ANTI-OXIDANT EVALUATION AND MOLECULAR DOCKING STUDIES OF PHYTOCOMPOUNDFROM MADHUCALONGIFOLIAAS POTENTIAL THYMIDYLATE SYNTHASE INHIBITOR

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Running Title: Anti-oxidant Evaluation and Molecular docking studies of Madhucalongifolia

ABSTRACT

Best alternative for cancer treatment is medicinal plants with numerous pharmacological properties which is used in many countries around the world. The present study was focussed to implement docking analysis of some phytocompounds present in Madhucalongifolia for anticancer action on thymidylate synthase to analyse potency of phytocompound. Madhucalongifolia leaves were dried and powdered. The powder was extracted with ethanol and water. In order to know the antioxidant potential of plant extract, phytochemical analysis followed by DPPH scavenging assay was done. The highest antioxidant activity was observed in ethanolic extract and therefore, this extract was chosen for further studies. The phytocompounds were functionally analysed by FTIR and GC-MS analysis. The GC-MS analysis determines the existence of various compounds in Madhucalongifolia ethanolic extracts. 5,5',8,8'-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthalene-1,1',4,4'-tetrone (C22H14O8) was one of the compound used for docking studies. Binding energy values showed the synthesized compound selectivity towards ATP-binding pocket of Thymidylate synthase, the enzyme target in cancer chemotherapy. The computational methodology such as molecular docking analysis is efficient in finding effective drugs made of natural origin against these diseases. It's evident that Madhucalongifolia contains various phytocomponents and considered as a plant of medicinal value against cancer.

Key words: Antioxidant activity; FTIR; GC-MS analysis; Madhucalongifolia; Phytochemical screening; Total phenolic content.

INTRODUCTION

Oxidative stress results out of increased free radicals are responsible for the development of various life threatening diseases including cancer. Haemorrhagic shock, arthritis, atherogenesis, Alzheimer disease, Parkinson’s disease and some gastrointestinal disorders are the diseases resulting from free radicals.1 Deleterious effects of free radicals such as oxidative damage of living cells are prevented by antioxidants, both endogenous or exogenous. This free radical scavengers can be synthetic and natural. Butylatedhydroxyanisole (BHA), Butylatedhydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are the synthetic antioxidants induces toxicity during long time usage2. Now, many in-depth studies are carried out in searching natural antioxidants from herbal sources.

Cancer is an abnormal growth of cells with potential speed in spreading to otherbodyparts. Cancer can affect different types of organs such as digestive, nervous, and circulatory systems, where hormones are released abnormally even to untargeted organ results in affecting the normal body function. Eventough there are many medicinal treatment available to treat cancer, they are not
effective in treating the disease to normal due to its high systemic toxicity and drug resistance. The final outcome may therefore not success. There is a continuous search of new herbal cures for cancer with enhancing efficacy and less toxicity to normal tissues.

In human cells, Thymidylate synthase (TS, EC 2.1.1.45), only source of enzyme for deoxythymidyne-monophosphate (dTMP) synthesis and this dTMP is an important precursor for DNA synthesis. When this enzyme is inhibited, replication process of DNA will be terminated and apoptosis occurs in fast dividing cells. This process is known as thymineless cell death. The molecular knowledge of such biological pathway note a unique option of mechanism using thymidylate synthase inhibitors to treat cancer where especially thymidylate synthase is involved. In cancer chemotherapy, thymidylate synthase enzyme is inhibited using 5-Fluorouracil (5-FU). This synthetic drug when used to treat cancer, results in the continuous exposure to Thymidylate synthase inhibitors on the site of tumour causing an increase in levels of intracellular Thymidylate synthase activity which may lead to resistance. The understanding of TS inhibition and its interaction with action of anticancer agents can explore new strategies with an effective TS inhibition without any resistance.

Madhucalongifolia comes from Sapotaceae family. Its pharmacological functions are well known for treating phlegm, inflammation, fractures, insect bite, diarrhoea, chronic tonsilitis, fever and rheumatism. This plant was reported to contain phytochemicals such as cardiac glycosides, proteins, starch, anthraquinone glycosides, phenolic compounds, tannins, terpenoids and saponins. These phytochemical compounds are responsible for their wound healing, antimicrobial, antioxidant, anti-inflammatory and anticancer activities.

Madhuca longifolia bark showed significant antioxidant potential and rule out lipid peroxidation when extracted with ethanol in a study was reported. Madhuca species also exhibits potent anti-inflammatory activity when extracted with crude alkaloid, ethanol and even saponin mixture in various studies. This reported action is due to the mechanism of phytochemicals present in Madhuca inhibits the synthesis of prostaglandin and it reduces the intercellular cell adhesion molecule-1 expression. Madhuca longifolia leaves were washed thrice in distilled water and dried at room temperature for about two to three weeks. The dried leaves were coarsely powdered using mechanical blender and stored in an airtight container for further use. Based on the successive solvent extraction method, Madhuca longifoliapowder were successively extracted with different solvents viz., ethanol and water using a Soxhlet apparatus. The extraction was carried out for 18 h with the selected solvents with a ratio 1:4 w/v, based on their polarity viz., ethanol and aqueous.

**MATERIALS AND METHODS**

**Preparation of plant extract**

Madhucalongifolia was identified and authenticated at Rapinat Herbarium, St. Joseph College, Tiruchirappalli, Tamilnadu. Madhucalongifolia leaves were washed thrice in distilled water and dried at room temperature for about two to three weeks. The dried leaves were coarsely powdered using mechanical blender and stored in an airtight container for further use. Based on the successive solvent extraction method, Madhucalongifoliapowder were successively extracted with different solvents viz., ethanol and water using a Soxhlet apparatus. The extraction was carried out for 18 h with the selected solvents with a ratio 1:4 w/v, based on their polarity viz., ethanol and aqueous.

**Phytochemical Studies**

The ethanol and aqueous extracts of leaves obtained by successive solvent extraction were subjected to various phytochemical analysis to detect the presence of various phytochemicals. Test for alkaloids, saponins, tannins, phenolic compound, flavanoids, steroids and triterpenoids were done according to the method reported in previous study.

**DPPH radical scavenging activity**

DPPH radical scavenging activity was used to test the antioxidant properties of any plant extract using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Briefly, methanolic DPPH solution (0.1 mM) was prepared and added to plant extract in ratio 1:3, at different concentrations (100-200
μg/ml). The absorbance value was noted using UV-visible Spectrophotometer at 517nm after 20 mins. Ascorbic acid was used as standard for comparison. Value in percentage was calculated according to the formula, which showed the DPPH inhibition of free radicals with the formula:

\[ \%RSA = 100 \times \frac{[A_c - A_t]}{A_c} \]

Where \( A_c \) control absorbance and \( A_t \), test sample absorbance.

**FTIR and GC-MS Analysis**

Fourier transform infrared (FTIR) spectrometry was carried out to identify the chemical compound responsible for Madhucalongifolia extract antioxidant potential. FTIR measures the chemical bond vibration related with their functional groups and creates a spectrum. By attaining IR spectra of particular plant extract, it is possible to identify the concrete structure of certain plant secondary metabolites. The compounds exist in Madhucalongifolia ethanolic extract was identified by GC-MS analysis. The principle of GC-MS associates an electron ionization system with them to use high energy carrying electrons (70 eV). The equipment has TR 5-MS capillary standard non-polar column, dimension: 30Mts, ID: 0.25 mm, Film thickness: 0.25μm. The carrier gas used is Helium with injection volume about 1 μL. The constituents present in Madhucalongifolia extract was identified by comparing the data available in the library (NIST and Willey) where the data was previously obtained from GC-MS instrument and the results obtained was tabulated.

**Molecular docking studies**

Docking studies was performed to get into the binding mode of the compounds. Here, the enzyme 3D structure (PDB ID: 1HVY) was restored and the study was conducted using Autodock version 4.2.6. Active constituent from Madhucalongifolia was restored in form of 3D structure and compound structure was generated as ligand. The protein and ligand binding were checked in the docking test. The compound was docked into the active site of binding pocket of Thymidylate synthase through positional root-mean-square deviation (RMSD) and results with less than 2Å was considered as best stabilizing energy form.

**RESULTS AND DISCUSSION**

**Phytochemical analysis**

The preliminary phytochemical test was carried out in Madhucalongifolia leaves extract as phytochemicals are direct medicinal agents. The phytochemical analysis was carried out in ethanolic and water extracts. Qualitative analysis of two different extracts confirmed the existence of alkaloid, flavonoid, tannin, terpenoid, quinine, saponin, phenols and coumarins as shown in Figure 2. The quinine was completely absent in both. The saponins and terpenoid were found to present only in ethanolic extract.

Flavonoids are antioxidant agents with multiple biological activities including anti-carcinogenic, anti-inflammatory, antibacterial, anti-allergic, antiviral and radioprotective effects. Tannins usually scavenge the hydroxyl radical toxins by directly react with it and forms tannin-protein complex. This complex act as a potential free radical scavenger in preventing the diseases. Saponin act as a mild detergent and in medicine, it is used in hypercholesterolaemia, hyperglycaemia, cancer treatments.
**Figure 1:** Phytochemical screening of 1a) water and 1b) ethanolic extracts of *Madhucalongifolia*

**DPPH radical scavenging activity**

**Table 1:** Scavenging effects of *Madhucalongifolia* extracts on DPPH

| Concentration (µg/ml) | % inhibition of aqueous extract | % inhibition of ethanolic extract |
|-----------------------|---------------------------------|----------------------------------|
| 100                   | 31.25 ± 1.52                    | 52.25 ± 1.44                     |
| 150                   | 52.91 ± 1.68                    | 60.91 ± 3.35                     |
| 200                   | 75.67 ± 1.84                    | 79.67 ± 2.25                     |

The extracts have inhibition activity against the DPPH in a dose dependent manner. In the DPPH assay, the percentage of inhibition of aqueous extract showed 31.25%, 52.91% and 75.67% at 100, 150 and 200 µg/ml concentration respectively as shown in Table 1. The ethanolic extract showed inhibition percentage of 52.25%, 60.91% and 79.67% at different concentration respectively as shown in Table 2. So the ethanolic extract of *Madhucalongifolia* have maximum antioxidant activity than aqueous extract. Phenolic compounds from different natural sources function as both primary and secondary antioxidants by different mechanisms. The existence of phytochemicals such as phenolics, tannins and coumarins in both the plant extracts may be a contributing factor for their potential free radical scavenging activity. The mechanism is that they exhibit antioxidant potential by their hydroxyl groups which function as hydrogen donor.

**FTIR and GC-MS Analysis**

**Figure 2:** FTIR spectrum of *Madhucalongifolia* thanolic extract

**Table 2:** FTIR spectral analysis of *Madhucalongifolia* thanolic extract

| No. | Frequency range | Type of bond | Functional group |
|-----|-----------------|--------------|------------------|
| 1   | 3366            | N-H Stretch  | 1°, 2° amines, amides |
Since the maximum antioxidant activity was observed in ethanolic extract, it was selected for further FTIR and GC-MS analysis. Characteristics functional groups responsible for the medicinal properties of plant were confirmed by FTIR analysis. FTIR spectrum with many absorption bands confirmed the presence of functional groups remained active in *Madhucalongifolia* extract as shown in Figure 2. The active groups and their types of bond in *Madhucalongifolia* ethanolic extract was shown in Table 2. The presence of band at 3366 correspond to N-H Stretching vibrations of 1°, 2° amines, amides. The peak at 2973 represents C-H Stretch related to aromatics. The peak at 2541 corresponds to H-C=O-C-H Stretch related with aldehydes. The band at 1924 was very weak and it showed -C=C- stretching vibrations that corresponds to the presence of alkynes in *Madhucalongifolia* extract. The peak for 1087 corresponds to C-N stretching linked to Aliphatic amines and the peak at 680 corresponds to C-Br Stretch related to alkyl halides. Fourier transform infrared (FTIR) spectrometry measures the bonds within its structural groups and creates a spectrum. This is similar as biochemical/metabolic fingerprint assessed by FTIR for the given sample. Through FTIR, we can detect the changes in primary and secondary metabolites of any given sample.
Figure 3: GC-MS chromatogram for Madhucalongifolia ethanolic extract.

Table 3: GC-MS spectral analysis of ethanolic extract of Madhucalongifolia

| No. | Retention Time | Compound Name                                                    | Peak area % |
|-----|----------------|-----------------------------------------------------------------|-------------|
| 1   | 4.292          | Oxime                                                           | 4.81        |
| 2   | 6.397          | Cyclotrisiloxane                                                 | 4.9         |
| 3   | 17.5           | 1-(+) - Ascorbic acid 2,6-dihexadecanoate                       | 3.01        |
| 4   | 19.21          | Oleyl alcohol, trifluoroacetate                                 | 3.99        |
| 5   | 22.54          | 5,5',8,8'-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthalene-1,1',4,4'-tetrone | 10.36       |
| 6   | 22.85          | 2,4,6-Tris-(1-phenylethyl)-phenol                                | 10.5        |
| 7   | 23.29          | 1-Methoxy-4-[(3,3,3-trifluoro-1-(pentfluoroethyl)-2-(trifluoromethyl)prop-1-en-1-yl]oxy]benzene | 1.35        |
| 8   | 23.45          | 2,3-Dicyano-4-[4-(1-imidazoyl)-butoxy]-phenol                    | 1.11        |
| 9   | 23.70          | 1-Dichloromethyl(dimethyl)silyloxybutane                         | 1.1         |
| 10  | 23.90          | 17-Oxo-6-pentyl-4-nor-3,5-seco-5-androsten-3-oic acid, methyl ester | 3.53        |
| 11  | 24.06          | Germacrene D                                                   | 2.48        |
| 12  | 24.12          | beta.-Sitosterol                                                | 5.35        |

GC-MS spectrum in addition to FTIR spectrum results, confirmed the existence of components using different retention times. The mass spectrometer identify the compounds structure and during analysis, the large compound fragmented into small compounds which appeared as peaks at different m/z ratios. These mass spectra fingerprint of compound can be identified from the data library. One of the structural compound identified was 5,5',8,8'-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthalene-1,1',4,4'-tetrone (C_{22}H_{14}O_{8}) which was used for docking studies. Other major components present in the Madhucalongifolia ethanolic extract were benzoic acid, diethyl Phthalate, oleyl alcohol, trifluoroacetate, ascorbic acid and various other compounds were identified as low level.

**Molecular docking studies**

**Table 4:** Details of phyto compound from Madhucalongifolia ethanolic extract for molecular docking

| Compound Names | Canonical SMILES | Compound structure |
|----------------|------------------|--------------------|
Table 5: Molecular docking analysis of phytocompound with binding pocket of Thymidylate synthase (1HVY)

| No. | Compound                                | Binding energy | H-bond interaction | H-bond distance Å° |
|-----|-----------------------------------------|----------------|--------------------|--------------------|
| 1.  | 5,5',8,8'-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthalene-1,1',4,4'-tetrone | -13.17         | TYR 132…O1        | 2.6                |
|     |                                         |                | GLN 125…O1        | 2.3                |
|     |                                         |                | GLU 225…O1        | 4.0                |

Figure 4: Docking pose of compound 1(5,5',8,8'-Tetrahydroxy-3,3'-dimethyl-2,2'-binaphthalene-1,1',4,4'-tetrone) on binding pocket of Thymidylate synthase (1HVY) where yellow line represents Hydrogen bond interactions.

Docking was carried out by AutoDock4 in ATP-binding pocket and the details are shown in Table 3 and 4. Through the grid map, the interaction between proteins and ligands through binding pocket was determined. The grid map was used with 60 points in each x, y, and z direction, equally spaced at 0.375Å°. Docking by Lamarckian genetic algorithm was studied with population size of 150; random starting position and conformation. The results of the molecular docking analysis indicate that the compounds were more selective towards the ATP-binding pocket of Thymidylate synthase (1HVY).

Lowest Binding Energy (LBE) and hydrogen bond interaction results during docking was shown in Table 4. The binding energy was between -9.38 and -5.29 kcal/mol. for Madhuca longifolia and its compound have expected binding energy was found between -13.17. These binding energy values showed fortunate selectivity towards ATP-binding pocket of Thymidylate synthase (1HVY) with synthesized compounds. Major docked poses exhibited well-established bonds with one or more amino acids in the binding pocket of thymidylate synthase. Like 5,5',8,8'-Tetrahydroxy-3,3'-...
dimethyl-2,2'-binaphthalene-1,1',4,4'-tetrone from Madhucalongifolia showed hydrogen bonds with less distance

**CONCLUSION**
Antioxidant activity of Madhucalongifolia ethanolic extract was confirmed by the presence of major phytochemicals such as flavonoids and tannins. In DPPH scavenging activity, significant difference between ethanol and aqueous plant extracts was observed and ethanol extracts exhibited excellent antioxidant activity. Therefore, for further study, ethanolic extract was selected. From the results of FTIR and GC-MS analysis, it could be concluded that Madhucalongifolia contains various bio-active compounds. Evaluation of pharmacological activity of various compounds are needed in future to know as it may help further to establish the application of isolated compound in treatment of various diseases and provide more assurance in application of such isolated compounds. From the docking analysis of the results of pharmacological studies, it was concluded that the synthesized compound accommodated in ATP-binding pocket of Thymidylate synthase.

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