same with 5ug/ml dose. Even though, the sensitivity differences between the chemical interferences and the back ground absorbance scores can interfere with the final output of MTT assay [39], the re-confirmation with SRB assay can validate the growth inhibition quotient from a cytotoxicity profile.

The Sulforhodamine B assay sensitizes the cytotoxicity with the reaction of the anionic dye binding with the amino acid from the exposed DNA fragments after the Test drug (AR) has been introduced. The Growth inhibition ratio of 31.7 µg/ml were compared with Standard drug Doxorubicin with significant cytotoxicity as expressed in the above quoted MTT assay. Therefore the inhibition quotient of the two assays can predict the AR influence in the apoptosis pathway with the Unpaired T test. The Table 3 relates the drug concentration and the inhibition rate in the SRB assay. The inhibition quotient and the non-significant p value (denoted in the Table 4) between the drug concentrations with the cell viability data from both the assay marks the cyto-toxicity and the subsequent apoptosis existence.

The Ethidium Bromide/Acridine Orange colorimetric assay reveals the clear image to apoptosis signals by orange coloration and the cell viability with green fluorescence, respectively. The IC_{50} inhibition range derived from the MTT assay was used for the colorimetric marking. The Acridine Orange diffuses into the viable cells to stain green and early apoptotic cells have bright green spotted nucleus from the chromatin condensation. The Ethidium bromide makes the cells having disrupted membrane integrity or the necrosis stained as orange. The images of MTT and EtBr/AO are in accordance with the cell viability and apoptosis signs featured in the Fig.5 in the MDA-MB-231 cell line.

The LC-MS data were channelized according to the hit score proportion [111] and the peak levels were with the Hespiridin, Neohespiridin, and Rutin, respectively. The source to the Hesperidin compound points the citrus dominance with lemon and Indian gooseberry included as levigation agents in the preparation. This reveals the peculiarity to classical Ayurvedic method of pharmaceutical processing where lemon juice was used as the triturating media for the incineration of the copper metal part. The apoptotic action of the Hesperidin was found as initiating the ROS load and regulating the apoptotic pathway like Bax/Bcl2 and the down regulation to PI3K/Akt/GSK-3β mechanism [17]. These compounds present the less polar solubility, but the natural sources are reserved with more bioavailability than the synthetic versions [40]. This small proportion of bioavailability has seen to brought forth the Caspase reactions in many in-vivo studies.

The flavonoid neohesperidin influences the apoptotic genes p53, Bax, and the experimentation on MDA-MB-231 cell line was found with influences to the mitochondrial pathway of apoptosis [41]. Rutin, the former Vitamin P and the natural polyphenolic compound exhibited the third highest peaking in the spectrum. The chemo resistance can be prevented with regulation to Akt/PI3k/mTOR phosphorylation from the conjugated treatment with the compound [42].

Therapy to tumour environment with nano-medicine faces the challenges like consistent circulation in blood and penetrative power to targeted tumour cells to bring about the changes like microtubule damages, intercalate DNA template functions, pH differences, etc. The Maleic acid amides are known to carry over these necessities and deliver the tumour micro-environment with optimum zeta potential aiding the cellular uptake when combined technically. Maleic acid can influence the apoptotic pathways with endoplasmic stress and mitochondrial mechanisms leading to programmed cell death [43].Maleic acid was conjugated experimentally to micelles and loaded with Docetaxel to promptly deliver the breast cancer cell lines [44]. The hydrated maleic acid is the phytochemical constituent in Emblica officinalis and Terminalia bellirica.

The Thiabendazole can be linked with the provisions to anti-angiogenesis and with upregulation to MCM2 (Mini Chromosome Maintanence) in glioblastoma cells [45]. The high expression of the MCM2, MCM4-7 and MCM10 in breast cancer patients are associated with high Relapse Free Survival (RFS) explained with data expression from Oncomine, GEPIA and cBioportal analysis [46]. The Abscisic acid (ABA) is known to create glucose tolerance and aids the uptake, simultaneously with insulin secretion and GLP-1 activity to accommodate the hepatocyte activity with the rapid glycolysis in TNBC cells [47]. The presence of ABA in citrus components [48] and also secretions from granulocytes [49], monocytes [50], macrophages, adipocytes [50], pancreatic β cells [51], etc might be preserving the glucose homeostasis. The ATP deprivation that affects the selectivity of a formulation and benefitting the cancer cells can thus be culminated with these compounds with diversification of energy source to normal cells and maintain the normalcy in the localised tumour regions.

Table- 5
LC/MS derived compounds and the related anti-cancer molecular targets.

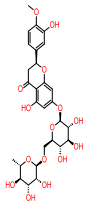
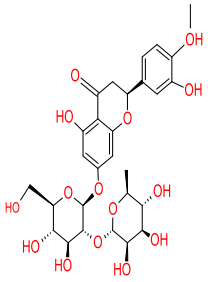
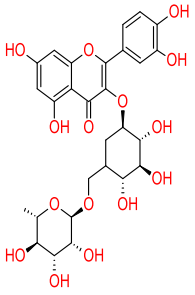
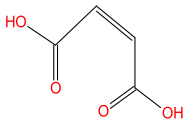
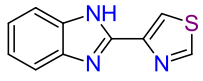
Constituent	Breast cancer cell line	Other cell line effects, in-vivo, clinical trials	Source	Probable mechanism of action	Reference
Hesperidin 	(a) MDA-MB-231 cell line (e) MCF-7 (f) In-vivo-DMBA induced	(b) Warburg effect inhibition with mRNA and proteins to GLUT1 transporter. (c) SNU-C4 Colon Ca cells (d) HeLa cells (f) In-vivo	Citrus fruits (Citrus lemon, Emblica officinalis)	(a) PD-L1 expression and the anti-metastasis via Akt and NFkB pathway. (d)Cell cycle arrest G0/G1, G2/M, Sphase with inhibition to Cyclin D kinases. (f) DMBA induced with Sprague Dawley and the Hesperidin corrected the gluconeogenesis, derangement with hepatic enzymes and maintained cell membrane integrity.	(a) [68], (b) [69], (c) [70], (d) [71], (e) [72], (f) [73]
Neohesperidin 	(a) MDA-MB-231	Osteosarcoma cell line	Citrus fruits (Citrus lemon, Emblica officinalis)	(a) Bcl2/Bax signalling pathway to apoptosis (b) ROS/JNK pathway	(a) [74], (b) [75]
Rutin 	(a) (b) MDA-MB-231 cell line	(c) A549, HT 29,Caco2	Citrus fruits (Citrus lemon, Emblica officinalis)	(a) Rutin-chitosan nano conjugates causes fragmentation of nuclear material in TNBC cells. (b) In-silico analysis revealed the ASK1 and JNK regulated signalling for apoptosis in MDA-MB-231 cell line (c) ROS mediated cytotoxicity	(a) [76](b) [77], (c) [78]
Maleic acid 	Targeted delivery	Precision medicine	Triphala	Micelle formed synergistic action for drug delivery	[79]
Thiabendazole 	Breast cancer	Glioblastoma cell	Citrus fruits (Citrus lemon, Emblica officinalis)	MCM2, MCM4-7 and MCM10 for relapse free interval and anti-angiogenesis effect	(a) [79] (b) [41]

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Table- 5 (*continued*)

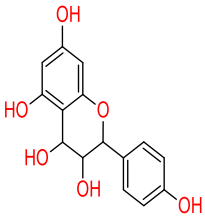
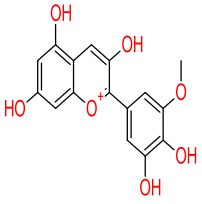
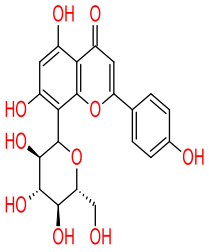
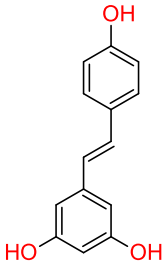
Constituent	Breast cancer cell line	Other cell line effects, in-vivo, clinical trials	Source	Probable mechanism of action	Reference
Kaempferol 	MDA-MB-231	(b) HeLa cells (c) Streptotozin induced diabetes	Triphala	(a) RhoA and Rac 1 down signalling with influence to microfilament rearrangement (b) H2AX histone phosphorylation with G2/M arrest with trigger to Caspase cascade (c) GLUT 4 receptor activation with stimulation to hexokinase activity	(a) [41], (b) [80](c) [81]
Petunidin 	MCF-7	Osteoblast and bone resorption	Calotropis procera	FasL activation and cytochrome *c* release for the processing towards the apoptosis. ROS scavenging and back up with anti-oxidant enzymes like SOD, GPX, Catalase, etc The prevention to DNA adduct formation in MCF-10 cells	(a) [82] (b) [83]
Vitexin 	MDA-MB-231	Glioblastoma cells	Citrus fruits (Citrus lemon, Emblica officinalis)	Antiproliferative and anti invasive effect	(a) [84], (b) [85]
Resveratrol 	MCF-7	GI Ca Cells	Citrus fruits (Citrus lemon, Emblica officinalis)	(a)POLD1 regulated caspase activation (b) extrinsic apoptosis pathway	(a) [86], (b) [87]

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Table- 5 (*continued*)

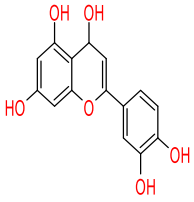
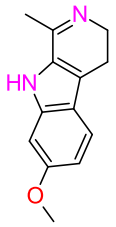
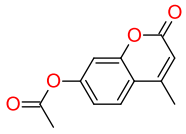
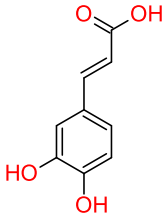
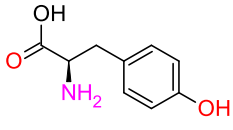
Constituent	Breast cancer cell line	Other cell line effects, in-vivo, clinical trials	Source	Probable mechanism of action	Reference
Luteolin 	Breast cancer	Liver, pancreas Ca	Terminalia chebula	(a)Notch signalling pathway and micro RNA incorporation to effect the anti-proliferative action (b)ROS mediated Pathway	(a) [88] (b) [89]
Harmaline hydrochloride dihydrate-Beta carboline 	1. IC50-30 μg/ml with MTT assay and Annexin-v-fluos staining Kit.	2. (1). Lewis Lung Cancer, Sarcoma 180 or Hep A (2). HepG2 HepAtumor in C57BL/6 mice with arm pit inoculation 2 × 10^6 (n = 8) (SC) cells/mouse and with 7.5 mg/kg of the test drug (IV) for 7 days. Sacrificed on 21st day Cytophosphane was the positive control with 30 mg/Kg dose. In-vitro- 1. Hep G2 = 0.011–0.021 μmol/ml (Harmane and its derivatives) In-vivo- 7.5 mg/Kg	Peganum Harmala, (Zygophyllaciae), Plumbago zeylanica	(a) P53, p21-highly expressed, Bcl2 decreased and the Bac, Puma, Bid, TRAIL, Caspase 8 upregulated (b) Bcl2 inhibition and the Fas upstream mechanism. No relation to P53 or Bax.	[90] (b) [91]
4 methyl umbelliferyl acetate 	Breast cancer invasion and progression enhanced with HA receptor over expression and CD44 level. -	(a) Ovarian cancer cell-Inhibition of HAS1 and HAS2 (HA synthases) -reduced the CBP chemoresistance- cell lines = OV-90, SKOV3 (b) Hep G2 cell line- DNA fragmentation (c) ES2, OV90 – P13/Akt pathway inhibition and ERK1/2, P38, JNK upregulation	Ferula communis, Triphala	(a) Inhibition of HAS1 and HAS2 (b) Cellular Apoptosis pathway (c) P13/Akt pathway inhibition and ERK1/2, P38, JNK upregulation	[92]
Ferulic acid 	Breast cancer-MDA_MB-231 cell line-regulates the Epithelial mesenchymal transition	Hela and Caski cells-Osteosarcoma, Human glioblastoma, prostate cancer	Angelica sinensis, Cimicifuga heracleifolia and Ligusticum chuangxiong. Ferula asafoetida, Plumbago zeylanica	COX 2 mediated antiproliferation, radioprotective effect, G1/S phase arrest with p53 and p21 upregulation. The down regulation of Cyclin D and E1	(a) [93] (b) [93]
L-tyrosine 	(a)L-tyrosine attached Cisplatin derivative medicine for breast cancer cell line	(b) B16F10 cells with L tyrosine loaded nanoparticles and the DC effect	Milk, Yoghurt (Panchamrutha)	(a) Erα targeted tumour action (b)Chloramine production and the cytotoxicity to cells.	(a) [94], (b) [95]

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Table- 5 (*continued*)

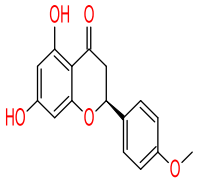
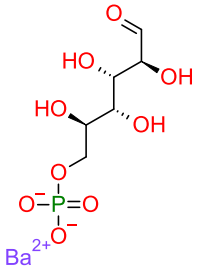
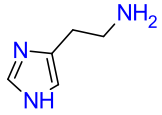
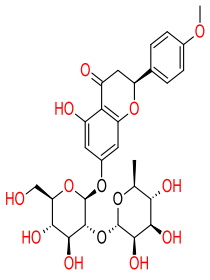
Constituent	Breast cancer cell line	Other cell line effects, in-vivo, clinical trials	Source	Probable mechanism of action	Reference
Isosakuranetin 	MDA-MB-231 cell line	Cervical cancer -HeLa cells	Ageratina altissima, Chromolaena odorata, Citrus fruits (Citrus lemon, Emblica officinalis)	(a) STAT3, IL-6 attenuation, Bax/Bcl2 pathway, Caspase 3 and 9 activation. (b) Loss of membrane integrity, decrease in MMP2/9 and reaction with RIP3, MLKL and Cyclin B.	(a) [96]. (b) [97]
D mannose 6 phosphate barium phosphate 	Breast cancer- Phase III clinical trial with oxidised Mannan-MUC1	Prostate cancer-	Panchamrutha	(a)Targeted ligands for increasing the selectivity (b) M6PR targeted theranostics	[98]
1-methyl histamine dihydrochloride 	Breast cancer	AML -Phase III trial	Vitis vinifera, Euglena gracilis, Citrus lemon, Emblica officinalis	Enhancement of cytokine IL-2, with no ROS generation. The activated T cells and Natural killer cells will thus able to surveil the immune system for the antigen cells.	[99]
Poncirin 	(a) MDA-MB-231 cell line	(b) AGS Gastric cancer cell line	Citrus foods (Citrus lemon, Emblica officinalis)	(a) c-Jun(NH) terminal Kinase and extrinsic kinases pathway, (b)FAS ligand upregulation and extrinsic apoptosis pathway.	[100]

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Table- 5 (*continued*)

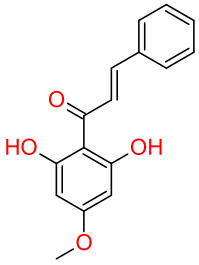
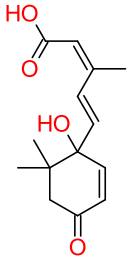
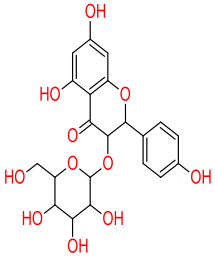
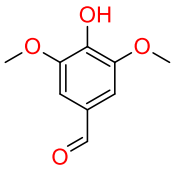
Constituent	Breast cancer cell line	Other cell line effects, in-vivo, clinical trials	Source	Probable mechanism of action	Reference
2′,6′-Dihydroxy-4-Methoxychalcone 	MCF-7	HL-60, K562, lymphocytes	Eugenia aque, Triphala	(a) Akt Pathway down regulation. (b) Structure activity relationship of the chalcones with amino group in the phenyl rings leading to the cytotoxicity	(a) [100](b) [101]
Abscisic acid 	Nil	Prostate Cancer cells- LNCaP, PC3, DUI145	Triphala	Regulation of the PPARγ and AkT down streaming with regulation to the dormancy of the cells.p27, p21, p16, upstreaming and Ki67 down streaming	[102]
Kaempferol 3 galactoside 	MCF-7 cells	LNCaP and Hepa-ICIC7	Tea, broccoli, tomato, Fruits and vegetables., Triphala	(a) P21 and Bax upregulation. Inhibition of Cuclin D1, Cyclin E, Cathepsin D. Downregulation of Akt, ERK pathway. Erα combination for suppressed action. Synergistic action with Triclosan. (b) Anti proliferative efffect with combination of flavanols and flavanones.	[103]
Syringaldehyde 	TNBC cell line	Peripheral blood mononuclear cells in Myocardial patients	Manihot esculenta and Magnolia officinalis. , Triphala	(a) [105].(b) [106]	[104]

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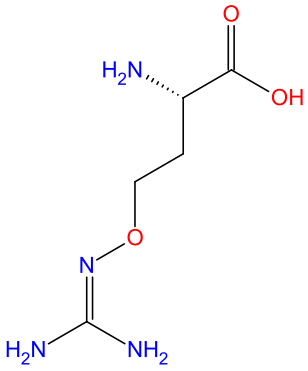
Table- 5 (*continued*)

Constituent	Breast cancer cell line	Other cell line effects, in-vivo, clinical trials	Source	Probable mechanism of action	Reference
L-Canavanine 	MCF-7	Pancreatic cancer cell - molecular docking	Sprouts, Alfalfa seeds, Triphala	(a) Arginine deprived breast cancer mangement and attenuates chemoresitance of vinblastin, Cisplatin, etc (b) Arginyl tRNA Synthetase -Substrate action	

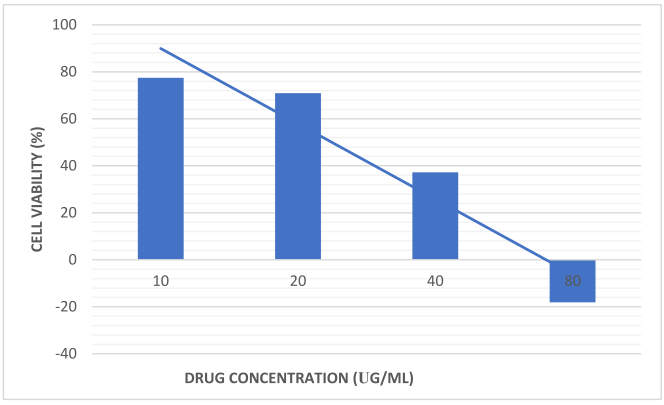


Fig. 3. SRB Assay on MDA-MB-231 cell line. Growth inhibition of the AR in concentration dependant manner on the MDA-MB-231 cells.

Kaempferol compounds are known dietary parts with varied target action to cancer cells [107]. The rutinoside [52],glucopyranoside [53], hexanoside [54] adjuvants are reported from Calotropis procera, Emblica officinalis, Terminalia bellerica respectively and the present formulation represents galactoside, glucoside, and glucuronide fractions of Kaempferol as a phyto-estrogen which are able to compete with the synthetic estrogens, DES, BPA for the Erβ site and therefore exerts the anti-proliferative effect seen in breast cancers [55]. The G2/M phase arrest seen in MDA-MB-231 cell lines when analyzed with MTS assay, DAPI staining and Western blotting expressed the cleavage to caspases 3, 9 and DNA condensation with double strand breaks, confirming the mitochondrial dependent pathway contributing to the cell cycle arrest with the compound [56]. The Rho signalling is connected with microfilament rearrangement which regulates the migration and invasion of cancer cells. The Low doses of Kaempferol proportion inhibits the evasion of TNBC cells with down-streaming RhoA and Rac 1 signalling as revealed in activation assay with the aggressive breast cancer cell line [57]. The streptozotocin induced diabetes was controlled with activation to GLUT4 receptors located at skeletal muscles with increased hexokinase activity. This activity regulates the hepatic glucose production and curtails the glucose availability to uncontrolled proliferation of cancer cells [58].The bioavailability to kaempferol is enhanced with Quercetin presence and the combination therapy with cisplatin can increase the anti-oxidant enzymes and control the pro-inflammatory factors like TNF-α, Interleukins ruling out the nephrotoxicity [59].

The Anthocyanins are potent anti-oxidants with the hydroxyl groups as the known scavengers for the ROS and the de-glycosylation to the core molecular skeleton producing the anthocyanidin like Petunidin, Malvidin, Delphinin, Cyanidin, etc. [60] These flavonoids assist the plants to thrive different abiotic stress such as drought responded changes with increased levels to Petunidin-3- glycoside like anthocyanin in Calotropis procera samples [61]. The adduct formation with DNA were prevented in the benzopyrene induced carcinogenesis in MCF-10 epithelial cells

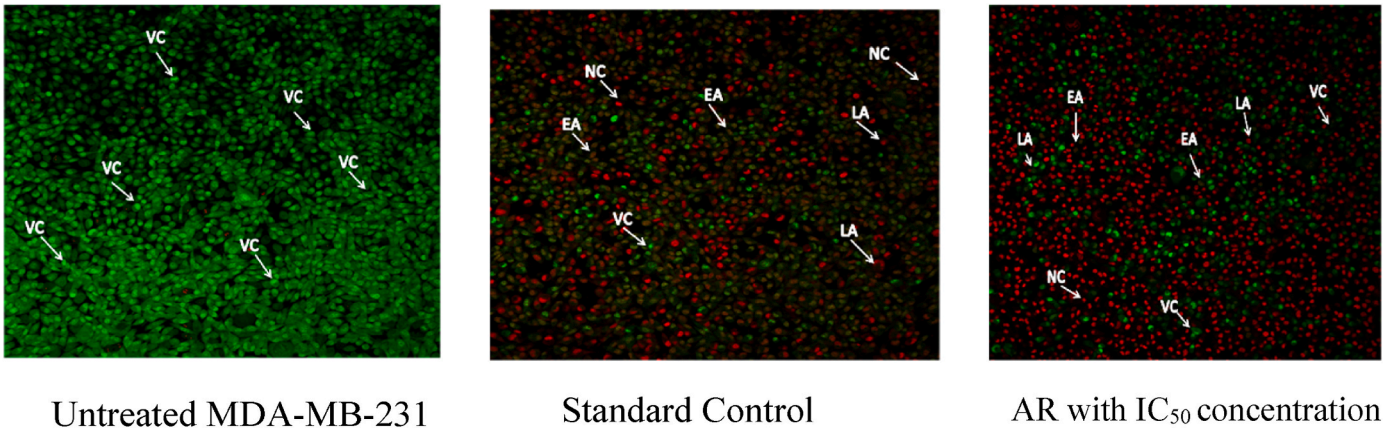


Fig. 4. Acridine Orange/Ethidium Bromide (AO/EtBr) Colorimetric images of AR treated MDA-MB-231 cell lines. The compared images of AR and Doxorubicin treated images are the 2nd and 3rd respectively.

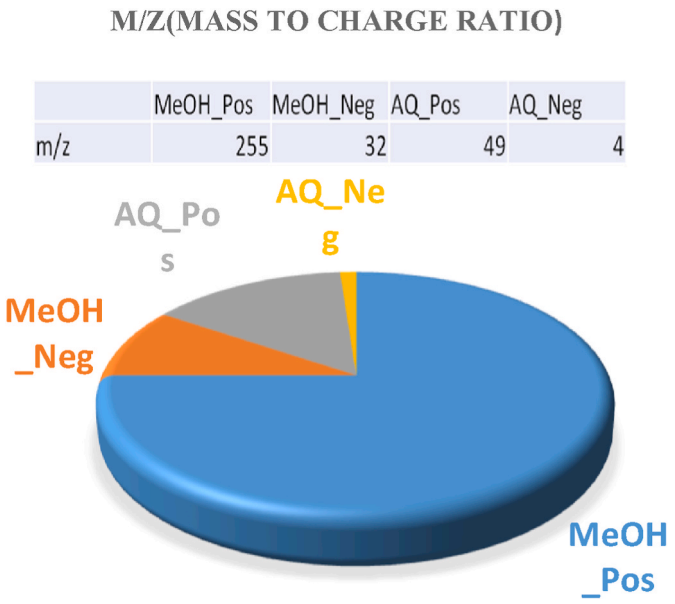


Fig. 5. LC/MS analysis of AR exhibiting the distribution of molecules.

when experimented with anthocyanidin [62]. The de-glycosylated forms are more potent compared to the conjugated sugar proportion anthocyanin from the lipophilicity and membrane solubility when introduced in many in-vivo animal experiments [63]. The apoptosis induction with these entities are dependent on both extrinsic and intrinsic pathway with FAS ligand and the cytochrome *c* released changes [64].

The Quercetin reaction with TNBC cells via multiple pathways includes the apoptosis induction, G2/M phase arrest and the propagative hindrance to proliferation, invasion, metastasis with p53 mediated factors such as GADD45 (DNA damage inducible protein) and p21 with restriction to the cyclin dependent kinase functions. The Foxo3a increment suggests the JNK pathway enclosing the cell death and uncompleted cell cycle phases. The pro-apoptotic FasL mRNA expression is linked with the Foxo3a transcriptional destination and the silencing of the factor [65]. The Quercetin presence can aid the upstreaming of the factor to bring about the apoptosis changes.

Vitexin, Vitexin-2-o-rhamnoside are the flavone glycoside from Calotropis procera [66],and Terminalia bellerica [54]. The vitexin compounds from natural products can assist the Natural Killer cells detect the oncogenic malformations with the exhibit from cell surfaces like MICA, MICB,etc. The significance to low cost immunotherapy principle can be developed from this type of compound exposure. The acetylated vitexin treated vigorous cells didn't disclose the miR20a which will suppress the surface indication of deformed members and thus present the cells for immune surveillance [67].

The Resveratrol was causing the anti-metastatic property by levelling down the TGF-β1 and the anti-tumour activity was from the DNA polymerase subunit gene -POLD1. The POLDI can regulate the expression of the suppressor protein p53, trigger the Caspase 3, cleaves the Bcl-2 and lead to apoptosis signal [68].

The Luteolin fraction exists in Terminalia chebula [69]and the flavonoid is found to influence the Notch signalling pathway in breast cancer cells. The effect is seen with in-corporation to micro RNAs such as MiRNA-34a, MiRNA-181a, MiRNA-139-5p, MiRNA-24, MiRNA-246 and their increased levels. The decreased expression with Notch-1, Hes-1, VEGF and MMPs were involved to cease the proliferation and effect the programmed cell death [70]. The heat stability of the Luteolin like compounds can influence the p53 suppressor action and the cell proliferation rate with regulation to PI3K/Akt pathway also [71]. The NFkB regulation to apoptosis, angiogenesis and inflammatory maintenance with expression to IL6 components are also well addressed by the luteolin compound.

5. Conclusion

The cytotoxicity of AR against MDA-MB-231 cell line represents anticancer potential of this formulation comprised of phytochemicals and polynuclear metal targets. It is argued that the particulate tetrahedrite morphology and nano size of the constituents could synergize the sequence of biochemical changes associated with a number of cellular events like, anti-oxidant, anti inflammatory, anti-metastasis, anti-invasive, anti-angiogenesis effects, could account for induction of apoptosis in the affected cell line. The relapse free survival with glycolysis regulation and chemo resistance surpassing could be the added advantages seen with the phytochemicals constituents of the AR adding to QoL. The detailed probing with in-vivo examinations and in-vitro applications to different cell lines are expected to be covered in future by including in-silico docking studies as the starting tool for predicting a constituent associated anticancer roles.

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Author contributions

R.Jayakumar: Conceptualization, Manuscript original drafting, Investigation, Formal analysis, Revision and Editing. **N. Joshi-** Methodology, Study Design, Conceptual Validation, Visualization,Supervision. **M. Dash:** Study Design, Conceptualization, Analytical validation, Methodology, Editing, Software. **S.K Trigun:** Editing, Review, Methodology, Visualization. **S.Gulati**: Revision, Manuscript validation. **P. Kumar**: Data curation, Revision, Editing. **S. Sharma**: Data curation, Editing. **A.Pandey**: Data curation, Methodological analysis. **F. Zeeshan & Cholke**: LC-MS analysis.

Declaration of competing interest

None.

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Appendix A. Supplementary data

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