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Identification and quantitation of ursolic acid in *Plectranthus amboinicus* extract; molecular docking approach for its antiproliferative potential

Mona M. AbdelMohsen[a*], Ibrahim A. Salah[a], Heba Handoussa[b], Yasmine M. Mandour[c]

[a] *Chemistry of Medicinal Plants Department, National Research Centre, 33 El-Bohouth St., Former El Tahrir St., P.O.12622, Dokki, Giza, Egypt.*

[b] *Pharmaceutical Biology Department, Faculty of Pharmacy and Biotechnology, German University in Cairo, Cairo, 11835, Egypt.*

[c] *School of Life and Medical Sciences, University of Hertfordshire hosted by Global Academic Foundation, New Administrative Capitol 11578, Cairo, Egypt.*

Abstract

High performance liquid chromatography (HPLC) with UV detection has been employed for the quantitative determination of ursolic acid in the ethyl acetate extract of the aerial parts of *Plectranthus amboinicus.* The antiproliferative activity of the extract and ursolic acid against MCF-7 was tested *in vitro* by MTT assay. Molecular modeling using MOE was employed to check the binding mode of ursolic acid (UA) with the crystal structures of the Protein tyrosine phosphatase 1B (PTP1B) enzyme complexed with the orthosteric sulfamic acid inhibitor (PDB: 2F71). Ursolic acid was detected in the ethyl acetate extract at 203 nm with retention time 6.98 min and the HPLC analytical methodology for standard curve was developed and validated to quantify ursolic acid as 3.96 mg/g DW. The ursolic acid and extract were highly active against MCF-7 cell line with IC_{50} 22.4± 0.64 µg/ml and 43.1± 2.3 µg/ml respectively. Molecular study emphasized the influential ursolic acid fitting in the binding site of PTP1B enzyme suggesting its inhibition to be responsible for the *in vitro* anticancer activity.

These findings justify the use of *Plectranthus amboinicus* against cancer and strengthen its selection for the discovery of natural anticancer agents. Also, the results provide important guidance for the potential pathway of ursolic acid as anticancer compound.

Keywords: *Plectranthus amboinicus;* Ursolic acid; HPLC; Anticancer; Molecular modeling.

Introduction

Breast cancer is one of the most prevalent cancers worldwide. A variety of approaches has been studied in cancer chemoprevention, one of which is based on the intervention during the progression of carcinogenesis by administrating one or more naturally occurring and/or synthetic compounds as an adjuvant to treatment [1]. Triterpenoids represent a group of natural substances that have shown to possess a multiplicity of biological effects including anti-inflammatory and anti-carcinogenic properties combined with low toxicity [2]. These compounds have been evaluated to be used in functional foods, drugs and other healthcare products [3]. Ursolic acid (UA) is a pentacycle triterpene which can exist as a free acid, found commonly in Lamiaceae plants as secondary metabolite [4]. It is reported to have several beneficial bioactivities including anti-inflammatory, hepatoprotective and as an antitumor [5].

Plectranthus amboinicus (Lamiaceae) is a medicinal plant widely used in India, China, and Brazil, to treat several diseases [6] and from there is propagated and widely cultivated throughout the world tropics and Africa. It is a large succulent aromatic perennial herb with fleshy highly distinctive smelling leaves [7]. It is a popular house plant as it is easy to grow in gardens and can survive various conditions [8]. It is a folkloric medicinal plant used in the treatment of a wide range of diseases due to the presence of a variety of phytochemicals [9, 10]. In this study, we focused on the identification and quantitative estimation of ursolic acid in the aerial parts methanol extract of *Plectranthus amboinicus* by HPLC-photodiode array detector. Verifying breast anticancer activity of the crude extract and ursolic acid by MTT assay, and furthermore, point out a

*Corresponding author e-mail: monaamohsen@yahoo.com
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route of mechanism of its activity through molecular docking analysis.

Experimental

Plant material and extract preparation

Fresh aerial parts of *Plectranthus amboinicus* were collected from El-Orman Botanical Garden. The plant material was verified by Dr. Mohammed El-Gibali Senior botanist according to its taxonomy (a voucher specimen are deposited at the herbarium of the NRC). The plant parts were washed and air dried at room temperature and coarsely powdered. The powdered plant was extracted with methanol using ecofriendly ultrasonic-assisted extraction (UAE) technique to result in high yield in optimal time [11, 12]. The extract was filtered and concentrated under reduced pressure at 45 °C. The crude extract was dissolved in 50% methanol and subjected to liquid-liquid extraction with hexane followed by ethyl acetate (3 times each) using a separating funnel. After re-extraction for three consecutive times, the combined ethyl acetate extracts were concentrated, lyophilized for further analysis.

Preparation of standard solution and crude extract

Ursolic acid (> 95% purity, obtained from Sigma Aldrich) was used as an analytical standard for high performance liquid chromatography (HPLC). A stock solution of 1 mg/mL was prepared in methanol for UA standard. A serial dilution was made on the stock solution with methanol to prepare standard solutions at concentrations of 5, 10, 15, 20 µg/mL, from each of which 20 µL was used for plotting analytical chromatograms to obtain the standard curve for UA. The ethyl acetate residue was dissolved in 1 mL methanol and transferred to an Eppendorf tube. The crude extract was filtered through a 0.22 µm micro-filter before HPLC analysis.

HPLC Analysis of UA

Standard concentrations were injected into HPLC system to establish standard calibration curve. Analyses were developed by HPLC system (Agilent Technologies, Waldbronn, Germany, modular model 1200 series instrument), equipped with Eclipse DBC_{18} column (5 µ m, 4.6 x 250 mm i.d.) . Isocratic elution was carried out with a mobile phase consisting of acidified methanol and acetonitrile in the ratio of 90:10 (v/v), at a flow rate of 1 ml/min. Detection was done using a diode array detector (DAD) at 203 and 210 nm. Ursolic acid was detected at 203 nm at room temperature with an eluent flow rate of 1.0 ml/min and an injection volume of 20µL, as previously described [13].

Cytotoxic activity against cancer cell line

All materials and reagents for the cell culture were purchased from Lonza (Verviers, Belgium). Breast carcinoma (MCF-7) was obtained from VACSERA Tissue Culture Unit. The cell line was maintained as monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The monolayers were passaged at 70–90% confluence using a trypsin-EDTA solution. and maintained at 37◦C with 5% CO_2 in a humidified atmosphere. The cytotoxic activity of ursolic acid and ethyl acetate extract were measured by MTT assay [14]. Cytotoxicity was determined according to the percent cell viability using MTT assay, and half maximal inhibitory concentration (IC_{50}) was calculated from the growth inhibition curve.

Statistical analysis

The IC_{50} values (concentration of sample causing 50% loss of intact cells of the vehicle control) were presented as mean values ± (SD) and were calculated using the concentration-response curve fit to the non-linear regression model using GraphPad Prism® v6.0 software (GraphPad Software Inc., San Diego, CA, USA).

Computational study of the isolated compound

Protein and ligand preparation

The crystal structures of the Protein tyrosine phosphatase 1B (PTP1B) enzyme complexed with the orthosteric sulfamic acid inhibitor (PDB: 2F71) was downloaded from the Protein Data Bank (PDB) database [15]. The proteins were prepared with the structure preparation wizard in MOE (version 2019.01) and saved as a mol2 file [16]. The 3D structure of the compound (ursolic acid) was built and minimized using the MMFF94x force field in MOE using a gradient of 0.0001 kcal/mol Å).

Molecular docking

Docking was performed using GOLD (version 5.8) [17, 18]. Binding site residues were defined by specifying the inhibitors' crystal structure coordinates using the default cutoff radius of 6 Å, with the "detect cavity" option enabled. GOLD docking experiments were performed using the GoldScore scoring function. The search efficiency of the genetic algorithm was at 200% setting with the receptor kept rigid. For ursolic

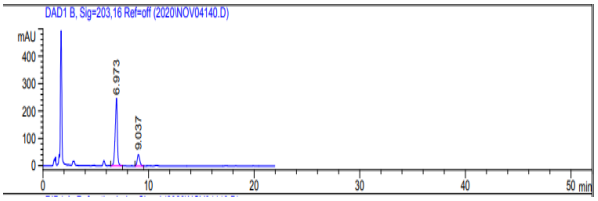
acid, 50 complexes were generated and clustered based on its RMSD with the threshold set at 0.75 Å using the complete linkage method. The best-ranked pose from the most populated cluster was selected as the final pose. The quality of pose prediction was assessed by calculating the heavy atom RMSD between the docked poses and the original PDB coordinates of the sulfamic acid inhibitor (Table S1, Supporting Information). The figures were prepared using PyMol [19].

Results

HPLC analysis

HPLC-DAD was applied for the analysis of ursolic acid in ethyl acetate extract. Figure 1(A) depicts a reprehensive chromatogram of the standard ursolic acid at 203nm, the chromatographic retention time was 6.97 min, and 1(B) depicts the chromatogram demonstrating the detection of ursolic acid in the extract under the optimized conditions. Quantitation was achieved against a standard curve of ursolic acid obtained from the analytical chromatograms at 203 nm. The linear regression data from the calibration curve was plotted over the range of 5- 20 µg/ml, r^2 =1. The yield of ursolic acid was 3.96 mg/g DW of plant material.

A



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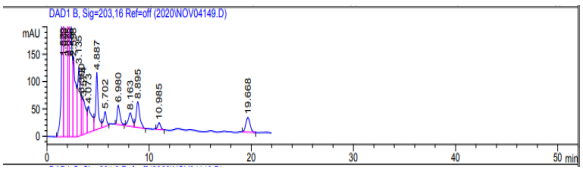


Figure 1. HPLC chromatograms. A: Ursolic acid standard; B: Ethyl acetate extract of aerial parts of *Plectranthus amboinicus.*

Cytotoxic activity

The cytotoxic activity of ursolic acid and ethyl acetate extract against breast carcinoma cells (MCF-7) was measured by MTT assay. The IC_{50} values were calculated using the concentration-response curves as demonstrated in Figure 2. The results showed high activity with IC_{50} values 22.4± 0.64 µg/ml and 43.1± 2.3 µg/ml for UA and the extract respectively, while Doxorubicin, the Reference Standard, showed IC_{50} value 0.35 ± 0.03 µg/ml.

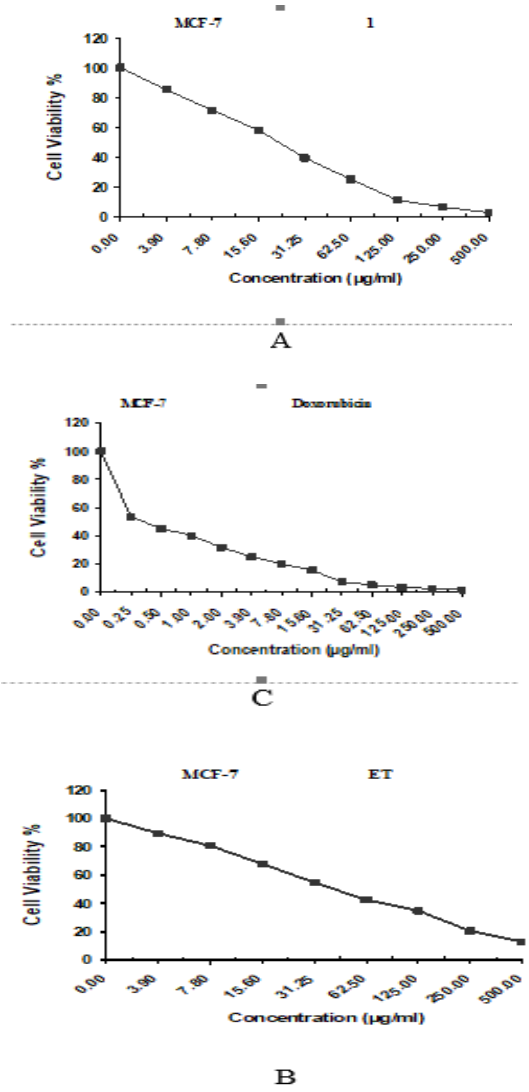


Figure 2. The concentration-response curves of A: Ursolic acid; B: Ethyl acetate extract; C: Doxorubicin (Reference Standard).

Molecular docking

Overexpression of PTP1B enzyme in tumor tissues was shown to contribute towards the pathogenesis of cancer implying a great potential of PTP1B enzyme inhibitors in cancer therapy [20]. Molecular docking analysis was performed in an attempt to explain the observed anticancer activity of ursolic acid. The crystal structure of PTP1B showed the presence of a catalytic binding site (site A) and a second binding site (site B). Site "A"; having the catalytic Cys215 residue; is defined by the P loop (residues His214-

Arg221) and is surrounded by four other loops: the WPD loop (residues Thr177-Phe185), the phosphotyrosine recognition loop (residues Asn40-Tyr46), the Q loop (residues Gln262-Phe269), and the R loop (residues Leu110-Cys121). Site "B" lies adjacent to site "A" and is defined by residues: Tyr20, Arg24, His25, Ala27, Phe52, Arg254, Met258 and Gly259. It is difficult to achieve inhibition selectivity against PTP1B by targeting the catalytic site "A" due to its high sequence similarity with other proteins. On the other hand, site "B" appeared more promising for designing selective ligands. Structural docking studies were conducted to check if there is preferential binding of ursolic acid for one site over the other.

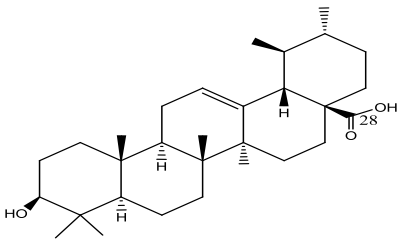
The structure of PTP1B enzyme complexed with the orthosteric sulfamic acid inhibitor (PDB: 2F71) was used to check the binding mode of ursolic acid. To validate the docking protocol, the cocrystallized sulfamic acid inhibitor was docked into the orthosteric site of the PTP1B enzyme (Figure 3A). All the resultant poses converged to a binding mode similar to that of the experimentally determined position with the best ranking pose having a root mean square deviation (RMSD) value of 0.983 Å (Figure 3A). Docking studies revealed ursolic acid binding into site "B" with no binding poses found binding into "site A". In this binding model, the carboxylic group of ursolic acid at C_{28} (Figure 3) formed ionic interactions with the basic sidechains of Arg24 and Arg254 in addition to a water-mediated hydrogen bond with the backbone carbonyl of Gln262. Other residues that form van der Waals contacts with the triterpene are Val49 and Met258 (Figure 3B). These favorable interactions suggest that PTP1B enzyme might be a key player for the observed anticancer activity of ursolic acid.

Discussion

Despite a lot of improvements in the management of cancer in the last decade, prophylaction, treatment and cure from cancer is still under question, therefore, it is imperative to elucidate novel therapies with fewer side effects. A large number of potential cancer chemo preventive agents which are chemicals or dietary compounds have been identified and functioned by different mechanisms to block, inhibit or reverse the development of cancer in normal or pre-neoplastic tissues and interfere different stages of carcinogenesis [21]. Investigations have revealed the anticancer properties of medicinal herbs and plant extracts through different pathways including immune activation, induction of DNA repair systems, carcinogen metabolism and suppression of cell cycle progression or induction of apoptosis [22]. Herbs and spices have been utilized in traditional medicines for thousands of years and the majority of population in Africa and developing countries still depends on these medicines for their primary health care. Besides, the scientific studies of plants used in ethnic practice have led to the discovery of many valuable drugs [23, 24]. The Lamiaceae or Labiatae consists of 210 genera, 3500 species and widely used culinary herbs, such as rosemary, sage, oregano, peppermint and many others from which several herbal products have shown potential anticarcinogenic properties [25, 26]. *Plectranthus amboinicus* is an edible, nontoxic, well-known plant belongs to family Lamiaceae, it contains flavonoids, rosmarinic acid, phenolic compounds and has been demonstrated for the treatment of various diseases [27, 28].

In our study, the aerial parts of *Plectranthus amboinicus* were extracted in terms of green and environmental chemistry with methanol using UAE technique which constitutes one of the simplest and most convenient extraction processes employing mechanic vibrations generated by sound waves (>20 kHz) for extracting bioactive compounds in high yield [12]. The methanol residue was successively fractionated with hexane followed by ethyl acetate. This two-step procedure was applied to separate possible less polar triterpenes (aldehydes and alcohols) from the more polar (acids). Ursolic acid was analyzed quantitatively in the ethyl acetate extract by RP-HPLC using a diode array detector (DAD) at 203 nm. Typical calibration curve was prepared using serial dilutions of standard (5- 20 µg/ml) from stock solution and plotting peak area (y) against injected amount (x, µg). The calibration curve had correlation coefficient, R_2 =1. The content of UA was quantified as 3.96 mg/g DW of the plant material which is about the same as *Salvia* species (4.34 ± 0.1 to 3.71 ± 0.08 mg/g DW) [29]. In accordance, with the results that *Plectranthus amboinicus* is an antitumor agent when used in combination with other anticancer drugs [30] and in an effort to investigate the effect of UA on the inhibition of the growth of MCF-7 cells [31, 32], the antiproliferative activity of the ethyl acetate fraction of *Plectranthus amboinicus* against MCF-7 human cancer cell line was investigated and it exhibited IC_{50} value (43.1± 2.3 µg/ml). Also, the antiproliferative activity of ursolic acid as an influential constituent of the crude extract was investigated and the results confirmed that ursolic acid exhibited significant activity with IC_{50}

value (22.4± 0.64 µg/ml) on comparison with doxorubicin which exhibited IC_{50} value (0.35± 0.036 µg/ml).



Ursolic acid

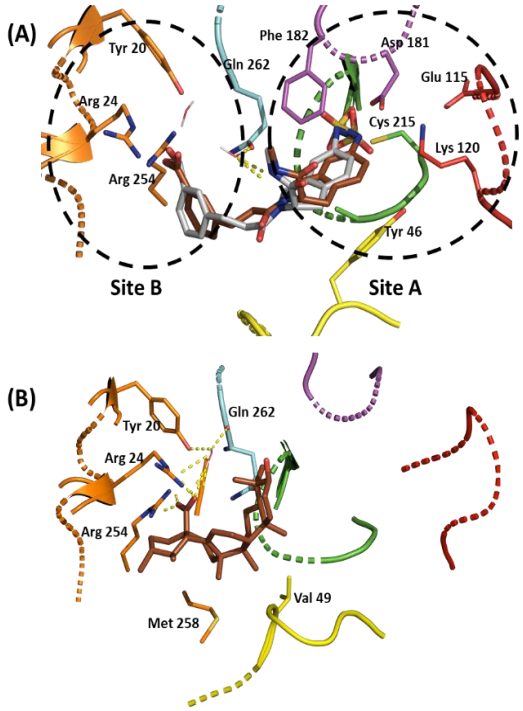


Figure 3: Ursolic acid. Graphical representation of the orthosteric binding pocket of PTP1B enzyme, showing residues from the P loop B (green), WPD loop (magenta), phosphotyrosine recognition loop (yellow), Q loop (cyano) and R loop (Red) of site "A" and residues of site "B" (brown). (A) Overlay of the docked pose (brown) and PDB coordinates 2F71 (grey) of sulfamic acid inhibitor. (B) Docked pose of ursolic acid. Only side chain atoms are shown for clarity.

Several pharmacological studies have demonstrated different pathways of ursolic acid to inhibit proliferation of breast tumor cells, induce apoptosis and autophagy of the cells without normal cell damage [33- 35]. In this study, molecular docking revealed the potential of ursolic acid to bind at the active site of PT1B enzyme, a key player in breast cancer. Ursolic acid showed preferential binding to the less conserved "site B", making it a promising lead for designing selective PT1B inhibitors. Molecular docking is a computer simulation method for studying the binding mode of ligands and receptors and the mechanism of interaction between molecules. This network pharmacological approach has been recommended for carrying out definite studies on the interactions between active compounds of natural products and diseases to explore drug targets [36, 37].

Conclusion

Therefore, these results emphasize the importance of *Plectranthus amboinicus* as a potential non-toxic adjuvant to anticancer therapy and support the pledge of ursolic acid as a promising chemotherapeutic agent for breast cancer.

Conflict of interest statement

There are no conflicts to declare.

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