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Molecular Docking Studies of *Alpinia galanga* Phytoconstituents for Psychostimulant Activity

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Abstract

Dopamine is a neurotransmitter responsible for sending signals from the central nervous system. It allows human beings to stay attentive and focused. Caffeine, the most widely consumed psychoactive substance in the world, is known to improve alertness by enhancing dopamine signaling in the brain. EnXtra®, an *Alpinia galanga* extract has been clinically proven to promote alertness however the mechanism for such action required elucidation. The current study was designed to explore the mechanism for the neurocognitive enhancing property of EnXtra® by the *in-silico* interaction of its potential compounds with various targets involved in such process namely Dopamine and Acetylcholinesterase (AchE). As evident by the outcomes of the study, active compounds of EnXtra® can block the dopamine reuptake thereby increasing the dopamine levels which further can enhance the visuospatial performance and mental clarity, leading to improved mental alertness. At the same time, its strong effect on Acetylcholinesterase target protein is indicative of its nootropic potential.

Keywords

EnXtra®, Caffeine, Dopamine, Enovate Biolife, Energy Drink, Cognitive, *In-Silico*

1. Introduction

Caffeine is a psychostimulant that is known for its ability to activate dopamine release especially in the prefrontal cortex [1]. Dopamine, in turn, increases levels of alertness by blocking adenosine receptors in the forebrain. However, in large doses, caffeine has deterring effects on the cardiovascular and the nervous system. Several attempts have been made to develop another natural product which

strikes the same effect without producing those daunting side effects.

The process of development of the natural products is being modernized, to better understand the mechanism of action responsible for their different physiological and pharmacological effects. Computational methods have been recently shown to be a scientifically valid tool for the study of the pharmacological activity *in vitro* [2] [3] [4]. One of such approaches is the inverse virtual screening, which has been extensively used in facilitating novel bioactive ligand discovery [5] [6] [7].

We describe herein the molecular docking of a database of probable molecules of high chemical diversity present in the aqueous extract of *A. galanga*, against a panel of target proteins responsible for enhancing attention network components.

The extract marketed as EnXtra® has been clinically proven to improve the mental alertness [8] [9]. The current research was conducted by Enovate Biolife, to discover the mechanism of potential nootropic compounds through their interaction with various targets (Dopamine and Acetylcholinesterase) involved in psychoactive functions.

A large number of *in-silico* models are useful for studying the molecules with variable biological activity out of which the inverse virtual screening is one of the most useful methods to provide information regarding ligand-protein interaction, potentially affecting the physiology.

2. Materials and Methods

2.1. Extract

2.1.1. Preparation of Extract

EnXtra®, a dried *A. galanga* rhizome extract, has been standardized using a battery of tests such as microscopy, HPLC and DNA fingerprinting. DNA fingerprinting authenticated the current *A. galanga* source and very well differentiated it from the *A. calcarata*. The raw material was subjected to various quality control (QC) checks such as physical identity, moisture content, water-soluble extractives, oil content and other group actives. The QC-cleared dried rhizomes were pulverized to a specific particle size and then taken for extraction. All oversized rhizomes were rejected and the qualifying raw powder was extracted in an aqueous medium under pressure with a targeted cold and hot temperature cycles. The extraction process was repeated until complete extraction was ensured. Post extraction, the solvent was distilled off and the residue was dried *in-vacuo* to ensure gentle drying and avoid the degradation of active principles. Thus processed extract was further assessed for microbial load and total bioactive components to ensure a compliance with established specifications.

2.1.2. Qualitative Assessment of Bioactive Components

The extract was analysed qualitatively by colour tests for the presence or absence of phytoconstituents belonging to different chemical classes using appropriate tests: Dragendroff's test (Alkaloids), Legal's test (Glycosides), Braemer's test (Tannins), Libermann-Burchard test (Steroids), Shinoda test (Flavonoids), So-

dium bicarbonate test (Saponins), Benedict's test (Carbohydrates), Terpenoid and Polyphenols [10].

2.1.3. GC-MS Analysis

Major fragments were reported in the chromatogram as well as in the mass spectrum using Gas Chromatography coupled-Mass Spectrometry (GC-MS). A comparison of the mass spectral data was made with that of the available molecules in the coupled database. Based on the similarity index of the compounds present in the library, we identified the presence of the probable chemical moieties in EnXtra®.

The gas chromatographic analysis was performed on a Thermo MS DSQ II gas chromatograph coupled to a mass spectrometer instrument. A DB 35-MS capillary standard non-polar column was utilised in the following conditions: GC Oven Temperature program: Initial temperature was 75˚C (held for 2 min), increased to 150˚C at a rate of 10˚C/min (held for 2 min), then to 220˚C at a rate of 10˚C/min, (held for 2 min), and finally to 260˚C at a rate of 10˚C/min and held for 3 min. Helium was used as carrier gas and the sample was injected in a split-less mode with a column flow of 1 mL/min. The Column oven initial temperature was 70.0˚C and was raised to 260˚C at 6˚C per minute. The individual components were identified by computerized matching of their mass spectra of peaks with those gathered in the WILEY 9-Mass Spectral library of the GC-MS data software system.

2.2. Molecular Modelling

The present inverse virtual screening was designed to perform a reverse pharmacological evaluation of the compounds from EnXtra®. A library of 97 probable compounds from EnXtra® was predicted from GC-MS by comparing their mass fragmentation patterns with standard reference spectra. These molecules were examined for their biological activity [11] and ADME profile, [12] out of which 25 compounds were found to obey the requisite limits. These were subjected to *in silico* docking assessment at selected active sites of the target proteins. Caffeine being a universally accepted stimulant was used as an active comparator [13].

The compounds were selected on the basis of their probability value (*i.e.* the extent to which the mass matched to the highest m/e in MS) and the area percentage (the indicative of approximate quantity) as assessed by GC-MS. The binding efficacies of these compounds were computed for the various target proteins responsible for mental alertness.

2.2.1. Target Preparation

4M48-Dopamine transporter and 4TVK-Acetylcholinesterase were considered for docking. The crystallographic structures of the above-specified proteins were downloaded from the RCSB Protein Data Bank, which is the only available global repository of information about the 3D structures of large biological molecules, including proteins and nucleic acids. The targets were prepared for

docking by removing the non-catalytic waters, the inhibitors and all the other molecules present in the pdb files using Pymol [14]. The proteins alone were neutralized at pH 7.4 by PROPKA. All the missing fragments and other errors present in the crystal structures were corrected using the Wizard Protein Preparation implemented in Maestro 11-Beta (2016) suite [15].

2.2.2. Ligand Preparation

The chemical structures of the shortlisted ligands were either downloaded from Zinc databases [16] or drawn using embedded tools. The ligands were neutralized at pH 7.4 by Epik and minimized by force field OPLS3 [17]. These ligands were analyzed for drug-like properties using Lipinski's rule of five.

2.2.3. Lipinski's Rule of Five

Also known as the Pfizer's rule of five [11], it is used to evaluate whether a bioactive compound has chemical and physical properties that would make it orally active in humans.

2.2.4. ADME Profile

Absorption, distribution, metabolism, and excretion properties are important predictors of the product's efficacy. QikProp tool was used to predict ADME properties of these ligands through property profiling. SMILES notations of the selected compounds were fed in the online Molinspiration software version 2011.06 (www.molinspiration.com) for the prediction of bioactivity score (GPCR ligands, kinase inhibitors, ion channel modulators, enzymes and nuclear receptors).

2.2.5. GlideScore

Docking of the selected ligands was performed for each target protein using Glide module of Schrödinger suite [18]. The GlideScore was calculated utilizing the extra precision (XP) mode for all the target proteins, which ensures enhanced docking accuracy. GlideScore simulates a binding free energy with more negative values represent tighter binders. The GlideScore for each compound docked to the selected target was considered as the best ranked. Compared with other scoring functions, GlideScore SP combines the empirical-based and force-field-based scoring function to make the score more accurate.

3. Results

3.1. Bioactive Composition

The qualitative evaluations exhibited majorly the presence of glycosides, flavonoids, terpenoids, and polyphenols in the current extract. The color tests also indicated the presence of alkaloids and saponins to a lesser extent as indicated in Table 1.

3.2. Lipinski's Rule of Five

In the present study, 43 ligands out of total 117 qualified the Lipinski's rule of five thereby suggesting to be orally active.

Table 1. Qualitative evaluation of phytoconstituents.

Phytochemical group	Presence (+) or Absence (−)
Glycosides	+++
Flavonoids	+++
Terpenoids	+++
Polyphenols	++−
Alkaloid	+−−
Saponins	+−−
Carbohydrates	−−−
Tannins	−−−

+++ Abundantly present; ++− Present; +−− Sparsely Present; −−− Absent.

3.3. ADME Profile

For all the dockable molecules, the aqueous solubility (Log S) critical for estimation of absorption and distribution of the drug within the body were well within the acceptable range. The bioactive scores can be interpreted as active (bioactivity score > 0), moderately active (bioactivity score: −5.0 - 0.0) and inactive (bioactivity score < −5.0). GPCR ligand, ion channel modulator, protein kinase inhibitor, nuclear receptor ligand, and protease inhibitor ligand-based signaling cascade was used for determining the bioactivity scores. The highest bioactivity score was observed for Withaferin (0.07) and Pestalone (0.07) followed by 2R3-O-Acetyl Pinobanksin (0.04) and 3 S Pinobanksin-3-cinnamate (0.03). Overall the pharmacokinetics parameters analyzed for the docked compounds were fitted well with the acceptable range defined for human use. They were also assessed for *in silico* bioactivity against target proteins such as GPCR, ion channel, kinase, a nuclear receptor, and protease. The results demonstrated that the investigated compounds were biologically active and produced the physiological actions by interacting with GPCR, Ion channel, nuclear and protease receptors (**Table 2**).

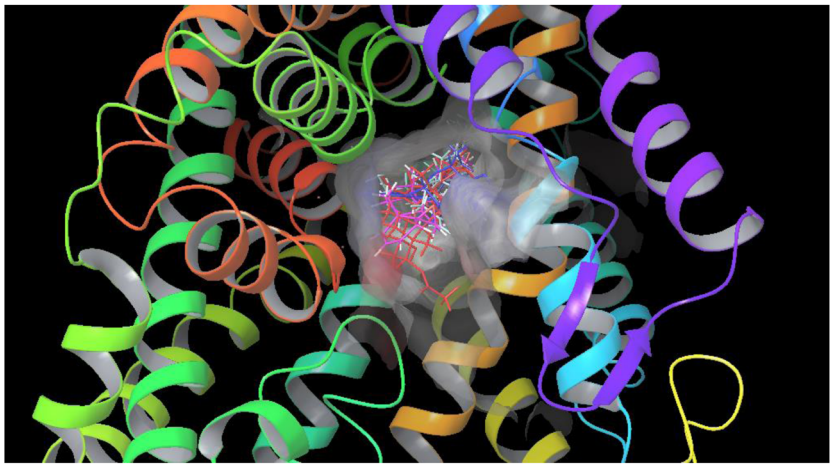
3.4. Docking

3.4.1. Interaction with Dopamine Target Protein

Dopamine receptors regulate neural networks that are involved in selective and involuntary attention. DAT mutant mice exhibit better responses to wake-promoting molecules such as modafinil, and caffeine. The target protein 4M48-Dopamine transporter crystal structure is the molecular basis for depression and is a known mechanism for regulation of dopamine uptake at chemical synapses [19]. Out of 43 docked ligands, thirteen ligands were found to be high scorers with a drug like property, obeying Lipinski's rule of five. Compounds 1 to 6 scored higher on Glide score as compared with caffeine which was used as an active comparator. The compounds which are high scoring against Dopamine target protein produces the agonistic effect leading to antidepressant/stimulant effect. Docking poses for top scoring compounds at the active site of Dopamine transporter have been presented in **Figure 1**.

Table 2. Bioactivity score for the docked compounds.

No.	Compounds	GPCR Ligand	Ion channel Modulator	Protein Kinase Inhibitor	Nuclear Receptor Ligand	Protease Inhibitor
1	Withaferin A	0.07	0.14	−0.49	0.76	0.15
2	Flavone 4'-oh-di-o-glucoside	−0.11	−0.63	−0.23	−0.2	−0.09
3	Toosendanin	0.36	−0.04	−0.3	0.35	0.37
4	-4-hydroxychroman	−0.48	−0.23	−1.24	−0.53	−1.04
5	Vitamin E	0.25	0.4	−0.2	0.41	0.28
6	Pestalon	0.07	−0.06	0.04	0.56	−0.23
7	Iso-propyl phenyl ether	−0.73	−0.41	−0.02	−0.58	−1.06
8	Epoxugedunin	0.17	−0.02	−0.32	0.52	0.19
9	Tetradecanoic acid	–	–	–	–	–
10	Desulphosinigrin	0.05	−0.23	−0.48	−0.6	−0.09
11	Cyclohexanecarboxylic acid	−1.97	−1.34	−2.67	−1.96	−1.85



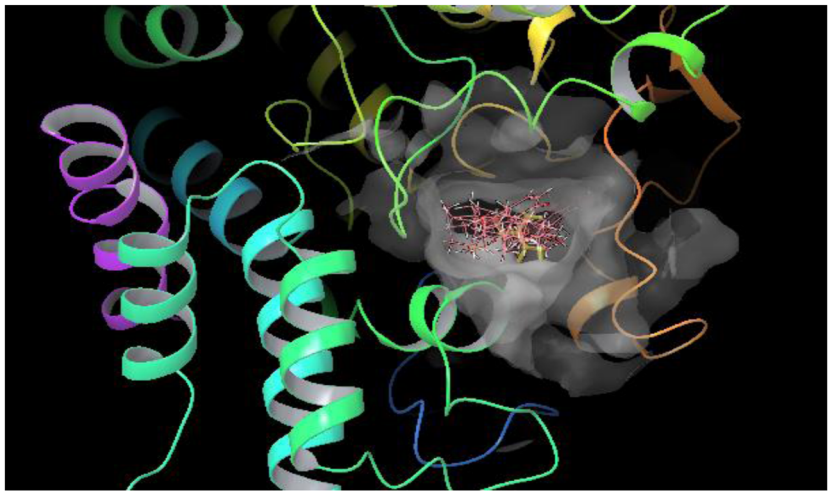
Compound	CID	Color
Decanedioic acid	31218	Orange
Withaferin A	265237	Blue
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl	141557	Grey
Flavone 4'-OH-di-O- glucoside	MOL FILE	White
Toosendanin	115060	Yellow
4-hydroxychroman	92890	Red
13-Docosenamide, (Z)	5365371	Pink
Caffeine	2519	Purple

Figure 1. Docking poses for top scoring compounds docked at the active site of 4M48.

3.4.2. Interaction with Acetylcholinesterase Target Protein

The selected crystal structure (4TVK) has been explored for multi-target drug designing of Acetylcholine esterase inhibitors [20]. AchE is a key enzyme responsible for the hydrolysis of acetylcholine, an essential neurotransmitter re-

sponsible for learning and memory [21]. The complement system, an important part of the innate immune system with a wide range of effects in multiple disease states in the CNS, could play such a contributory role [22]. The compounds which are high scoring against Acetylcholine-esterase target protein act by inhibiting the active site, thus suppressing the AchE action, leading to stimulant effect. Docking poses for top scoring compounds at the active site of 4TVK are given in Figure 2.



Compound	CID	Color
Pestalone	10071878	Fluorescent green
Withaferin A	265237	Yellow
Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl-	141557	blue
Flavone 4' OH – di –o-glucoside	MOL FILE	red
Vitamin E	14985	pink
4-Hydroxychroman	92890	Dark green
Cyclo hexane carboxylic acid	53401233	white
Caffeine	2519	Purple

Figure 2. Docking poses for top scoring compounds docked at the active site of 4TVK.

3.4.3. Glide Score

The results of the docking studies in terms of Glide Docking Score for both the target protein have been summarised target protein-wise in Table 3, exclusively

Table 3. GlideScore for the docked compounds—EnXtra®.

No.	Compound Name	Dopamine Transporter Protein	Acetylcholine Esterase target Protein
1	Decanedioic acid bis (2-ethylhexyl) ester	−8.11	−4.11
2	Withaferin A	−7.50	−6.39
3	Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl-	−7.45	−6.39

Continued

4	13-Docosenamide, (Z)-	−7.38	−4.42
5	Flavone 4'-OH-di-O-glucoside	−7.22	−9.28
6	4-hydroxychroman	−7.07	−7.21
7	Caffeine	−7.00	−6.09
8	Buxandonine	−6.63	−4.02
9	17.alfa.,21a-28,30-Bionorhopane	−6.62	−5.48
10	Pestalone	−6.46	−9.28
11	Vitamin E	−6.21	−7.79
12	3,3,4-Trimethyl-1,3-dihydro-pyrrole-2-thione	−6.13	−5.67
13	Trans caryophyllene	−6.04	−5.76
14	Cyclohexanecarboxylic acid	−5.94	−7.16
15	9,19-Cyclolanostan-3-ol,acetate	−5.41	−4.83
16	Iso-propyl phenyl ether	−5.24	−6.54
17	Geranyl-a-terpinene	−5.20	−3.40
18	Transgeranyl geraniol	−4.61	−3.85
19	2,3-Dihydro -3,5-dihydro xy-6-methy l-4H-pyran-4-one	−4.47	−6.33
20	Epoxugedunin	−4.39	–
21	Geranyl-a-terpinene	−4.35	−3.40
22	Desulphosinigrin	−4.29	−6.79
23	1-Propanol-3dimethylamino	−4.19	−2.48
24	1,2,3-Propanetriol	−2.45	−1.68
25	(cis)-2-nonadecene	−2.07	0.56
26	Tetradecanoic acid	−0.86	−2.02

for the bioactives of the EnXtra® along with caffeine. Six compounds from the aqueous extract of A. galanga were found to be top scorers on the Dopamine target protein whereas, on Acetylcholinesterase target proteins, the Glide score of most of the compounds was higher as compared to the caffeine.

4. Discussion

Alpinia galanga (L.) Willd (Zingiberaceae) is widely distributed in India. It is a perennial, aromatic, rhizomatous herb. In India, it is traditionally used as nervine tonic and stimulant. Based on the reported neuroprotective [23] and the CNS stimulant [24] activities of *A. galanga*, Enovate Biolife explored the potentials of different extract for psychostimulant action via a pilot study [8]. The aqueous extract of *A. galanga* was found to be the most efficient in enhancing the mental alertness which was further assessed by a well-designed clinical study in the human population [9]. The findings indicated that the current extract (EnXtra®) is more efficient compared to caffeine to maintain the mental alert-

ness without inducing the caffeine-crash like symptoms. Hence, we decided to further fingerprint the exact phytochemicals responsible for the elicited pharmacological effect of phytochemical profiling, followed by virtual modeling studies.

As evident from docking scores for best docking poses: Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl-; 2,3-Dihydro; -3,5-Dihydroxy-6-Methyl-4H-Pyran-4-One; 4-Hydroxychroman; Flavone 4'-OH-Di-O-Glucoside; 13-Docosenamide, (Z); Decanedioic acid-bis (2-ethylhexyl) ester and Trans-caryophyllene can be postulated as the most promising bioactive molecules being the top scorers (Glide score less than minus 6).

The benzenoid derivative Naphthalene,1,2,3,4-tetrahydro-2,5,8-trimethyl-, which was one of the top scorers on both the target proteins. This observation suggests that the active sites of the target proteins favor the hydrophobic interactions. Three prominent pyran compounds which belong to flavonoid class were identified in the extracts such as 4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl, 4-Hydroxychroman and 2H-1-Benzopyran-3-ol, 3,4-dihydro-. The 4H-Pyran nucleus is a fertile source of biologically important molecules possessing a wide spectrum of biological and pharmacological activities including central nervous system activity [25]. Also, it is one of the phytoconstituents class being hypothesized to be responsible for improving the mental alertness and are known for their potentials to prevent oxidative stress which leads to CNS-related diseases. EnXtra® contains long chain fatty acid amides and esters such as 13-Docosenamide, (Z)- and Decanedioic acid, bis (2-ethylhexyl) esters. The overall structure of these fatty acids is long hydrocarbon chains of various lengths and degrees of unsaturation terminated with carboxylic acid groups. They possess hydrophobic properties, because of the lipids they contain and are able to form membranes within organisms [26]. A recent study by Arunagiri *et al.* [27] showed that the combination of lithium and aripiprazole supplemented with omega-3 fatty acids provide protective effects against the MPD-induced neuroendocrine system and multiple neurochemical abnormalities. In another study [28], supplementation with various fatty acids of natural origin in a specific proportion to ADHD patients would elicit in the improvements in hyperactivity, impulsivity, attention, visual learning, word reading, and working/short-term memory. Trans-Caryophyllene belongs to terpenoid class which is a large group of secondary metabolites which display many activities in the CNS [29].

The pronounced effect of bioactives from EnXtra on AchE protein reveals its strong potential as a nootropic agent. The cut-off for a "good" docking score may vary a little from system to system, and changing the van der Waals radii can change the scores significantly. The docking score < minus 6 was considered as a good binding capacity of the ligand to the target proteins. In this study, it can be justified on the grounds of the hydrophobic nature of the binding ligands and shallow cavity of the active site on the target proteins. Also, as these bioactive compounds are present in considerable amounts in the aqueous extract (EnXtra®), they can be further evaluated as prominent biomarkers for standard-

izing the extraction process to produce an extract with optimum psychostimulant potential.

Based on analysis of the docking poses, we shortlisted the target proteins that exhibit significant role in neurostimulation. As evident by glide score, active compounds of EnXtra® increase the dopamine levels, by means of blocking the uptake. This increase in dopamine levels can enhance the visuospatial performance and mental clarity, leading to improved mental alertness. Hence it can be concluded that bioactive molecules from EnXtra® decrease dopamine uptake and inhibit the active site of adenosine receptors and produce the attention-enhancing effect.

5. Conclusion

This study for the first time reports the potential of compounds of *A. galanga* extract to enhance the mental clarity. The docking study also revealed that few compounds with appreciable glide scores can serve as marker compounds for further standardization with respect to the phytochemical profile of EnXtra®. Further, *in vitro* investigations are warranted to probe into the proposed/possible mechanism of action of these marker compounds on the target proteins.

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Author Contributions

The study was conceptualized and designed by Santhy Sivanandana. The manuscript was prepared and refined through the collective efforts of all the listed authors. Dr. Surekha Pimple was substantially involved in the manuscript preparation. All authors read and approved the final manuscript for publication.

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