A New Potent Anti-cancer Corosolic Ester Identified from the Super Miracle Plant *Hippophae rhamnoides* (Sea buckthorn)

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Abstract

*Hippophae rhamnoides* L., commonly called Sea buckthorn, is native to Asia and Europe and is known for its nutritional and medicinal values. The aim of the present study was to investigate the anti-cancer constituents of *H. rhamnoides*. Among the three isolated compounds namely: 1-(2-hydroxynaphthalen-1-yl) ethan-1-one (1), Oleandric acid (2), and Hippocrasolate (3), compound 3 was a new corosolic ester derivative. The isolated compounds (2 and 3) displayed anticancer activity against lung (NCI-H460) and breast (MCF-7) cancer cell lines with IC₅₀ values of ~3 μM and ~6 μM, respectively. However, compound 1 was active only against breast cancer cells with IC₅₀ value of ~43 μM. These compounds displayed only weak interactions with minor groove of DNA in DNA-ligand conformational studies and therefore, structural DNA damage was not noted in electrophoretic mobility experiments. It was concluded that new compound 2 possessed more potent anticancer activity than that of known compound 3 against lung cancer cell line.

Keywords: Anti-cancer, Corosolic acid, DNA, *Hippophae rhamnoides*, Pentacyclic triterpene

Introduction

*Hippophae rhamnoides* L. commonly called Sea Buckthorn (SBT) is a valuable and unique natural sources gained attention worldwide not only for its medicinal properties but also for its nutritional properties. It is a multi-medical tree shrub of the genus Hippocrea belongs to the family Elaeagnaceae. *Hippophae rhamnoides* L. is mostly found in the moderate geographic locations of the north hemisphere, categorized into six species and 12 subspecies in the plant taxonomy [1]. It is a deciduous and nitrogen-fixing plant of cold arid region, native to Asia and Europe.

All parts of SBT are considered as a rich source of numerous bioactive constituents with excessive nutritional and medicinal properties [2-4]. Due to these properties, *H. rhamnoides* L. is domesticated around the globe [5,6]. It is a very important medicinal remedy which is considered to be a rich source of a large number of phytochemicals, nutrients and bio-active constituents [7,8]. The bio-active compounds include vitamins, carotenoids, phytosterols, poly unsaturated fatty acids, organic acids, mineral components and essential amino acids [7-10]. SBT has been used in Europe and Asia for pharmaceuticals, foods and therapeutic purpose for centuries. SBT leaves, berries and seeds are well known for their medicinal properties [11-14].

The known medicinal properties of SBT include immune-modulatory, anti-stress, radio protective, antioxidant, tissue regeneration, anti-atherogenic, hepato-protective property and improving functions of blood circulation and digestive system [4,15-26]. It is interesting to note that the aqueous extract of SBT has recently been reported to possess hypoglycemic activity [27]. SBT contains a lot of different bioactive constituents with multiple properties which have ability to prevent postprandial hyperglycemia [28]. Flavonoids from fruits and seeds of SBT can cause hypotipidemia and hypoglycemia [29]. The leaves of methanolic extracts of SBT contain compounds showing alpha-glucosidase inhibition activity which may be used in diabetes because it has the ability to control inhibition activity [30].

Methodology

General instrumentation

UV spectra were obtained using a Hitachi-U-3200 spectrophotometer. IR spectra were recorded on a Fasco A-302 spectrophotometer. 1D- and 2D NMR spectra were obtained on AM-500, Bruker spectrometers in CD₃OD and CDC₁₃, using tetramethylsilane (SiMe₄) as internal standard. Mass spectra (EI-MS and HR-EMS) were analyzed on a Mass AB SCIEX spectrometer QSTAR xl. Column chromatography was carried out by using silica gel 60 (Merck, 70–230 mesh), TLC was...
carried out on silica gel 60 PF254 (Merck), with detection by UV at 254 nm and 366 nm.

**Plant material**
The Sea buckthorn berries (8.5kg) were collected randomly in August-September from Gilgit, north of Pakistan in September 2019. The plant was identified by a botanist from Department of Botany, University of Karachi. A voucher specimen (No.G.H.No. 84346) was deposited in the Herbarium, Department of Botany, University of Karachi.

**Extraction and isolation**
The berries were dried at room temperature and soaked in methanol for 72h (3times). The extract solution was filtered and concentrated under vacuum. The extract was suspended in water (0.5L) and partitioned with ethyl acetate to give the ethyl acetate-soluble part (111.0g). The ethyl acetate fraction was then fractionated with 4% Na2CO3 and 30 % HCl to obtain acidic and basic fractions. The basic fraction was dried over Na2SO4 washed with water and evaporated the solvents under reduced pressure and obtained a neutral fraction. Both the acidic and neutral fractions were partitioned with hexane to get hexane insoluble and soluble fractions.

The ethyl acetate acidic hexane insoluble part (10g) was subjected to a silica gel column chromatography, using a gradient solvent system of Pet. ether:EtOAc: CH2Cl2: MeOH with increasing polarity to give 20 fractions which were combined on the basis of TLC and obtained eight sub-fractions (HRF 1-8). The fraction 2 (1.02g) was fractionated into four sub-fractions through normal phase CC. Compound 1 was purified from the fraction HRF-2D using hexane/ethyl acetate (5:5 v/v) as solvent system. Fraction 4 (1.48g) was further subjected to column chromatography using Pet. ether/EtOAc as solvent system (8.5:1.5 v/v) and obtained compound 2. Compound 3 was purified from fraction 4 using CH2Cl2: MeOH (9:1 v/v) as solvent system through normal phase CC (Figure 1).

![Figure 1: Structure of compounds 1, 2 and 3.](image)

**Sulforhodamine B assay**
The growth inhibitory activities of compounds 1, 2, and 3 were evaluated against human non-small cell lung (NCI-H460) and breast (MCF-7) cancer cell lines by using sulforhodamine-B assay for the determination of IC50 values. The concentration of the test agents that inhibits 50% of the cell growth was referred as IC50. For this, the cells (10000cells/100µL) from respective cell lines were incubated in 96 well plates for 24h at 37ºC in 5% humidified CO2 incubator. The stock solutions of compounds 1, 2, and 3 (20 mM) in DMSO, and doxorubicin (1mM) in distilled water were prepared. A range of dilutions for 1 (1, 10, 25, 50 and100µM), 2 (1, 2.5, 5, 7.5 and 10 µM) were added (100µL) in respective wells. The pre-determined IC50 value of doxorubicin (5µM) was used as positive control (data for range of dilutions are not shown). After completion of 48h, ice cold TCA (50µL, 50%) was added and left at room temperature for 30min.

DNA docking studies
To perform the DOCK calculations that predict the best orientations of the ligand in the binding site of the receptor we require preparing the receptor and ligand as inputs. UCSF Chimera is a visualizing tool used here to prepare the receptor and ligand with its Dock Prep tool option [31]. The structure of DNA PDB ID: 1d29 was selected as receptor and downloaded from Protein Data Bank (PDB) [32]. The three compounds are taken as ligands to check if they are making any interactions with the DNA moiety. Hydrogen atoms and partial charges were added to both ligand and receptor. AM1-BCC charges were added to receptor, gasteiger charges were added to ligand and files were saved in mol2 format as program DOCK read file in Mol2 format. Molecular surface of receptor was prepared by DMS program. The DOCK accessory program spgen was used to generate the spheres with a probe radius of 1.4 angstrom. It generated 12 clusters and the cluster 1 with maximum number of spheres i.e. 38 was selected for construction of box with the help of show box DOCK accessory program. The maximum 38 spheres in cluster 1 were retained for docking (Figure 2).

![Figure 2: Surface diagram of DNA (PDB ID: 1d29) representing the cluster 1 with maximum numbers of violet colored spheres.](image)
The maximum number of orientation was set as 1000 and maximum number of conformations was kept 10 for each compound.

**Electrophoretic mobility of plasmid pBR322 DNA**

Electrophoretic mobility of pBR322 plasmid DNA was performed to assess the direct interaction of compounds with DNA. Briefly, agarose gel (1%) was prepared in 1× TAE buffer (70mM) by heating for 1minfollowed by its casting in gel tray at room conditions. pBR322 DNA (500ng, 5µL/well) was mixed either with PBS (control) or with compounds 1, 2, 3 (2.5µM), and doxorubicin (250 nM) and placed at 37°C for 30min. Loading buffer (3µL, bromophenol blue, xylene cyanol and glycerol in a ratio of 1:1:120) was applied to the above reaction mixture. This sample was loaded in the wells of the agarose gel and electrophoresis was performed in TAE buffer at 70 V for 1.5h. The gel was dipped in ethidium bromide solution (5µg/µL) for 20min and washed with tap water. The mobility pattern of the circular DNA was observed under UV light and photographed.

**Figure 3: Grid box setting.**

**Statistical analysis**

Duncan’s multiple range test using SPSS 17 program. The data was analyzed by using one-way ANOVA with p<0.05level was considered as significant followed by.

**Results and Discussion**

Compound 1 was purified as white powder having the molecular formula C_34_36_38_40_42 H_50_52_54_56 O_9_11_13. The IR spectrum showed absorption peaks at 3486cm\(^{-1}\). The 1H-NMR spectrum showed characteristic bands of carbohydrates and hydroxyl groups. The UV spectrum showed maximum absorption at 260, 257, 254, 252, 249 nm. The mass spectrum showed the molecular ion peak at m/z 503.1 (M+1). The NMR spectral data of compound 1 was assigned on the basis of COSY, HMBC and HSQC correlations and are given in Table 1. The appearance of an ester carbonyl group at 175.8 ppm in the 13C NMR spectral data of compound 1 suggested the presence of an ester functional group and its location was identified at C-28 by the key COSY and HMBC correlations as shown in Figure 4.

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</table>

Table 1: NMR spectral data of compound 3 (1H-NMR 500 Hz, 13C NMR 125 MHz).

A search in literature found that the spectral characteristics of 3 were consistent to the reported literature values of Corosolic acid except the aromatic spectral data that confirmed by the key COSY and HMBC correlations as shown in Figure 3. The correlation between protons and carbon of compound 3 are summarized in table 1.

![Figure 4: HMBC correlation of compound 3.](image)

**Figures 5 and 6** depicts compound 1 was not effective against non-small cell lung cancer cell line (NCI-H460) while it displayed growth inhibition against breast cancer cells (MCF-7) with IC50 value of ~43 µM. In addition, compound 2 and 3 exhibited growth inhibition against both non-small cell lung and breast cancer cells. In lung cancer cell line (NCI-H460), HI-6 exhibited IC50 value of ~2.8 µM which was ~2x more potent than that of HI-7. However, in case of breast cancer cells (MCF-7), both were equipotent with IC50 value of ~3 µM (Figure 7).

![Figure 5: Growth inhibitory effects of HI-2, HI-6, and HI-7 on non-small cell lung cancer (NCI-H460) cell line.](image)

After successful docking runs, the best docked conformation for each compound was viewed in UCSF chimera by using View Dock tool option. All three compounds were docked in the minor groove of DNA model (Figure 8).

![Figure 6: Growth inhibitory effects of comp. 1, 2 and 3 against breast cancer (MCF-7) cell line.](image)

Structure analysis of each compound was done by Structural analysis tool. We did not find any hydrogen bond interactions between DNA and our top ranked conformations of three compounds. They formed weak interactions such as van der waal and hydrophobic interactions with the DNA. We also docked the Doxorubicin by following the same protocols. It produces its anticancer activity through intercalation mode. The doxorubicin was also docked in the minor groove but at the different position (Figure 9).

![Figure 7: Growth inhibition in the presence of test agents against human non-small cell lung (NCI-H460) and breast (MCF-7) cancer cell lines. Non-small cell lung cancer cells (NCI-H460): compound 2 (a) and compound 3 (b), breast cancer cells (MCF-7): compound 1 (c), compound 2 (d), compound 3 (e). IC50 values: compound 1 ( ), compound 2 ( ) and Compound 3 ( ).](image)

The docking of doxorubicin in minor grooves suggests that minor groove binding is the dominant pre-intercalation step (Lei, 2012 #17). The rigid scores of all compounds and doxorubicin are given in Table 2. The high negative value of doxorubicin indicates that it is a strong DNA binder than our compounds. The docking outcomes also correlate with the gel electrophoresis run where doxorubicin is showing high affinity as compared to our three compounds.
References


