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Isolation & Characterization of topo-poisons from *Arthrospira Platensis*: An *in-silico* approach

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ABSTRACT

Breast cancer is diagnosed in every 29 seconds around the world and the Indian Council of Medical Research (ICMR) said in 2016 the total number of new cancer cases is expected to be around 14.5 lakh and the figure is likely to reach nearly 17.3 lakh new cases in 2020. Hence there is a need for the discovery of novel anti breast cancer, dual human topoisomerase I & II leads. Hence the current research is focused to isolate and characterize a novel sulpha lipid, Sulpho quinovosyl diacyl glycerol (SQDG) from Arthospira platensis (Phormidiaceae) using flash chromatography and LCMS (ESI-MS). The percentage yield of isolated SQDG was found to be 20.5 % w/w. The Isolated SQDG showed significant in vitro anticancer activity on MCF-7 cell lines with CTC_{50} value of 0.46 µm in compare to standard quercitin. In order to propose the molecular mechanism of apoptosis, the isolated lipids (GLAME and SQDG) have been docked into the crystal structure of topoisomerase I (1K4T, 3AL2) and topoisomerase II (1ZXN, 3QX3) using Schrödinger suite, 2014-3. The in silico results showed that SQDG may be a potent Human topoisomerase I & II poison. Hence the current molecule may act as a lead molecule in anticancer therapy.

Keywords: Sulpho quinovosyl diacyl glycerol, Flash chromatography, Human Topoisomerase 1 & II Poison, MCF-7 Cell lines and in-silico molecular docking studies.

INTRODUCTION

Breast cancer is a major global health problem and the incidence is estimated to rise further over the next 20 years despite current alternative efforts to prevent the disease. The other risk factors which add to the burden of breast cancer are the increase in obesity, alcohol consumption, inactivity, and hormone replacement therapy (HRT) [1].

Breast cancer ranks as the fifth cause of death from cancer overall. Even though Significant advances in the prevention, diagnosis and management of breast cancer have been made in recent years based on the clinical application of scientific discoveries. However, breast cancer remains a complex disease process affecting millions worldwide, and further advances in scientific knowledge and clinical care could improve many lives [2].

It has been estimated that 30–40 percent of all cancers can be prevented by lifestyle and dietary measures alone. Obesity, nutrient sparse foods such as concentrated sugars and refined flour products that contribute to impaired

glucose metabolism (which leads to diabetes), low fiber intake, consumption of red meat, and imbalance of omega 3 and omega 6 fats all contribute to excess cancer risk. Supplementary use of oral digestive enzymes and probiotics also has merit as anticancer dietary measures. When a diet is compiled according to the guidelines here it is likely that there would be at least a 60–70 percent decrease in breast, colorectal, and prostate cancers, and even a 40–50 percent decrease in lung cancer, along with similar reductions in cancers at other sites. Such a diet would be conductive to preventing cancer and would favor recovery from cancer as well [3].

However, the globe is focused for the discovery of new anticancer lead molecules is from algal sources like *Spirulina* (*Arthrospira platensis*) belongs to the family Phormidiaceae[4]. It is a blue-green alga used as a dietary supplement because of its hypocholesterolemic properties. Among other bioactive substances, it is also rich in tetrapyrrolic compounds, glycolipids and sulpha lipids, a potent antioxidant and anti-proliferative agents [5]. The algae is a richest source of sulpha lipid known as Sulphoquinovosyl Diacylglycerol (SQDG) which is a compound of current interest in anticancer research reported for its various anticancer activities like anti leukemia, antiretroviral[6], inhibition of mammalian DNA polymerases[7] etc .

Lipids have the potential for development as anticancer agents. Endogenous membrane lipids, such as ceramides and certain saturated fatty acids, have been found to modulate the viability of tumor cells. In addition, many tumors over-express cyclooxygenase, lipoxygenase or cytochrome P450 enzymes that mediate the biotransformation of ω-6 polyunsaturated fatty acids (PUFAs) to potent eicosanoid regulators of tumor cell proliferation and cell death [8].

Sulfoquinovosyldiacylglycerol (SQDG), known trivially as plant sulfolipid, is a component of plant photosynthetic membranes. In contrast to the usual sulfate ester, as found in animal sulfatides, SQDG contains a sulfonic acid linkage. SQDG typically constitutes about 5% w/w of the total acyl lipid content of higher plant leaves. However, in certain algae SQDG is a major lipid component and levels of up to 29% of total lipids have been reported. Hence with the invention of flash chromatography, the isolation of lipids became easy [9-10]. However, the isolation of sulpha lipids is a very tedious process separated by only traditional gravity column chromatographic techniques where the yield, purity and recovery will be a challenging task [11].

Now a day's, an extensive research is taking place on Sulphoquinovosyl Diacyl Glycerol (SQDG) as an anticancer leads. However very few data is available on the isolation of lead molecule and the mechanism of anticancer action is unclear. Since the sulpha lipid belongs to the category of functional lipids/ nutraceuticals, the use of SQDG can be generally recognized as safe. As very less data is available on the promising anticancer molecule named SQDG.

The present research is focused on the isolation of Sulphoquinovosyl Diacyl Glycerol using flash chromatography, a novel method for the isolation has been developed using flash chromatography technique and the isolated lipid was subjected to cytotoxic studies against MCF-7 (breast cancer) cell lines and the results showed that the isolated compound is a potent cytotoxic agent in compare to standard rutin. In order to know the mechanism of cytotoxicity or apoptosis, the isolated lipid was docked into the crystal structre of topoisomerase I (1K4T, 3AL2) and topoisomerase II (1ZXN, 3QX3) and the docking results proved that the isolated lipid was a good dual topo poison in compare to standard Camptothecin and salvicine.

MATERIALS AND METHODS

All chemicals used were purchased from Fluka chemicals and their purity was checked by GC. All solvents were purified by distillation using Rotavap (Buchi R120) and if necessary residual water was removed. The components of solvents and elements are given in volume ratios of the components. The standard Sulpha quinovosyl diacyl glycerol (SQDG) was purchased from Avanti polar lipids, Inc. The mass spectra of sulpha lipid was recorded on JEOL GCMATE IIGC-MS.

2.1. Algae

Fresh cultures of *Spirulina platensis* were obtained from "Antenna Research Foundation Pvt Ltd.," Madurai, Tamilnadu, India. The cell paste was lyophilized and stored at −20 °C for further use.

2.2. HPTLC Quantification of SQDG in Ethyl Acetate Fraction

100 g of *Spirulina* was macerated in a volume of 600 ml (200:100) chloroform: methanol for 48 h. The collected filtrate was evaporated to give a crude lipid (7g) as a greenish brown gummy solid. The gummy solid was dissolved

with 30% methanol in water solution (50 ml) and then partitioned with 100 ml of hexane. The hexane layer was evaporated to give the hexane extract (2.7 g) and the aqueous methanol was then sequentially partitioned with ethyl acetate The obtained ethyl acetate fraction (4.5 g) was subjected to HPTLC analysis using Chloroform: Acetone: Water (30:6:2) and the Rf value was calculated and compared with the standard SQDG. It was found to be 0.41 (**Fig 1 & 2**).

2.2.1. Preparation of Standard and sample solution

250 mg of the ethyl acetate fraction was dissolved in 10 ml of ethyl acetate and the obtained clear solution was used for application on to the aluminum pre coated silica gel 60 GF_{254} TLC plate. 100 µg of standard SQDG was dissolved in 1 ml of ethyl acetate to obtain the clear solution which was used for application on to the aluminum pre coated silica gel 60 GF_{254} TLC plate.

2.2.2. Chromatographic conditions

Stationary phase: Silica gel 60 F_{254}
Mobile phase: Chloroform: Acetone: Water (30: 06:02)
Mobile phase volume: 8 ml
Band length: 7 mm
Application rate: 12 s/µl
Development chamber: Camag twin trough development chamber (10 × 10 cm)
Development distance: 6 cm from the application position
Scanner: Camag TLC scanner III
Detection wavelength: 366 nm
Slit dimension: 4.00 × 0.30 mm
Scanning speed: 20 mm/s
Data resolution: 100 µm/step
Measurement mode: Absorption
Peak area of standard SQDG = 14320
Peak area of SQDG in ethyl acetate fraction = 3348

2.3. Isolation of SQDG (SL) from ethyl acetate fraction using Biotage (Isolera one) flash chromatography

Based on the HPTLC profile, a flash chromatographic method was developed (Fig 3 & Fig 4). 0.5 g of ethyl acetate fraction obtained above was directly applied on 10 g samplet and samplet was dried under vacuum in rotary evaporator (Buchi R 120).The dried samplet was packed in 50 g KPSil Biotage SNAP Cartridge. A constant flow rate 50 ml/min of mobile phase (30% Chloroform: 6 % Acetone in 2% water) is used. A total no of 105 fractions, each 22 ml was collected in different test tubes at the wave length of 366 nm. Each individual fraction was subjected to TLC analysis using the same mobile phase in comparison to that of standard Rf of SQDG (SL). Fraction no 15 – 20 were found to be as SQDG and the percentage yield was calculated. The structure of isolated compound was determined and confirmed by its HPTLC and LCMS analysis.

2.4. LC-MS Analysis of isolated SQDG

Shimadzu HPLC, Japan with system controller (SCL -10A), twin pump (LC-10AT VP), UV-Vis detector (SPD-10A VP) and rheodyne injector with 100 µl injection loop and the separation was done using lichrosorb micro bondapack C-18 Column (Merck). The data processing was done by using shimadzu HPLC Software class –VP (V5.03). The LC-MS experiment was carried out on a micromass quadro II triple quadrupole mass spectrometer. And the ESI capillary was set at 3.5 KV and the voltage was 40 V (Fig 4 & Fig 5) [12].

2.5. *In vitro* Cytotoxic Studies Using Sulphorhodamine B (SRB) assay

The cell cultures (MCF-7) used in this research were procured from National Center for Cell Sciences, Pune, India. The cells were grown in Earls Minimal Essential Medium supplemented with 2 mmol L-glutamine, 10 % Fetal Bovine Serum, Pencillin (100 µg/ml) and amphotericin B (5µg/ml) and the cells were maintained at 37^0C in a humidified atmosphere with 5 % CO_2 and subculture twice a week. The SRB assay was carried out following by the method developed by Jubie S *et al.*, 2015[13].

2.6. Molecular docking studies and ADMET studies were performed by GLIDE integrated Maestro (9.3) of *Schrödinger* Suit.

The isolated compounds SQDG and GLA-ME were docked in to crystalline structures of topoisomerases I & II, in order to know the molecular mechanism of cytotoxicity or apoptosis. The GLA-ME was isolated and characterized by the method developed by (Jubie S *et al.*, 2015) [5]. The ADMET profile of the isolated compounds was also determined followed by the method developed by (Chaitanya MVNL et al., 2015) [14].

2.7. Molecular Mechanics/Generalized Born Surface Area (MM/GBSA)

The binding free energy of inhibitors in the catalytic domain enzyme (1K4T) was calculated by Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) VSGB 2.0 method. The calculated inhibitor-enzyme complex was relaxed using the local optimization feature in Prime, version 4.1, Schrödinger 2014, and the energies of the system were calculated using the VSGB 2.0 (solvation model) method available in Schrödinger suite 2014 (Schrodinger 9.9/2014-3 suite) [15 -17].

RESULTS AND DISCUSSION

3.1. Isolation of SQDG from *Spirulina platensis* using Flash chromatography

The Ethyl acetate fraction was quantified for SQDG content using HPTLC quantification, the amount of SQDG present in the given sample of ethyl acetate fraction was found to be 20.76 % w/w. The HPTLC method was developed first time and found to be economical and accurate in compare to other expensive methods like HPLC. A novel method was developed to separate SQDG from ethyl acetate fraction using flash chromatography and the percentage yield was found to be 20 % w/w, which is a very good yield when compared to the traditional methods. The isolated SQDG was confirmed by mass spectra (m/z; 827.11) (Fig 3, 4 & 5).

3.2. Cytotoxic studies of SQDG on MCF-7 cell lines

The isolated compound was subjected for cytotoxic activity against MCF-7 (Breast cancer cell lines) using SRB assay method and the isolated compound proved as a significant cytotoxic agent with a CTC_{50} (concentration of the sample required to kill 50 % of the cells) value of 39.59 (µg/ml) in compare to standard quercitin with CTC_{50} value of 36.29 (µg/ml) (Table 1).

3.3. Molecular docking studies

In order to know the molecular mechanism of the isolated compounds, they were docked in to the topoisomerase I & II enzyme targets (PDB ID: 3ALN, 3QS3, 1ZXN, 1K4T). The two compounds (GLA-1 & SQDG) have given good poses for all selected enzymes domains and given similar G-scores as that of standard camptothecin and Salvicine (Table 2). Due to these similar G-scores, it may be hypothesized that all the compounds bind to the human topoisomerase I & II domain in a similar manner. The *in silico* studies proved that the cytotoxicity shown by SQDG and GLA-ME is may be due to the induction of apoptosis of tumor cells by inhibiting the human DNA topoisomerase I & II which can be one of the possible mechanisms of the apoptosis induction in cancer cell (MCF-7). Both the compounds showed highest XP G Scores than the standard topo II poison, Salvicine as a result of docking of two compounds and Salvicine with 1ZXN (GLA-1 -9.11, SL -10.81, Salvicine -7.19) The good G-scores of the isolated compound (SL) may be probably due to more hydrogen bonding interactions of the compound SL (Hydrogen Bond length-1.89) than the topo I poison Camptothecin (Fig 6 &7).

Drug likeliness, log P, log S, molecular weight and toxicity risks may be used to judge the compound's overall potential to qualify a ligand as potential drug candidate. The two compounds ,GLA-1 and SL have appropriate log P (octanol/water) value for biological efficacy. GLA-1 had one lipinsky violation and SL-1 had three lipinsky violation and satisfying pharmacological properties of 95% available drugs with high to medium predicted oral absorption availability. Molecular weight of each ligand falls within the range of 334 to 770 Daltons. The ligands are having no toxic functional groups. Log S values of these ligands are within the acceptable range of 95 % of existing drugs. Also, the lipophilicity data suggested that the compounds were most lipophilic in nature.

The Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) studies proved that the molecule having good binding energy on the selected targets (Table 4). The detailed study of molecular mechanism is in progress.

The developed flash chromatographical method can be used as an important method for the isolation of sulpha lipids from natural sources. As the method is fast and economical giving a good yield of sulpha lipid when compared to

the traditional conventional methods, it can be implemented as a commercial method for the isolation of sulphalipids. Although, many literatures are available that SQDG has anticancer property, they failed to focus the cytotoxic activity of SQDG on MCF-7 (human breast cancer cell ines). Hence the current research has proved that SQDG is having good cytotoxic activity against breast cancer cell lines. Therefore the isolated molecules can be a good lead source for discovery of novel anticancer drugs against breast cancer and lung cancer. As there is a current demand for the novel dual human topo-poisons (I & II), the *in-silico* studies proved that the apoptosis induction in breast cancer cell lines may be due to dual human topopoison activity. However these molecules have to be taken further for extensive research in order to prove the *in vitro* topo I &II inhibitoty activities.

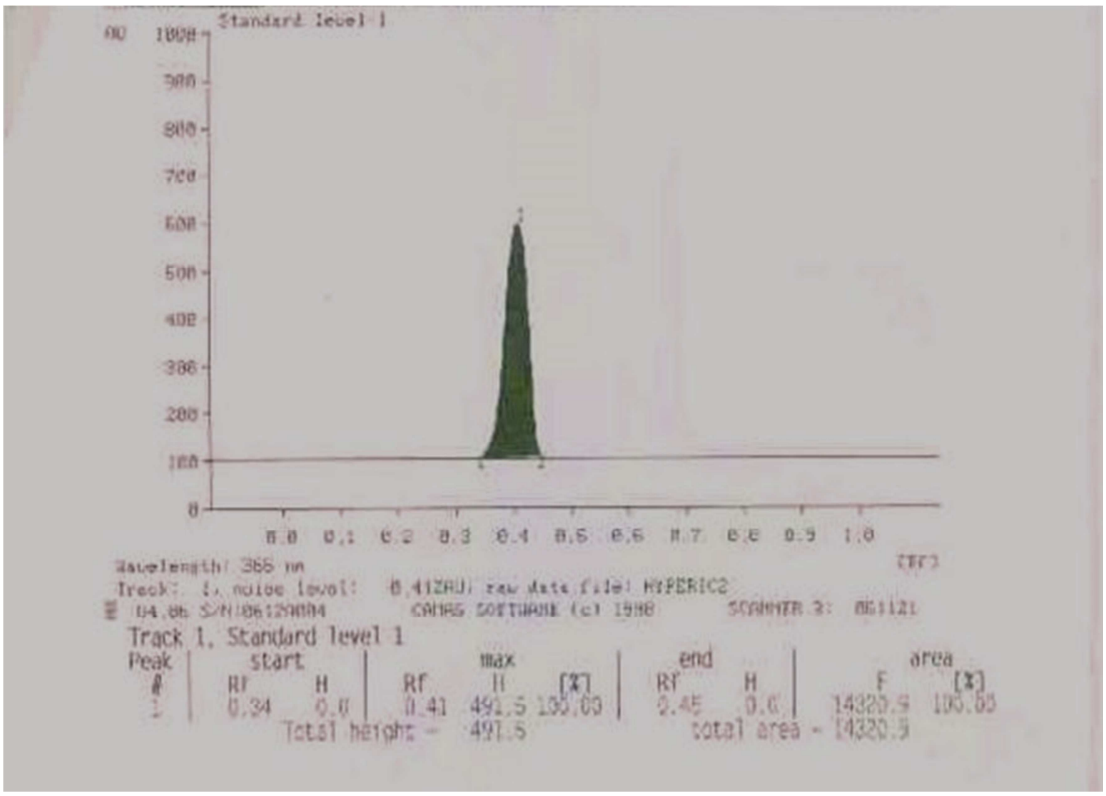


Fig 1. HPTLC Chromatogram of Standard SQDG (SulphoquinovosylDiacylGlycerol)

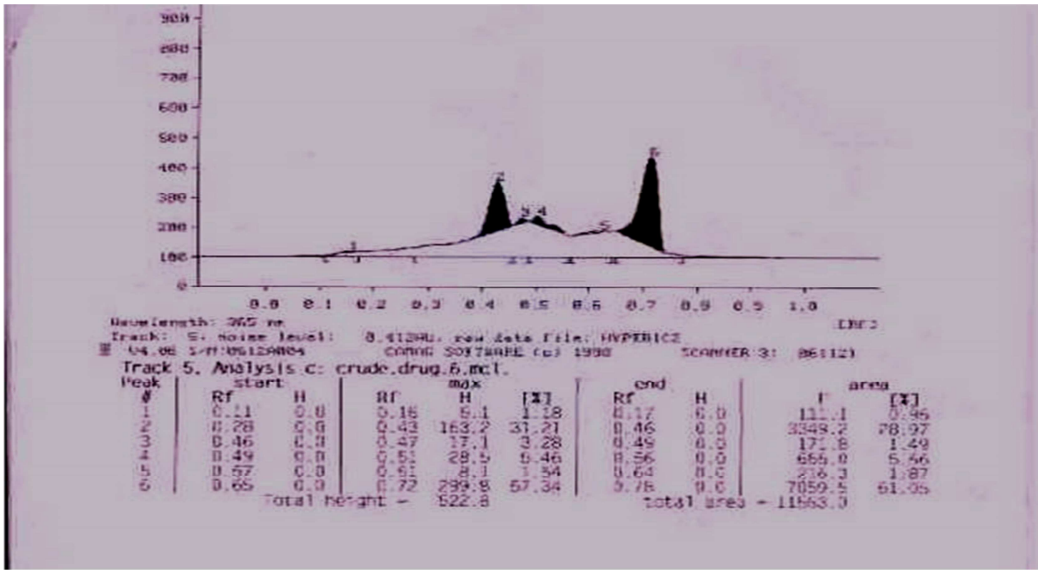


Fig 2. HPTLC Chromatogram of Standard SQDG (SulphoquinovosylDiacylGlycerol)

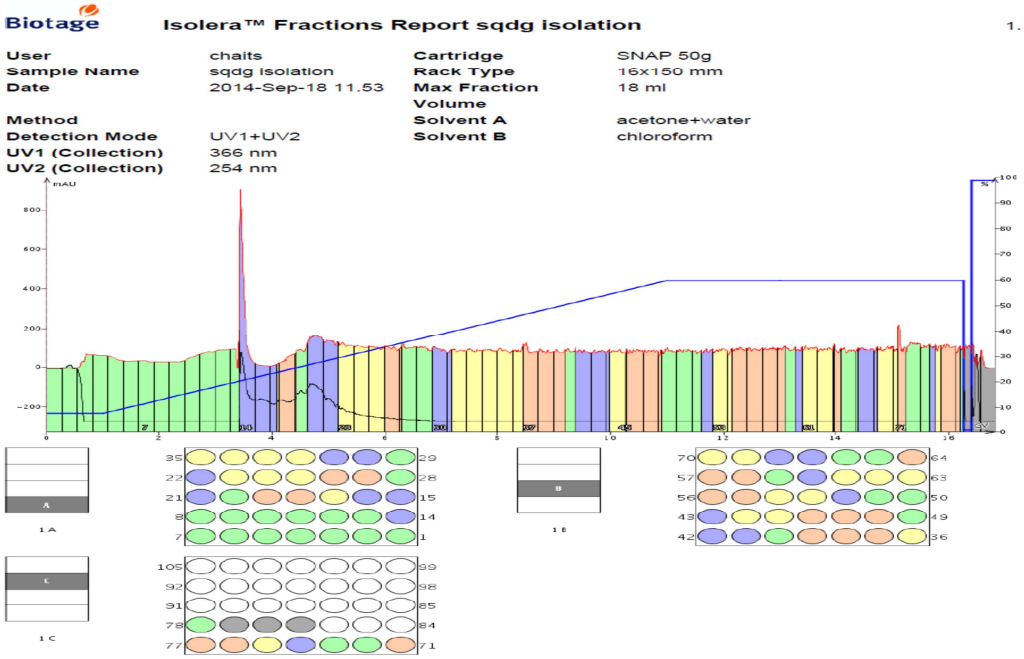


Fig 3. Flash Chromatogram of isolation of SQDG (SulphoquinovosylDiacylGlycerol)

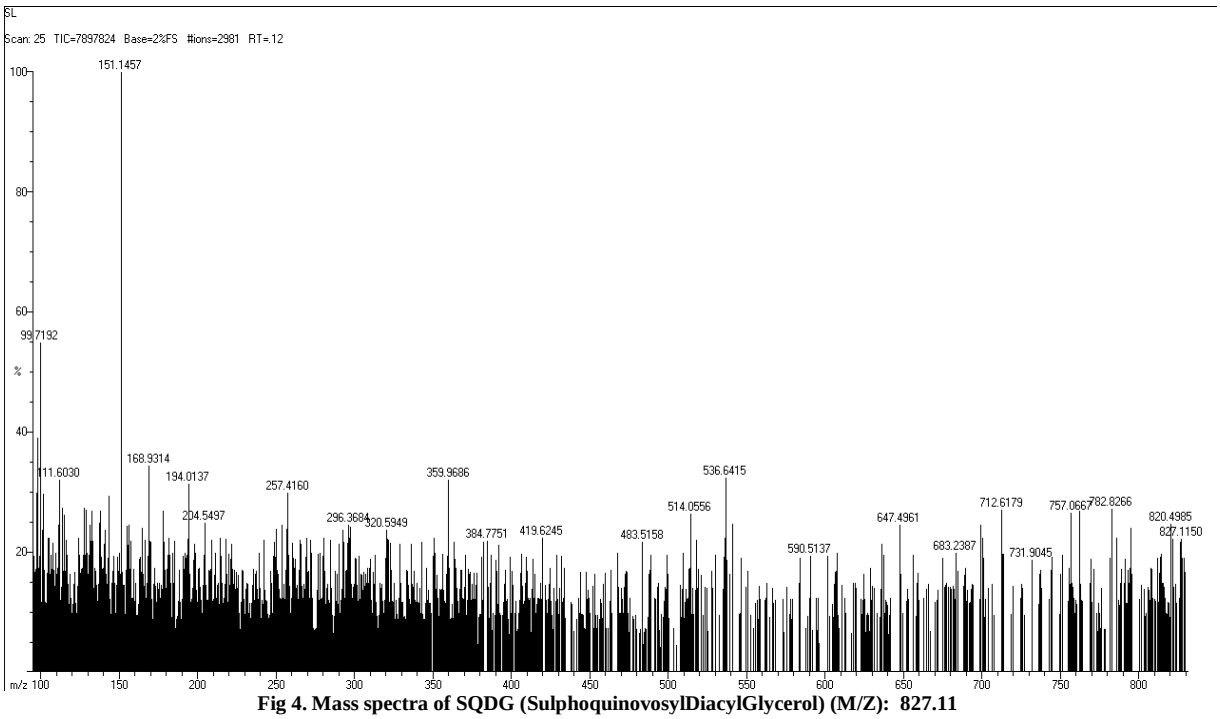


Fig 4. Mass spectra of SQDG (SulphoquinovosylDiacylGlycerol) (M/Z): 827.11

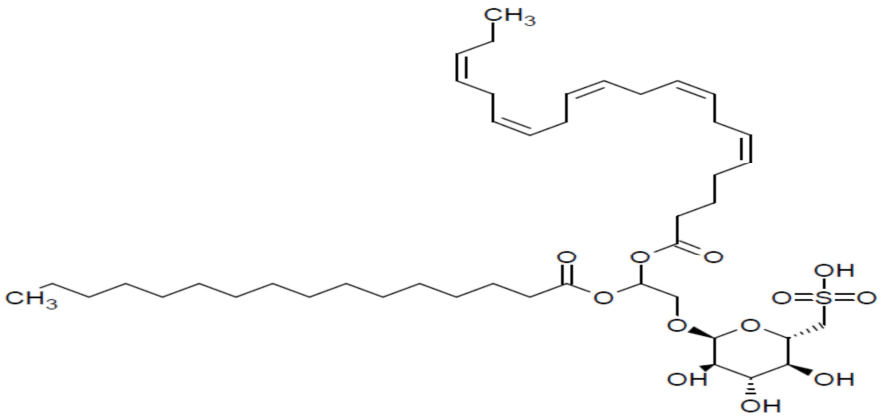


Fig 5. Chemical Structre of SQDG (SulphoquinovosylDiacylGlycerol)

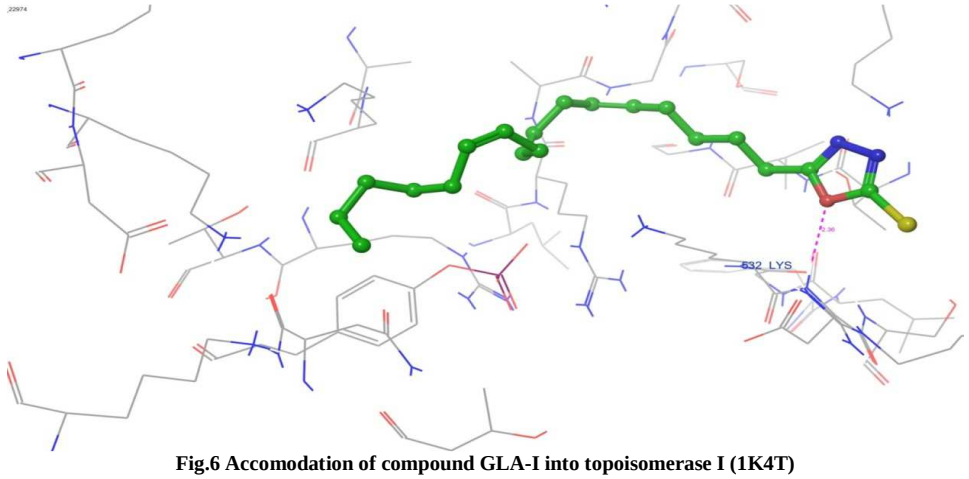


Fig.6 Accomodation of compound GLA-I into topoisomerase I (1K4T)

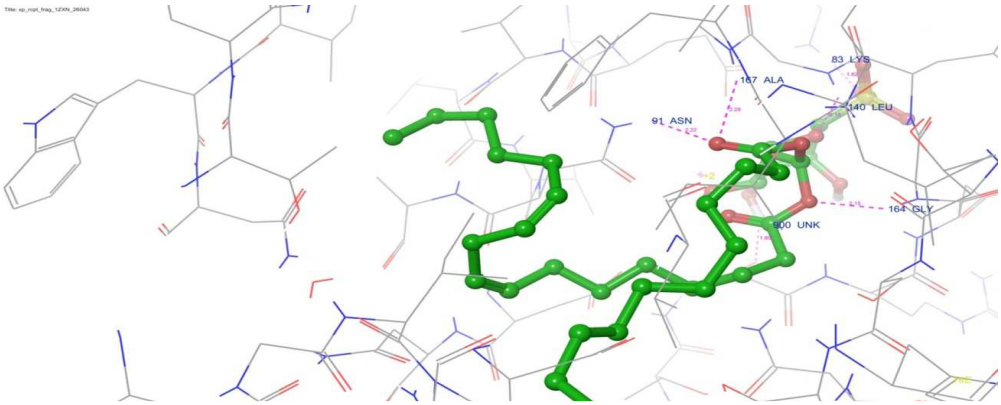


Fig.7 Accomodation of compound SL into topoisomerase I (1K4T)

Table 1. Cytotoxic activitiy of Standard Quericitin and SQDG (Sulphoquinovosyl DiacylGlycerol)

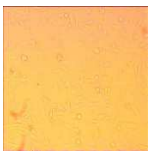
S. No	Name of the Compound	SRB assay CTC_{50} Values (µg/ ml ± SEM)		
		Cell lines		
		Concentration (µg/ml)	MCF-7	VERO
1.	Std Quercitin	1000 500 250 125 62.5	36.25± 1.85 	390.6±0.53 
2.	SQDG (Sulphoquinovosyl DiacylGlycerol)	1000 500 250 125 62.5	39.59± 0.95 	675.5±0.87 

Table 2: G-Scores from the glide XP docking run of novel compounds with different Top 1&II Enzymes

Compound	G-Score	Lipophilic EVdw	H –bond	Electro	Low MW	Penalties	HB Penal	Rot Penal
Target 1K4T								
Camp	-4.98	-3.95	-0.89	-0.38	-0.33	1	0	0
GLAME (GL)	-4.20	-3.39	-0.24	-1.91	-0.38	0	0	0.79
SQDG(SL)	-7.45	-2.45	-2.30	-1.60	0	0	0	0.40
Target 3AL2								
Camp	-2.85	-1.75	-1.38	-0.54	-0.33	1	0	0
GLAME (GL)	-2.47	-2.24	-0.75	-0.75	-0.38	0	0	0.79
SQDG(SL)	-2.48	-3.64	-3.50	-3.50	0	1.90	0	0.40
Target 1ZXN								
Sal	-7.19	-3.68	-2.38	-2.38	-0.39	0	0	0
GLAME (GL)	-9.11	-3.73	0	0	-0.38	0	0	0
SQDG(SL)	-10.81	-4.50	-4.30	-4.30	0	0	0	0
Target 3QX3								
Sal	-7.35	-15.55	-1.69	-1.69	-0.39	0	0	0
GLAME (GL)	-5.89	-19.64	0	0	-0.38	0	0	0
SQDG(SL)	-9.81	-36.69	-1.68	-1.68	-4.50	0	0	0

Camp - Camptothecin
Sal - Salvicine
G-Score - GLIDE Score
Lipophilic Evdw - Lipophilic Evidence
H-bond - Hydrogen bond
Electro - Electrostatic potential
Low MW - Low Molecular Weight
Penalties
HB Penal - Hydrophobic Penalty
Rot Penal - Rotational Penalty

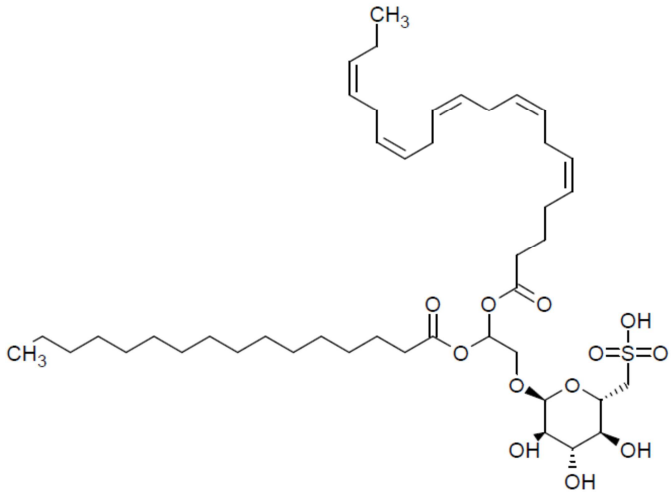
Table 3. QIKPROP 3.4 predictions of ADMET for the compounds GL-1 and SL

Property	GLAME (GL)	SQDG (SL)	Acceptable Range
CNS	0	-2	-2 to +2
Mol wt	292	777.06	130 to 7.25
Dipole	3.47	9.63	1to 12.5
SASA	642.25	1363.86	300 to 1000
Volume	1213.74	2600.18	500 to 2000
Donor HB	0.8	3	0 to 6
Accept HB	2.5	13.8	2 to 20
Q P Log Poct	13.31	36.47	8 to 35
Q P Log PW	2.63	15.04	4 to 45
QP Log Po/W	5.97	8	-2 to 6.5
QP Log S	-5.20	-9.36	-6.5 to -0.5
QP Log BB	-0.51	-5.90	-3.0 to -1.2
QP Log KP	-0.85	-3.09	-8 to -1
IP	9.51	9.72	7.9 to 10.5
Rule of 5	1	3	4
Rule of 3	1	3	3

CNS Predicted central nervous system activity on a -2 (inactive) to +2 (active) scales
mol.wt Molecular weight of the molecule
dipole Computed dipole moment of the molecule
SASA Total solvent accessible surface area (SASA) in square angstroms using a probe with a 1.4 A◦ radius
volume Total solvent accessible volume in cubic angstroms using a probe with a 1.4 A◦ radius
donor HB Estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution.
acceptHB Estimated number of hydrogen bonds that would be accepted by the solute to water molecules in an aqueous solution
QPlogPoct Predicted octanol/water partition coefficient
QPlogPw Predicted water/gas partition co-efficient
QPlogPo/w Predicted octanol/water partition co-efficient
QPlogS Predicted aqueous solubility
QPlogBB Predicted brain/blood partition coefficient
QPlogKp Predicted skin permeability
IP PM3 calculated ionizationPotential
Rule of 5 Number of violations of Lipinski's rule of five
Rule of 3 Number of violations of Jorgensen's's rule of three

Table 4. MM/GBSA studies of isolated compounds on selected enzyme targets

S.NO	Name of the Compound	Molecular Mechanics/Generalized Born Surface Area (MM/GBSA)			
		1K4T	3AL2	1ZXN	3QX3
1.	Std camptothecin	-74.80	-43.59	-6.07	-1.63
2.	Std Salvicine	-28.21	-67.36	-63.60	52.90
3.	SQDG (SL)	-82.96	-58.25	-12.52	-44.44
4.	GLAME (GL)	-28.21	-67.36	-63.60	-52.90



SQDG (SulphoquinovosylDiacylGlycerol)

PubChem CID: 56842030
Chemical Names: Sulfoquinovosyl diglyceride; SQDG lipid; 207976-87-2; Diacylsulfoquinovosyl glyceride; 6-Sulfoquinovosyldiacylglycerol; 6-Sulfoquinovosyl-diacylglycerol; More...
Molecular Formula: C45H76O12S
Molecular Weight: 841.151 g/mol
InChI Key: IIDPUWZBYVLQEU-GMJMQRIKSA-N
Modify Date: 2016-10-29
Create Date: 2012-03-21
InChI=1S/C45H76O12S/c1-3-5-7-9-11-13-15-17-18-19-20-22-23-25-27-29-31-33-40(46)54-35-38(36-55-45-44(50)43(49)42(48)39(57-45)37-58(51,52)53)56-41(47)34-32-30-28-26-24-21-16-14-12-10-8-6-4-2/h5,7,11,13,17-18,20,22,25,27,38-39,42-45,48-50H,3-4,6,8-10,12,14-16,19,21,23-24,26,28-37H2,1-2H3,(H,51,52,53)/b7-5+,13-11+,18-17+,22-20+,27-25+/t38?,39-,42-,43+,44-,45+/m1/s1.

CONCLUSION

The developed flash chromatography method can be used as an important method for the isolation of sulpha lipids from natural sources. As the method is fast and economical giving a good yield of sulpha lipid when compared to the traditional conventional methods, it can be implemented as a commercial method for the isolation of sulphalipids. Although, many literatures are available that SQDG has anticancer property, they failed to focus the cytotoxic activity of SQDG on MCF-7 (human breast cancer cell line). Hence the current research has proved that SQDG is having good cytotoxic activity against breast cancer cell lines. Therefore the isolated molecules can be a good lead source for discovery of novel anticancer drugs against breast cancer and lung cancer. As there is a current demand for the novel dual human topo-poisons (I & II), the *in-silico* studies proved that the apoptosis induction in breast cancer cell lines may be due to dual human topopoison activity. However these molecules have to be taken further for extensive research in order to prove the *in vitro* topo I &II inhibitory activities.

Acknowledgements
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Abbreviations
SQDG: SulphoquinovosylDiacylGlycerol, SL : Sulpha lipid, GLA-ME: Methyl gamma linolenate ester

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