Evaluation of the Role of Merromoside from *Ipomoea aquatica* Forsskal Hydroalcoholic Extract in the Downregulation of ROS Species in Overcoming MDR in Breast Cancer

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Abstract

**Purpose:** The medicinal plant *Ipomoea aquatica* belonging to *convulvulaceae* family is an effective natural herb for treatment of various ailments and possesses effective anticancer activity. The aim of the work is to characterize a secondary metabolite merromoside (a resin glycoside) for anti-breast cancer activity through down regulation of ROS species.

**Methods:** The Extract of the whole plant has been prepared by maceration method using 50%v/v ethanol in distilled water to get a hydroalcoholic extract. The phytochemical evaluation reveals that the active secondary metabolite was isolated by using column chromatographic technique. The isolated compound was evaluated for its anticancer properties through invitro method such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay on Michigan Cancer Foundation-7 Cell lines. The purity and structural characterization were done by high-performance thin layer chromatography, Fourier Transform Infrared Spectroscopy, Proton and $^{13}$C Nuclear Magnetic Resonance spectroscopy and Liquid Chromatography–Mass Spectrometry.

**Results:** The isolated compound (W04) from the derived extract showed Rf value of 0.79 that showed IC$_{50}$ of 182.8μg/ml. The chemical structure of W04 has been confirmed as 

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\text{[4,5-dihydroxy-6-[5-hydroxy-2-methyl-4-(2-methylpropanoyloxy)-6-[(24,25,26-trihydroxy-5,23-dimethyl-9-oxo-19-pentyl-2,4,8,20,22-pentaoxatricyclo[19.2.2.13,7]hexacosan-6-yl]oxy]oxan-3-yl]oxy-2-methylpropanoate with the molecular weight of 979.15268.}
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The isolated compound merromoside from hydroalcoholic extract of *Ipomoea aquatica* has been evaluated for anti-breast cancer properties. The down regulation of ROS species will prevent reverse signalling and angiogenesis. This indicates that merromoside will overcome MDR in breast cancer especially DOX-resistant.

**Keywords:** Merromoside- resin glycoside- breast cancer- MCF-7 cell lines

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Introduction

Plants are rich sources of medicinal compounds that provide preventive and curative therapy than synthetic drugs. Hence, there is an increased focus towards assembling and contributing herbal remedy for meeting the global exigency through drug development and research (Apu et al., 2012). The International Agency for Research on Cancer (IARC) estimates that globally, 1 in 5 people develop cancer during their lifetime, and 1 in 8 men and 1 in 11 women die from the disease. Breast cancer represents 1 in 4 cancers diagnosed among women globally. Colorectal, lung, cervical, and thyroid cancers are also common among women (Mantha Saxena et al., 2013; Marvibaigi et al., 2016). As per the report on the plant, *Convulvulaceae* plants would have resin glycosides like aquaterins, convolvulins and jalapins (Ono, 2017). Aquaterins (I to XIX) present in the *I. aquatica* plant had evaluated for aquaterin II induced G0/G1 arrest regulated by related proteins CDK4/6, cyclin D/E and p21 used mitochondria-mediated apoptosis featured by MMP decrease, ROS accumulation, caspase cascade activation and Bax/Bcl-2 alteration (Bo-Yi Fan et al., 2015). Reversal of multidrug resistance (MDR) by thirty resin glycosides from the morning glory family (*Convulvulaceae*) was evaluated in vinblastine-resistant human breast carcinoma cells (MCF-7/Vin) (Figueroa-González et al., 2012). The mechanism of resin glycosides in improving the MDR reversal activity was revealed through a structure–activity relationship study that substituting Rha’ C-3 with a trans-cinnamoyl group (Li et al., 2017). *I. aquatica* was screened for its activity against fibroblast cell lysis after *Heterometrus laoticus* scorpion venom treatment but the result was clearly negative. The purified compound from the methanolic extract of *I. aquatica* showed cytotoxicity towards cell cultures with CTC50 values of 387 mg/ml against normal Vero cell line, (normal African green monkey kidney) and HEP-2 (human larynx epithelial carcinoma) cell and A-549 (human small cell lung carcinoma). The crude extract was
more potent than that of the purified compound probably due to the combination of anthocyanin and other phenolic compounds. Isolation of a free radical-scavenging antioxidant from water spinach (*Ipomoea aquatica* Forsk) was reported (Parimala K et al., 2013). Anticancer activity of *Ipomoea aquatica*, a common leafy vegetable in prostate cancer and reduction of cisplatin-induced hepatotoxicity in vivo were reported. From the above literature information, it is clear that *I. aquatica* plant extract has potential as anti-tumor active while its effect has not been explored with breast cancer cell lines. Resin glycosides, as members of glycolipids, have shown anticancer activity. The activities include mammalian cytotoxicity of tricolorin, antimetastatic activity of caicicoside, HepG2 inhibition by aquaterin-II (Chen et al., 2017; Fan BY et al., 2015; Pereda-Miranda et al., 2010; Renecruesi et al., 2004). Dichordrins, resin glycosides isolated from *Dichondra repens*, have shown to induce MDR and increase the cytotoxicity of vincristine (Song et al., 2015). In radiotherapy, blockage of HIF1 response has been shown to minimise lactate levels that worked as an aid to suppress anaerobic metabolism resulting in improved efficacy (Reuter et al., 2010). The role of resin glycosides in inhibiting P-gp has been reported by the use of resin glycosides from *Merremiatamnmosa* (Lour.) Hallier. F (Convolvulaceae) (Kim HR et al., 2004; Kitagawa I et al., 1997; Kokate CK et al., 2015; Lande et al., 2015). Efflux pump inhibitory ability of resin glycosides towards combating drug resistance in combinatorial therapy has also been reported that indicate the potential of the glycolipids in cancer treatment (Corona-Castañeda et al., 2016). Hence, the research was focused on exploring the anti-breast cancer potential of isolated compound from the opted plant extract for anti-breast cancer activity *Ipomoea aquatica* (Hu et al., 2014).

**Materials and Methods**

**Materials**

**Plant Sample**

The common name of the plant is Water Spinach, River Spinach. The botanical name is identified and authenticated as *Ipomoea aquatica* Forssk från belonging to the family Convolvulaceae. The plant was collected from the location Parambikulam – Aliyar Riverine, Pollachi. The part of the plant used for the study was whole plant. The plant was authenticated at BSI, Coimbatore-641003, Tamil Nadu, India, with the authentication number of BSI/SRC/5/23/2017/Tech./3269.

**Chemicals/Reagents used**

The chemicals and reagents used for the study were Petroleum Benzene boiling range 60.0°C-80.0°C GR, (Merck Specialities Private Limited, Mumbai – 400 018), Pyridine GR, Trichloro acetic acid, Sodium nitroprusside dehydrate purified, Silica gel G 60 – 120 mesh for column chromatography, o-toluidine, (Nice chemicals, Kochi – 682 024, Barfoed’s reagent, (Oxford Laboratory, Mumbai – 400 002), Ethanol AR 99.9%, (Jiangsu Huaxi International Trade Co., Ltd.China), Zinc (metal) Powder, Laboratory Rasayan, Gelatin, Extra pure, (Hi Media Laboratories Private Limited, Mumbai – 400 086), Potassium dichromate, Qualigens Fine Chemicals, Mumbai – 400 030. Sulphur powder, Distilled water, Selwynoff’s reagent LR, (Reachem Laboratory chemicals Private Limited, Chennai – 600 098), Molsch’s reagent LR, 1-naphthol LR, Dimethyl sulfoxide LR, Toluene (Sulphur free), Formic acid LR, Phenyl hydrazine hydrochloride LR, Sodium oxalate LR, Dimethyl formamide LR, Ammonia solution LR, Benedict’s quantitative reagent LR, Barium chloride LR, Fehling Solution A LR, (Chemsure, Chennai – 600 098), Acetic acid Glacial LR, Acetone LR, Biuret reagent, (LobaChemie Private Limited, Mumbai – 400 005), Mayer’s solution, Fehling Solution No.2, Bial’s reagent, Picric acid, Ferric chloride, Ammonium oxalate LR, Methanol LR, (S d Fine Chemicals Limited, Mumbai – 400 030), Potassium iodide, Oxalic acid, Sodium hydroxide pellets purified, Silica gel G for TGL, (LobaChemie Private Limited, Mumbai – 400 005), Potassium bromide for IR, MTT Powder, DMSO, Cell lines, ATCC, DMEM/F12, (Invitrogen), Fetal Bovine Serum, Penicillin,Streptomycin, Trypsin, EDTA, Glucose in PBS and DMBA, (Sigma Chemicals Co. St. Louis, MO, USA).

**Instruments used**

The analytical instruments utilized for the research were Precision Balance (Wensar), Hot plate (Cinetix), Ultra Sonicator (Labman), Electrical Water bath (Technico), UV cabinet (CAMAG and Deep Vision), FTIR (Shimadzu), LCMS (PE Sciex API3000), FT-NMR (BRUKER AV400), Double beam UV/Visible spectrophotometer (Shimadzu UV 1800), CO, incubator (Thermo Fischer Scientific) and Microplate reader (Tecan).

**Methods**

**Collection and Extraction**

**Maceration – a conventional extraction**

In this process, the whole or coarsely powdered crude drug was placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then was strained, the marc (the damp solid material) was pressed, and the combined liquids were clarified by filtration or decantation after standing (Apu et al., 2012).

**Preliminary Phytochemical Screening**

Phytochemical process of the hydroalcoholic extract was carried out to determine the presence of secondary metabolite such as flavonoids, tannins, terpenoids, steroids, alkaloids, saponins and reducing sugars using standard phytochemical methods. Medicinal properties of the plants are determined by their phytochemical constituents (JB, 1973; Khadabadi SS et al., 2013; Shaikh., 2020).

**Isolation and purification of bioactive compounds from plant samples**

Completely dried plant extract sample should be mixed with silica gel to make a fine powdered form for easy distribution of sample in already packed silica gel.
column. Sample powdered mass should be placed on the top of the pre-packed silica column and sample should be covered with a layer of cotton. The solvents of different polarities were passed through column at uniform rate under gravity to fractionate the sample extract (Bajpai et al., 2016; Corona-Castañeda et al., 2016; JB, 1973; Lande et al., 2015). Each fraction was collected separately in a test tube and numbered consecutively for further analysis on thin layer chromatography. The visualized spots of the components in the chromatogram are marked and the Rf value of each spot is calculated by the formula: $R_f = \frac{d}{D}$, where $d$ is the distance travelled by the sample (cm) and $D$ is the distance travelled by the solvent (cm) (Kokate CK et al., 2015). Based on the nature of the compounds, further spectral analyses such as infrared (IR), mass spectrometry and nuclear magnetic resonance (NMR) can be performed to elucidate the chemical structure of target compounds (L et al., 2012).

**Anticancer Evaluation**

**Determination of effect of plant extract on cell proliferation by MTT assay**

**Cell culture and drug treatment**

Human breast carcinoma cell lines MCF-7 and MDA-MB-231 were obtained from ATCC through NCCS, Pune, India. The cells were cultured and maintained in Modified Eagle’s Medium (MEM) and Dulbecco’s Modified Eagle’s Medium (DMEM), respectively, supplemented with 10% Fetal Bovine Serum (FBS) and 100 IU/mL Penicillin and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO$_2$ at 37°C. Stock solutions of AZA (Sigma), TSA (Sigma), and SFN (Sigma) were prepared in dimethyl-sulphoxide (DMSO) whereas SAM (Sigma) was dissolved in milli-Q water. Stock solutions were further diluted to working concentrations in DMEM prior to use. Cells were harvested by trypsinization and cell number was counted by hemocytometer. The number of living cells was calculated by Trypan blue staining (0.2% v/v). For determining the concentration of drug that inhibited cell proliferation by 50% ($IC_{50}$), 5 × 10$^4$ cells per well were seeded in a 96-well micro-titer plates and after 24 h incubation, were treated with the epigenetic modulators at different concentrations (AZA, SAM, SFN (1, 5, 10, 15, 20, 50μM) and TSA (10, 20, 50, 100, 250, 500 nM)) mixed in respective medias supplemented with 5% FBS. Control cells were treated with DMSO only (Grigalius et al., 2017; JB, 1973; Juan-García et al., 2015; Raj et al., 2014).

**Cell viability analysis by colometric MTT assay**

The effect of the epigenetic modulators, DNMT and HDAC inhibitors on cellular proliferation was assessed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-DiphenyltetrazoliumBromide (MTT) assay, using standard protocol. Briefly, the drug-treated cells in each of the 96 wells were washed twice with PBS. 0.8 mg/mL MTT solution was prepared from stock MTT solution (5 mg/mL PBS, pH 7.2). A total of 100μL MTT solution was added to each well and incubated at 37°C for 4 h in dark. The supernatant was re-moved and 100 μL of DMSO was added into each well to dissolve the formazan crystals. The absorbance was measured at 570 nm and results were expressed as the mean of three replicates as a percentage of control (taken as 100%). The extent of cytotoxicity was defined as the relative reduction of the optical density (OD), which correlated to the amount of viable cells in relation to cell control (100%). The cell viability was plotted in a graph and the $IC_{50}$ was calculated accordingly to decide the optimum dosage of the drugs for further studies (Liaudanskas et al., 2021; Vijayarathna et al., 2012).

**Statistical analysis**

All data are presented as means ± SD. Statistical analysis was performed using the Student’s t-test by SPSS software. Values of $P<0.05$ were considered as significant value.

**Structural characterization**

**FTIR (KBrPressed Pellet Technique)**

Sample is mixed with potassium bromide (0.1%w/v) and compressed into a thin transparent pellet using a hydraulic press at 3,000 psi or 200 bar pressure. The scanned spectra are meant for interpretation (Tossaton Charoonratana et al., 2014; YR, 2013).

**Nuclear Magnetic Resonance Spectroscopic Analysis**

D$_2$O was used as solvent for NMR analysis using both $^1$H and $^13$C (Tossaton Charoonratana et al., 2014).

**Spectroscopic Parameters**

**Mass Spectroscopic Analysis**

Liquid chromatography and mass spectroscopic analysis using electron spray ionization method was sued and the spectra for two compounds were evaluated for the various ion peaks to get complete information about chemical structure (Song et al., 2015; Willard et al., 1986).

**Results**

The cold maceration was conducted with hydroalcoholic menstruum for 7 days taking 350.0g of drug and 2000.0ml of menstruum. The percentage yield of hydroalcoholic extract was found to be 28.91 % w/w.

From the phytochemical tests, it was found that the primary metabolites such as carbohydrates, proteins & amino acids and the secondary metabolites like alkaloids, glycosides, flavonoids, tannins & triterpenoids were present in the prepared extract.

FTIR experiments for W04 as O-H Alcohols (3545

### Table 1. HPTLC Analysis of Isolated Compound W04

| Sample | Concentration | At 254 nm | At 366 nm |
|--------|---------------|-----------|-----------|
|        | Rf value      | Peak Area | Rf value  | Peak Area  |
| W04    | 1.0 μg/ spot  | 0.79      | 2305.6    | 0.79     | 2647.4    |

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& 3473 cm\(^{-1}\)); C-H Alkanes (2936 cm\(^{-1}\)); C=C Alkenes (1646 cm\(^{-1}\)); C=O Aromatics (1610 cm\(^{-1}\)) and C-C, C-O Primary/Secondary Alcohols (1070 cm\(^{-1}\)). FT – NMR spectral studies for proton NMR spectra of compounds

Figure 1. FTIR, 1H NMR, 13C NMR and LC/MS Spectral Data of Isolated Compound W04 Separately

Figure 2. Possible Predictable Structure for W04
were concluding the expected set of protons with active functional groups. The 1H spectrum resulted for W04 existed with 25-CH3 (δ-0.927), 5-CH3-sugar (δ-1.426), 11-CH2 (δ-2.224), 5-H-sugar (δ-3.852), 4-H-sugar (δ-4.509), 3-H-sugar (δ-5.999), 6-OH (δ-2.522 & 3.560), 9-H (δ-2.653) and 7-H (δ-2.675). FT – NMR spectral studies for 13C NMR spectra of compounds were finalizing the predictable set of carbons with active functional groups. The spectrum gives rise to following groups for W04, C-6 C=O (δ-153.75), C-8 C=O (δ-143.49), C-1 (δ-132.95 & 132.74), C-4-sugar (δ-78.97), C-5-sugar (δ-65.25), C-7 C=O (δ-40.13), C-11 (δ-39.71 & 39.72), C-23 (δ-39.50, 39.29, 39.09 & 38.87), C-12 (δ-30.42) and C-24 (δ-25.02 &22.43). LC/MS spectral data revealed that the compound found with the molecular weight of 979.1527 and the molecular formula of C48H82O20. The spectra are collected in Figure 1.

Finally, all the spectral data including FTIR, 1H NMR, 13C NMR and LCMS give rise to the prediction of possible chemical structure through the advanced instrumental software for compound (W04) as merremoside with IUPAC name of [4,5-dihydroxy-6-[5-hydroxy-2-methyl-4-(2-methylpropanoyloxy)-6-[(24,25,26-trihydroxy-5,23-dimethyl-9-oxo-19-pentyl-2,4,8,20,22-pentaoxatricyclo[19.2.2.13,7] hexacosan-6-yl]oxy]oxan-3-yl]oxy-2-methyloxan-3-yl] 2-methyl propanoate (Figure 2). HPTLC chromatogram of isolated compounds Rf value showed the single peak at specific values, compound 1 at 0.37 and compound 2 at 0.79. The results are shown in the Figure 3 and Table 1 respectively.

EIA and its isolated compounds were evaluated for their in vitro anticancer activity using MCF – 7 cell lines by colorimetric assay which showed noteworthy inhibitory action on the proliferating cells.

The analysis of half maximal inhibitory concentration (IC<sub>50</sub>) of all samples concluded the action of W04 was significant with IC<sub>50</sub> value of 182.8 µg/ml when it was compared with the extract 220.9 µg/ml, individually, as shown in the Table 2.

### Discussion

Role of natural compounds in cancer treatment has been attributed to increasing concentration of drugs that resulted in suppressing cancer cell viability, and migration in addition to causing increased cell death (Nanayakkara et al., 2018). Biochanin A and other natural compounds have exerted MDR reversal through P-gp inhibition (Chung et
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Presence of ortho-dihydroxy function in flavone C ring has been suggested to be important for antioxidant and anticancer activity (Raj et al., 2014). Resin glycosides with acyl sugar coupled with amphiphilic nature have been reported to show P-gp inhibition leading to overcoming MDR in cancer (Lira-Ricárdez J et al., 2020; Rybalkina et al., 2021; Syed et al., 2016).

The present results of higher antioxidant activity leading to anticancer activity against breast cancer cell lines are in line with reports of plant phenolics in herbal medicine with strong antioxidant activity (Liaudanskas et al., 2021). Oxidative stress in causing changes in metabolic processes that trigger cancer cell proliferation and the role of quenching of free radical species has been found to be critical to control cancer (Pandey et al., 2009). Higher phenolic content from Scurraferruginea (Jack) Danser had shown to enhanced apoptosis in breast cancer cell lines, due to high level of radical quenching ability (Iriti et al., 2017; Marvibaigi et al., 2016; Rybalkina et al., 2021; Syed et al., 2016; Veena Sharma et al., 2017). In view of the above, the anticancer activity of fraction W04 from Ipomoea aquatica, could be correlated with high phenolic content through its antioxidant activity.

In conclusion, the isolated resin glycoside (merromoside) from the Ipomoea aquatica has been evaluated for its anticancer activity against human breast cancer cell lines using MCF-7. From the chemical and biological characterization of a lead molecule from Indian water spinach containing flavone hydroxyl group and the acyl groups in resin glycoside W04 might be responsible for the antioxidant activity coupled with inhibiting P-gp efflux through down regulation of ROS and inflammatory cytokines, thereby resulting in inhibiting tumorigenesis. The anticancer effect was highly significant and it correlates with standard anticancer drug Vinblastine. And its potential will be further examined in MDR cancer cell lines.

Author Contribution Statement

SM and SS contributed to the study conception and design. Material preparation, data collection and analysis were performed by SM and SS. MS and NA supervised the process of data collection and analysis. The first draft of the manuscript was written by SM and NA and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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This work is a part of approved Ph.D thesis of an author SM.

Ethical Approval Statement

As only in vitro cell lines studies are reported in this manuscript, ethical approval statement has not been enclosed.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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