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INVITRO AND *INSILICO* ANALYSIS OF POTENTIAL ANTIOXIDANT FROM *BERBERIS ARISTATA* AND ITS SUBSTITUTES

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ABSTRACT

Antioxidative properties of extracts from plants have become a great interest due to their possible uses as natural additives to replace synthetic ones. The antioxidant potential of the plant extracts resulted by DPPH, ABTS and FRAP assays indicated that the extracts not only possess antioxidant activity but also the free radical scavenger capability. The utility and choice of *in silico* screening tools are dependent on the reason for their use and/or the nature of the output generated. The interaction profile of PKC alpha protein and Berberin complex indicates that the Berberin interacts at Phenylvaline and valine residue of protein showing that the Berberine has hydrophobic, non polar interaction whereas non covalent interaction via hydrogen bonding at the mentioned active site residues of protein. The results were further performed regarding the protein-ligand complex stability by performing a Molecular Dynamics simulation run of the complex for 100 ps using MacroModel module of Schrodinge suite. The simulation run showed that the complex is stable with time evolution. The *in silico* study of protein-Berberine complex rendered clear picture of effective interaction of Berberine with the protein.

KEYWORDS: Berberine, antioxidant, PKC-alpha, Docking, Molecular dynamics, DPPH.

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INTRODUCTION

Antioxidant assays differ from each other in terms of substrates, probes, reaction conditions, and quantitation methods. It is extremely difficult to compare the results from different assays [1]. In the meantime, new assays claiming to measure antioxidant capacity continue to be reported [2]. Medicinal plants have been explored therapeutically in traditional medicines and are a valuable source for drug discovery. Insufficient knowledge about the molecular mechanism of these medicinal plants limits the scope of their application and hinders the effort to design new drugs using the therapeutic principles of herbal medicines. This problem can be partially alleviated if efficient methods for rapid identification of protein targets of herbal ingredients can be introduced. Efforts have been directed at developing efficient computer methods for facilitating target identification [3]. Berberine, isolated from *Berberis aristata* is an isoquinoline alkaloid and is the chief active principle implied for the various biological activities [5] and is present in all market samples sold as 'Daruharidra'. Ethanobotanical studies indicate that the decoction of the leaves is commonly used to treat skin disease, menorrhagia, cholera, jaundice, ear infection and urinary tract infections [4]. The root employed as an antiperiodic, diaphoretic antipyretic and its action was believed to be as powerful as quinine, while the bark is used as a tonic and antiperiodic. The medicinal value of *Berberis* has also been proved by various pharmacological studies. The computing resources considered included the various databases currently available and the software that has been or might be used in analyses these data. The various different kinds of databases identified as relevant included those holding ethnobotanical and/or chemical and/or pharmacological and/or toxicological data on the herbs used in medicine, as well as those that hold data on known or potential molecular targets for the herbal constituents. The software tools considered as relevant included programs that provide for (a)Virtual screening of natural product libraries and chemical libraries; (b) pattern recognition and (c) bioinformatics tools. This present approach focused on the pharmacological studies yielding berberine as a potent therapeutic agent effective as an antioxidant under laboratory conditions and through computational biology.

MATERIALS AND METHODS

1. Plant collection

The authentic drugs (*B. aristata*, *B. asiatica*, *B. lycium*, *C.fenestratum* and *M. umbellate*) were obtained from the Repository of Medicinal Resources, Foundation for Revitalisation of Rural and Local Health Traditions (FRLHT), Bangalore, Karnataka, India. Stem samples sold as 'Daruharidra' procured from eight different raw drug markets in South India (Table 1).

Table 1
Sources of the plants used in this study

Sample No.	Type of the Sample obtained	Place of Collection
P1	Market sample	Trivanthapuram, Kerala, India
P2	Market sample	Coimbatore, Tamilnadu, India
P3	Market sample	Trishur, Kerala, India
P4	Market sample	Coonoor, Tamilnadu, India
P5	Market sample	Chennai, Tamilnadu, India
P6	Authetic sample (*B. aristata*)	FRLHT, Bangalore, India
P7	Authetic sample (*B. asiatica*)	FRLHT, Bangalore, India
P8	Authetic sample (*B. lycium*)	FRLHT, Bangalore, India
P9	Authetic sample (*Coscinium fenestratum*)	FRLHT, Bangalore, India
P10	Authetic sample (*Morinda umbellata*)	FRLHT, Bangalore, India

2. Extraction of plant material

The extracts of both the authentic and the market samples were carried out using methanol [6].

3. Antioxidant study of berberine

Free radicals are highly reactive chemical species indicated species indicated in various diseases and ailments. There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effect of free radicals in the human body, and to prevent the deterioration of fat and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources [7]. There is therefore a parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants [8]. One such method that is currently popular is based upon the use of the stable free radical diphenylpicrylhydrazyl (DPPH). It concerned with the actual sufficiency of these substances either as antioxidants or antioxidants or as “life-style enhancers” in humans [9].

3.1 DPPH Assay

The molecule of 1, 1 –diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare election over the molecule as a whole, so that the molecules do not dimerize, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour , characterized by an absorption band in ethanol solution centred at about 520nm. When a solution of DPPH is mixed with that donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Representing the DPPH radical by Z°and the donar molecule by AH, the primary reaction is

$$Z^\circ + AH = ZH + A^\circ$$

Where ZH is reduced form and A° is free radical produced in the first step. The stoichiometry, that is, the number of molecules of DPPH reduced (decolorized) by one molecule of the reductant. The reaction is therefore intended to provide the link with the reactions taking place in an oxidising system, such as the autoxidation of the lipid or other unsaturated substance; the DPPH molecule Z° is thus intended to represent the free radicals formed in the system whose activity is to be supposed by the substance AH. Equal volumes of sample volume and the DPPH were added to an equal amount of water and the optical density is measured at 517 nm.

3.2 ABTS Assay

The Zen-Bio ABTS Antioxidant Assay Kit can be used to determine the total antioxidant capacity of biological fluids, cells, and tissue. It can also be used to assay the antioxidant activity of naturally occurring or synthetic compounds for use as dietary supplements, topical protection, and therapeutics. The assay measures ABTS.+ radical cation formation induced by metmyoglobin and hydrogen peroxide. Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control inhibiting the formation of the radical cation in a dose dependent manner. The antioxidant activity in biological fluids, cells, tissues, and natural extracts could be normalized to equivalent Trolox units to quantify the composite antioxidant activity present. This assay measures radical scavenging by electron donation and when combined with Zen-Bio’s ORAC antioxidant assay kit, provides a comprehensive analysis of a test sample’s antioxidant activity. A ferryl myoglobin radical was formed from metmyoglobin and hydrogen peroxide. The ferryl myoglobin radical can oxidize ABTS (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, ABTS.+, that is green in color and can be measured by absorbance at 405nm. Antioxidants suppress this reaction by electron donation radical scavenging and inhibit the formation of the colored ABTS radical. The concentration of antioxidant in the test sample is inversely proportional to the ABTS radical formation and 405nm absorbance. The ABTS solution cooled at 4^{o}C and allowed it to come to room

temperature. The contents of the 1.5 mM Trolox standard tube after thawing were spun briefly. 80µl of Assay Buffer is vortexed with 1.5 mM Trolox standard tube provided. This produces a diluted stock Trolox standard of 300µM. 50µl of assay buffer into 6 tubes is pipetted. The 300µM stock dilution serves as the highest standard, and the assay buffer serves as the zero standard. The Myoglobin Working Solution is prepared by mixing 25µl Myoglobin stock solution to the Dilution Buffer bottle (contains 2.475 ml) and gently inverted. 10µl of samples or Trolox standards to individual wells of the assay plate provided to which 10µl of assay buffer was added to individual wells as a negative control. 20µl of the myoglobin working solution is added to each of the wells containing standards and samples. To begin the assay, 100µl of the ABTS solution per well is added and placed on a plate shaker at room temperature. The reaction is allowed to proceed for 5 minutes after which 50 µl of Stop Solution is added to each well. The absorbance was read using a plate reader at a wavelength of 405 nm.

3.3 FRAP Assay

The DetectX® Ferric Reducing Ability of Plasma (FRAP™) Assay Kit was designed to quantitatively measure antioxidant status in a variety of samples. The assay measures the antioxidant ability of all species. A Ferrous Chloride standard is provided to generate a standard curve for the assay and all samples should be read off of the standard curve. Samples were diluted in the provided assay buffer and added to the wells. The FRAP color Solution is made by mixing Reagent A and B with Assay Buffer. The FRAP Color Solution is added to all wells and the plate incubated at room temperature. Antioxidant power in the samples reacts with the FRAP Color Solution to generate a blue colored product which is read at 593 nm.

The percentage of scavenging activity was measured by the following formula

$$\text{Scavenging Activity} = \frac{[\text{Absorbance of standard} - \text{Absorbance of Sample}] * 100}{\text{Absorbance of sample}}$$

3.4 Insilico analysis

PKC-α has been observed to be involved in many biological responses such as differentiation, cell migration and adhesion, angiogenesis ad tumourigenesis. An effect involving inflammation has been seen in nitric oxide production involved as an inflammatory mediator is enhanced by the overexpression of PKC-α. In order to screen the atomic and molecular effects of berberine as an antioxidant, PKC-α has taken up as a target.

3.4.1 Protein and ligand source

The 3-D crystal structure of Protein Kinase C alpha complex with NVP-AEB071 bearing PDB ID: 3IW4 has obtained from open source Protein Data Bank database [10, 11]. The crystal structure of Berberine compound has obtained from open source NCBI-PubChem compound database [12] (CID: 2353)

3.4.2 Molecular docking

Molecular docking has emerged as a reliable tool for drug discovery and virtual screening. The principle of molecular docking relies on the fact of determining the exact orientation and conformation of ligand while binding to the protein [13]. To examine the binding mode and affinity of Berberine we performed molecular docking study. Berberine compound has docked with protein kinase C alpha using Glide module of Schrödinger Inc. [14]. A ligand may possess several orientations and conformational arrangements. For an efficient binding, a ligand must possess an accurate conformation that fits to the binding surface of the protein. Hence, Berberine compound was prepared prior to docking using the Ligprep tool of Schrödinger Inc. All possible stereoisomers, tautomers, conformers and ionisation states of Berberine were enerated by Epik module [15, 16]. The retrieved crystal structure of protein was preprocessed using a PrepWizard tool of Schrödinger Inc by adding hydrogen atoms, assigning bond order, deleting heteroatoms, protein optimisation and energy minimization These parameters for charge and potential energy calculation

for protein energy minimization were incorporated from MacroModel application of Schrödinger Inc (Schrödinger, LLC, NY, 2013) for processing. The crystal structure of protein obtained from PDB database is a complex with NVP-AEB071 having three chains (A, B and C), four missing side chains and no water molecules. The protein crystal structure was refined by removing the chains B and C and adding missing side chains. A grid for receptor centred at all residues was generated of 22, 22 and 22 Å (x, y and z) dimension. An extra precision, flexible ligand docking was performed using the generated grid.

3.4.3 Protein-ligand complex stability

The dynamical behaviour and its evolution with time can be well evaluated by molecular dynamics simulation of a system, which gives state of a system at each interval of time by generating position and velocities. The stability of a system can be studied by means of molecular dynamics simulation. Hence dynamics simulation of best ligand pose and protein complex obtained after molecular docking was performed for 100 Picoseconds (ps) using MacroModel module of Schrödinger Inc. (Schrödinger, LLC, NY, 2013). The energy minimisation of the system to a low gradient was done using PRCG (Polak-Ribiere Conjugate Gradient) method for 1000 maximum iterations and at 0.05 convergence threshold. The Potential energy was calculated using OPLS_2005 force field [17] and the charges were also taken from OPLS_2005. The Van der Waals and electrostatic calculation were performed at extended cutoff. Total trajectory for 100 samples were generated. The atoms, bond, angles and dihedrals were not subjected to any constraints. After an equilibration run for 10 ps , a total dynamics simulation run for 100 ps was performed at 300 K temperature, in water solvent.

RESULTS AND DISCUSSION

The common feature of all mentioned medicinal plants is their antioxidant activity. The property of an antioxidant is ascribed to the presence polyphenols. Natural antioxidants present scavenge harmful free radicals from our body by either enhancing the body's natural antioxidant defences or by supplementing with proven dietary antioxidants resulting in reduced risk of chronic diseases [18]. However, popular and the most frequent form in which those plants are used is an aqueous infusion or decoction. Its preparation can induce the degradation of polyphenols. Therefore, in this study, we compared the antioxidant properties of authentic and market samples of Daruharidra and investigated their relations to the phenolic content.The antioxidant properties were studied with DPPH and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sul phonic acid) radical scavenging and ferric reducing antioxidant power (FRAP) tests. All the assays were compared with the percentage of scavenging property. The antioxidant assay using DPPH confirms the scavenging percentage of the plant extract to be over 90% confirming the antioxidant potential of the extract. ABTS radical scavenging assay proves the antioxidant capability of the plant extract. Although it is over 70%, the confirmation with the DPPH assay confirms the extract has medicinal uses. FRAP values are not only dependent on species but also the geographical origin and harvest time. Although of different geographical locations all the samples show FRAP antioxidant capability at an average of over 60% (Fig 1, 2, 3). The pose viewer analysis of molecular docking showed that Berberine interacted with Protein kinase C alpha with GlideScore of -5.203 kcal/mol. It possessed two site interactions with protein kinase C alpha. The molecular representation of Berberine pose with protein is depicted in Fig. 3 and the spatial interaction of the same in Fig. 4. It can be observed that compound shows hydrophobic interaction with π electrons of phenyl ring of Phenylalanine residue of Protein kinase C alpha protein having residual atom types PHE 307 (C) and PHE 308 (C) whereas it shows weak non covalent hydrogen bond interaction with valine residue of Protein kinase C alpha having VAL (O) residual atom types. The major binding mode of Berberine with Protein kinase C alpha was identified as π interaction and non-covalent

interaction for its antioxidant activity. Molecular dynamics simulation gave insights of the structural behaviour and stability of Protein kinase C alpha protein and Berberine complex in water solvent and 300 K temperature. After 10 ps equilibration a trajectory for 100 ps was generated for 100 samples (Fig. 6). Root Mean Squared Deviation (RMSD) analysis showed that the complex attains stability after 60 ps and was found stable thereafter throughout the simulation run with constant RMSD of about 6.7 to 6.9 Å. This indicates that the compound Berberine after binding to protein kinase C alpha does not deform the protein and the complex is stable, which is an essential drug factor for a compound to be physiologically stable.

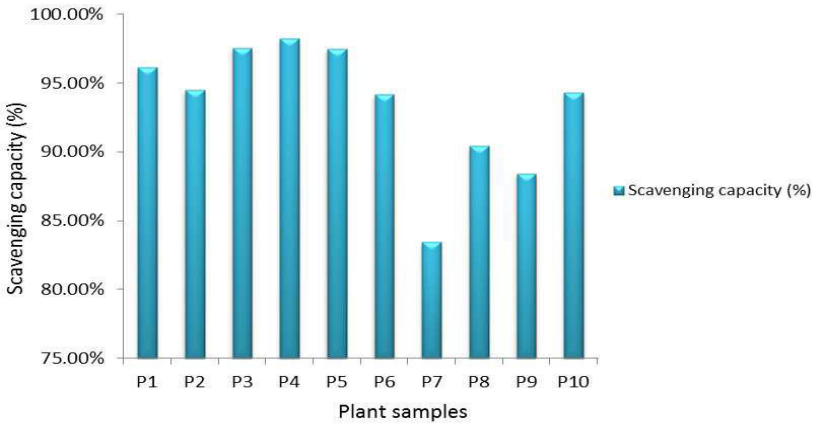

Figure 1
Graphical representation of the DPPH Scavenging Assay

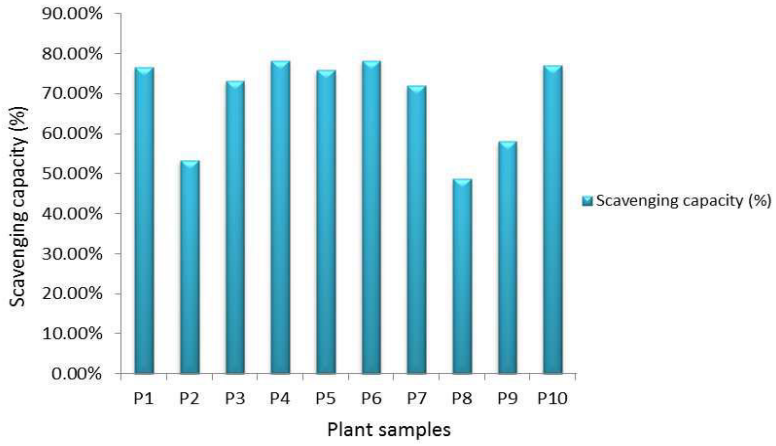

Figure 2
Graphical representation of the ABTS Assay

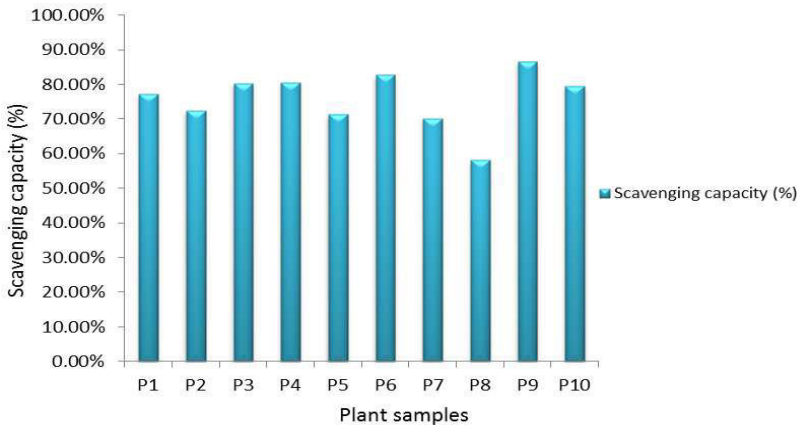

Figure 3
Graphical representation of the FRAP Scavenging Assay

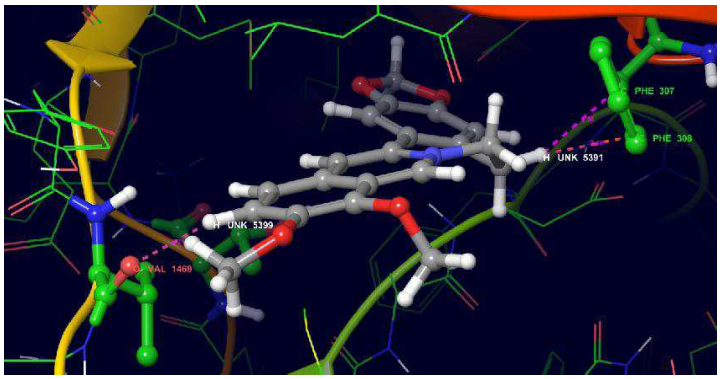

Figure. 4
Interaction profile for Protein kinase C alpha-Berberine Complex obtained after molecular docking.

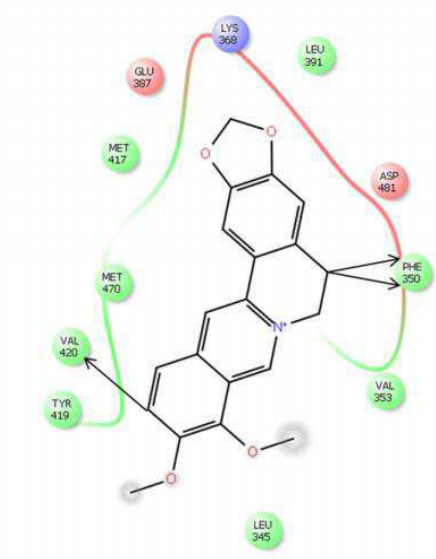

Figure 5
Two-dimensional spatial interaction profile of Protein kinase C alpha and Berberine complex.

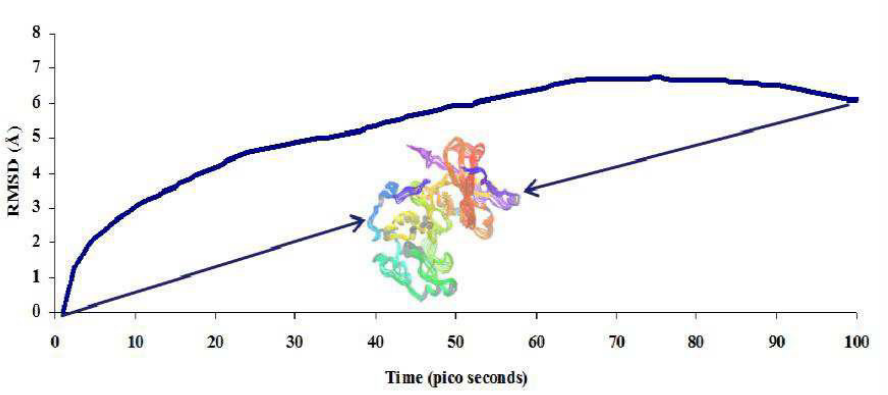

Figure 6
RMSD plot of Berberine-Protein kinase C alpha complex for 100 ps MD simulation run

CONCLUSION

An *in-silico* approach was opted in order to investigate the binding affinity and molecular stability of Berberine with the Protein kinase C alpha protein. The molecular docking study predicted Berberine bound to the protein with the GlideScore of -5.203 kcal/mol. The two major interactions, the π interaction and non-covalent hydrogen bonding were found as Berberine's chief active binding modes with the protein. The protein-ligand complex was found to be stable for 100 ps molecular dynamics simulation run which, further confirmed the stability of the compound after binding with the protein. Designing new compounds by modifying the interacting residues of the compound may enhance binding affinity with the protein and hence probably may lead to compound with better antioxidant property.

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