Multidrug resistance reversal activity of extract and a rare dimeric naphthoquinone from *Diospyros lotus*

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Abstract: A dimeric naphthoquinone namely dihydrodyspyrole R (1) was purified once more from *Diospyros lotus.* Dihydrodyspyrole R and chloroform fractions were evaluated for their effects on the reversion of multidrug resistance (MDR). The compounds (1) and extract exhibited promising MDR reversing effect in a dose-dependent manner against mouse T-lymphoma cell line. Molecular docking of compound 1 revealed the correlation between *in-silico* with *in-vitro* results. The molecular docking results showed that compound 1 is bind closely where co-crystal ligand of P-gp is present. But usually, computational investigation predicts that, if a compound gives lesser score then compound will exhibit good activity. Hence, the docking scores of compound 1 are the near to the Rhodamine. It is conclude that there are certain important structural features of compound 1which are responsible for the inhibiting potency of P-gp from mice. The computational Petra/Osiris/Molinspiration (POM) analysis confirms the possibility of use of compound 1 without side effect or less toxicity risks.

Keywords: *Diospyros lotus,* Di-naphthodiospyrol R, anticancer, molecular docking.

INTRODUCTION

Diospyros lotus belongs to family Ebenaceae which comprises of 500 species spread in United kingdom, sub-tropicalregions, Japan and Asia (Uddin *et al*., 2011). *D. lotus* grows up to 9 meter in tallness and grown in semi shade area (Chittendon, 1956). Various parts of *Diospyros* genus are used for different aliments as anti-microbial agent, hiccough, internal hemorrhage, febrifuge, as astringent, as sedative, and for the treatment of biliousness (Tezuka *et al.,* 1973; Ganapaty *et al.,* 2006). Bioactive compounds including triterpenoids, oleanane, ursane series have been reported from *Diospyros* with documented anti-inflammatory properties (Watt and Breyer-Brandwijk, 1932; Chopra and Nayar, 1956). *D. lotus* crude extracts and its compounds have also been reported as excellent muscle relaxants and sedative potential (Chopra *et al.,* 1956; Rauf *et al.*, 2015d).

During the past few decades, a remarkable success in basic cancer research and clinical oncology lead to the development of cancer chemotherapy but still the treatment of cancer patients is limited choice. The drug therapy frequently leads to non-satisfactory consequences with incurable results for patients because of limitations like the severe side effects and an increase of drug resistance (Kuete *et al*., 2015).

The multidrug resistance (MDR) is an essential rapid resistance and one of the largest obstacles to productive chemotherapy for the cure of cancer. This resistance normally caused due to greater expression levels of membrane efflux. The proteins belonging to the ATP-binding cassette (ABC) family of transporters that are capable for aggressively transporting an extensive variety of drugs expelled out from cells. The most important studied MDR proteins, is that the ABC transporter P-glycoprotein (P-gp). The P-gp expression has been shown to rise after chemotherapy treatment and is intensely associated with reduced response to chemotherapy (Higgins, 2007). Therefore, the P-gp is frequently targeted for the treatment of cancer and other human diseases, thus, it can be measured as a model for this family of transporters (Jones and George, 2004). The polypeptide

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chain of P-gp casus is 1280 amino acid residues. The p-gp is comprised of six transmembrane domains (TM) and a hydrophilic domain containing an ATP-binding site, known as nucleotide binding domain (NBD) (Jara *et al.*, 2013). The main binding pocket for Rhodamine123 in mouse P-gp was found to be a hydrophobic pocket involving residues such as Ser218, Phe299, Val334, Leu335, and Phe339. These residues were proposed to play a vital role for substrate binding on experimental bases (Li *et al.,* 2010).

Keeping in view the importance of p-gp in the field of cancer drug discovery research, there is an urgent need for the development of drugs to overcome these limitations and move toward for safe therapy for the treatment of cancer. The current study deals with *in-vitro* and *in-silico* MDR studies of a rare dimeric naphthoquinone from *D. lotus* (fig. 1).

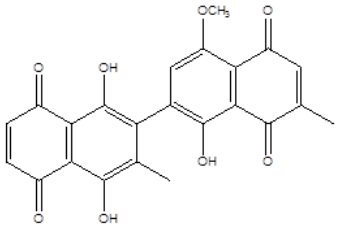


Fig. 1: Chemical structure of rare naphthoquinone (1).

MATERIALS AND METHODS

Plant material

The roots of *Diospyros lotus* were collected in local area of Malarasolpati, Razagram, Distt, Lower Dir, KPK, Pakistan. The plant specimen was identified by Prof. Dr. Abdur Rashid Department of Botany University of Peshawar, Peshawar, Pakistan. The voucher specimen No (Bot. 20036 (PUP) was deposited in herbarium of said department.

Extraction and isolation

The plant material (14 kg) of *D. lotus* were crushed to fine powered form using local grinder machine and subjected to cold extraction using alcoholic (MeOH) solvent. The extract was concentrated by using rotary evaporator (55 ^{0}C) to yield a dark residue (202g) as per reported methods (Uddin *et al*., 2012). The dark residue was suspended in water and partitioned with various polar and non-polar solvents including; *n*-hexane, chloroform, ethyl acetate successively. After fractionation chloroform fraction 30 g was subjected to normal column chromatography using silica gel60 (0.062-0.200 mm; Merck). The column was eluted with *n*-hexane: EtOAc as the solvent phase. Eluting column with hexane-EtOAc(100:0 → 18:82), red fraction was yield which exposedone compound (1) on pencil column chromatography. The structure of dihydrodyspyrole R (1) was confirmed by comparing their spectra data with reported (Rauf *et al*., 2015d).

Assay for reversal of MDR in mouse lymphoma cells

Parent cells lines (L5178 MDR and L5178Y) were grown in McCoy`s 5A medium comprising 10% heat-in-activated horse serum, were skillful with antibiotics and L-glutamine. Cell lines was made up to a density of $2\times10^6$ mL re-suspended in serum-free McCoy`s 5A medium and spread in 0.5mL aliquots into Eppendorf (centrifuge) tubes. Compound 1 was combining at 2µg/ml concentrations; then samples were incubated at 25 C^0 for 10 mints. Verapamil was applied as a positive control. The biological screening was performed exactly followed by recently published protocol (Rauf*et al.,* 2016). On the basis of the dignified fluorescence values were calculated using below formula:

$$FAR = \frac{MD_{treated}/MD_{control}}{Parental_{treated}/Parental_{control}}$$

Docking studies

Retrieval of X-ray crystallographic structure of mice P-glycoprotein (P-gp) has a four letter PDB code 4Q9L from protein data bank (PDB (Berman *et al*., 2000). Energy refinement of the structure was carried out by swiss PDB viewer v4.1.0 program (Guex and Peitsch, 1997). The compound 1 and Rhodamine123 structures were prepared through Chem sketch (Li *et al.*, 2004) and Avogadro's software (Hanwell *et al*., 2012) by adding hydrogens and geometry optimization. The docking studies were carried out through two docking programs such as Autodock Vina (Trott and Olson, 2010) and i-GEMDOCKv 2.1 (Hsu *et al.*, 2011). Docking procedure was optimized by a co-crystal ligand of P-gp. All the defaulting docking constraints were used for both software (Rauf *et al.*, 2015a; Rauf *et al.*, 2015b; Rauf *et al*., 2015c). The docking analysis was carried out through LIGPLOT+ version v.1.4.5 (Wallace *et al*., 1995), PyMOL(DeLano, 2002) and Discovery studio visualizer software (Visualizer, 2005).

RESULTS

On the basis of the previous anti-cancer potencies of *D. lotus* (Rauf *et al.*, 2015d), crude extract and compound **1** was selected for flow-cytometric cell cycle screening at a concentration (2µg/ml). The effect of multi drug resistance mouse lymphoma c is displayed in table 1. Compound 1 is also screen for docking and POM analysis study the results are given in fig 2 and 5.

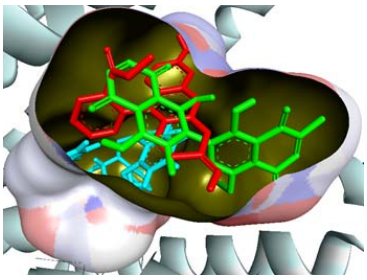


Fig. 2: The overview of predicted binding pocket of the compound 1(shown by sticks green colors) and the

standard Rhodamine-123 (red color). The P-glycoprotein (light cyan color) and the co-crystallized ligand are shown by the stick dark cyan color.

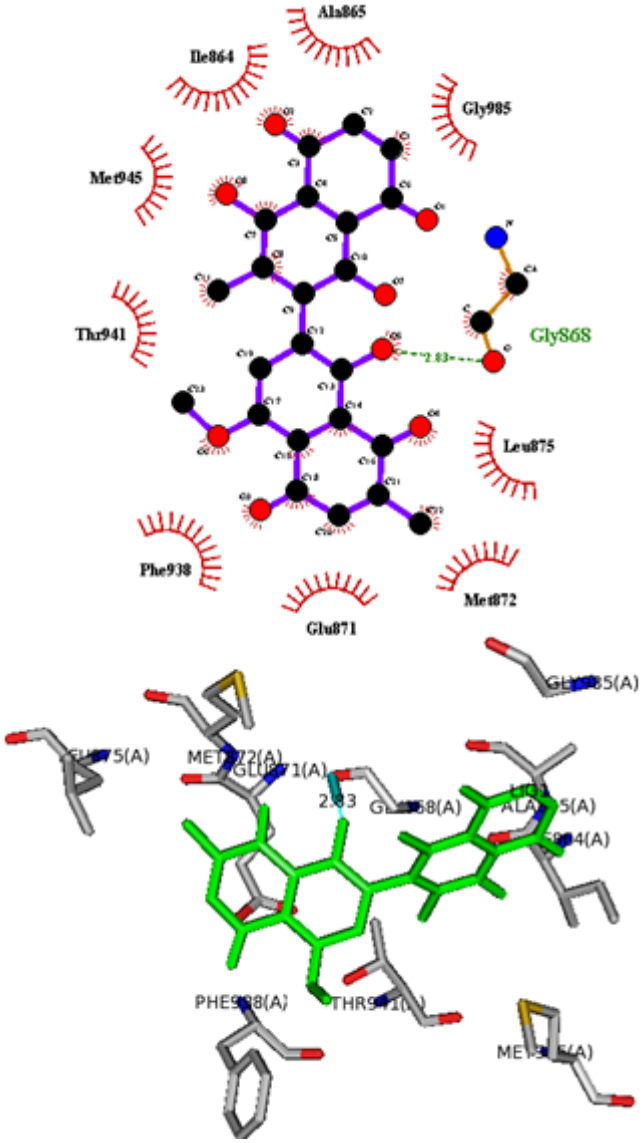


Fig. 3: The 2-D and 3-D interaction profile of compound 1 in the pocket of p-gp.

DISCUSSION

The fluorescence activity ratio (FAR) values were used to assess the *ABCB1* transporter modulating properties. The values of SSC (side scatter count) and FSC (forward scatter count) were increased in the flow cytometry which showed that the extract/compounds (1) had membrane effect and the granulation of cytoplasm was increased. The FAR values obtained by using extract/1, indicating that extract and its isolated compound 1 is very effective MDR modulator in a short time experiment. Verapamil, which is a calcium channel blocker and chemosensitizer, was used as a positive control. The results obtained showed that compound 1 was strong modulator of the efflux pump activity (FAR 10.22, 2µg/ml) (table 1).

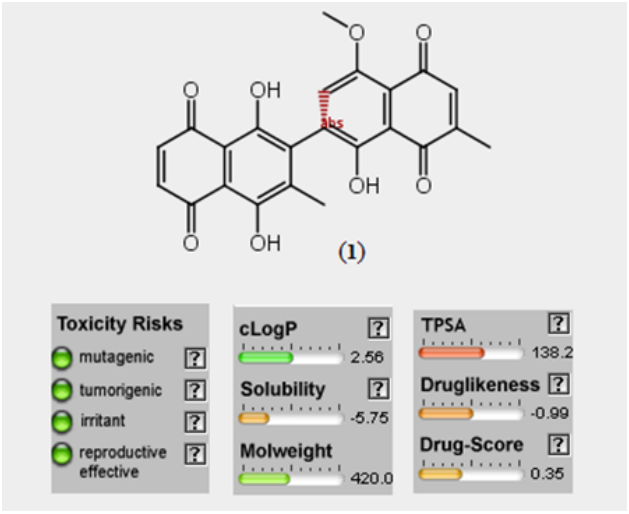


Fig. 4: Osiris calculations of druglikeness of compound 1. Toxicity Risks (:not toxic, : slightly toxic, : highly toxic).cLog*P* and Molecular Weight are in perfect accordance with Lipinski 5 rules (cLogP <5 and Molecular weight <500 g/mole).

Computational approaches in the field of drug discovery have taken an important role. It primarily predicted the inhibiting potency of new compounds against the targeted enzymes. Molecular docking investigated the correlation between *in silico* with *in vitro* results of compound 1. Here, we carried out the docking studies of compound 1and the standard Rhodamine123 against the mice P-gp. The docking of compound 1 gives good results as it is confirmed from the docking table 2 and fig. 2. The compound 1 is binds nearly where already co-crystal ligand of P-gp is present. But usually, computational investigation predicts that, if a compound gives lesser score then compound has good activity. Hence, the docking scores of compound 1are close to the Rhodamine (table 2). Therefore, hence may be conclude that there are certain important structural features of compound 1 which are responsible for inhibiting the potency of P-gpin mice.

The detailed interactions (fig. 3) such as hydrogen bonding and hydrophobic contacts of the compound 1 against the P-gp reveals that there is a total of nine hydrophobic interactions observed from the residues i.e Ile864, Ala865, Glu871, Met872, Leu875, Phe938, Thr941, Met945, and Gly985. But only one hydrogen bond interaction has been observed residues Gly 868 with a distance of 2.83Å in the binding pocket of P-gp. Hence, these interactions may be responsible for such a good binding capacity of compound 1.

POM Analyses of compound 1

cLog*P* (octanol/water partition coefficient) is calculated by the procedure advanced by Molinspiration and Osiris

Table 1: The effect of dihydrodyspyrole R (1) and crude extract on the Rhodamine123 accumulation assay in L5178 MDR mouse lymphoma cell

Test	Sample	(Final concentration) µg/mL	FSC	SSC	Mean	FAR	Peak Ch
1	PAR	-	2315	684	70.8	-	69.8
2	PAR	-	2134	603	65.5	-	67.3
3	MDR	-	2339	753	2.01	-	1.6
	MDR MEAN	-	2326	914	1.64	-	1.54
4	Verapamil	10	2329	711	21.9	13.35	27.4
5	Crude extract	20	1926	1107	0.921	1.16	0.75
8	1	2	1844	1133	9.53	12.06	15.4
18	DMSO	0.2%	2247	759	1.02	0.62	0.931
19	MDR	-	2313	1076	1.27	-	1.49

Table 2: The docking score of compound 1 and standard Rhodamine123 against mice p-gp.

Compound	Autodock Vina	i-GEM DOCK			
	B. Affinity	Total Energy	VDW	HBond	Elec
1	-8.0	-84	-72	-12	0
Rhodamine123	-8.2	-87	-86	-1	0

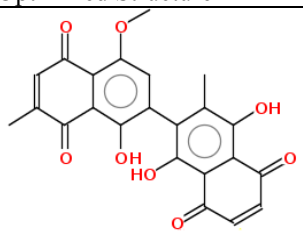
Molecular Properties Calculation		Optimized Structure	Bioactivity Scores Calculation	
TPSA	138		GPCR ligand	-0.14
MW	420		Ion channel modulator	-0.01
nON	8		Kinase inhibitor	0.04[a]
nOHNH	3		Nuclear receptor	-0.04
nviolations	0		Proteaseinhibitor	-0.24
volume	347		Enzyme inhibitor	0.19[a]

Fig. 5: Molinspiration calculations of Bioactivity Scores (BS) of compound 1.[a] Compound 1 is able to have potential bioactivity as kinase inhibitor and other enzyme inhibitor (BS = 0.04 and 0.19 respectively).

as a sum of fragment-based contributions and correction factors (Ertl*et al*., 2000). The technique is strong and is brilliant to process almost all organic and organo metallic based compounds. Molecular polar surface area TPSA is calculated by the procedure reported by Ertl *et al*. since 2000 as a sum of fragment contributions. O and N centered polar fragments are measured. PSA has been exposed to be a very excellent descriptor symbolizing drug absorption, counting intestinal absorption, bio-availability, Caco-2 permeability and blood brain barrier penetration.

Prediction results of screen compound 1 with molecular properties (TPSA, GPCR ligand and ICM) are given in figs. 4 and 5. Compound 1 has no violation of five rules of Lipinski. This explains why most of the natural compounds exhibit good bioavailability. Drug likeness of compound 1 seems correct in the same range of some standard drugs.

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