

Original Article

LEAD MOLECULE IDENTIFICATION FROM *VITEX TRIFOLIA* LINN FOR HELMINTHIASIS USING *IN VITRO* AND *IN SILICO* METHODS

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ABSTRACT

Objective: The study was an attempt to discover a lead molecule to treat helminthiasis using *Vitex trifolia*. Linn (*V. folia* Linn) through sterile effect, *in vitro* and *in silico* evaluation.

Methods: The antibacterial activity was done by Kirby-Bauer disc diffusion method in three different concentrations of extract and *in vitro* anthelmintic activity was carried out by petri dish and organ bath method. Further, the *in silico* docking studies were carried out by 11 phytoconstituents against phosphoethanolamine methyltransferase (4FGZ) using Auto Dock 4.2, it was working based on the principle of Lamarckian genetic algorithm. In docking studies, three important parameters such as binding energy, inhibition constant and intermolecular energy are determined.

Results: The extracts showed an antibacterial effect in three different concentrations. At 16 mcg/disc a significant effect was observed when compared to blank and ciprofloxacin 5 mcg/disc. The anthelmintic activity in the petri dish method, means paralyzing time of *Pheretima posthuma* with the dose of 25, 50 and 100 mg/ml were 13.78, 5.79 and 4.57 min respectively and Piperazine citrate (10 mg/ml) showed paralysis in 21.58 min. In the organ bath method, the time for paralysis of the worm was recorded on a slow-moving Sherrington rotating drum and the study report showed that paralyzing time was decreased at increasing concentrations of the extract. The results of *in silico* studies exhibited a binding energy of -10.25kcal/mol, inhibitory constant (Ki) 30.91nM, intermolecular energy, -10.84kcal/mol for abietatriene-3-ol which is lesser than the standard ligand phosphoethanolamine (-6.03kcal/mol, 38.29µM, -7.82kcal/mol) respectively.

Conclusion: The study reports conclude that the active constituents in *V. folia* Linn having better anthelmintic activity, thus the active constituents may be optimized and make way to a new moiety for the treatment of helminthiasis.

Keywords: *V. trifolia*, Helminthiasis, Binding energy, Inhibitory constant, Intermolecular energy, Phosphoethanolamine methyltransferase, Abietatriene-3-ol, Phosphoethanolamine

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INTRODUCTION

Helminthiasis is one of the major public health issues across many countries. Anthelmintic drugs have been the only effective method of controlling worm infestations, but there is now widespread parasite resistance to most of the commercially available drugs [1, 2]. The development of resistance to a group of anthelmintics poses a challenge to identify novel molecular targets for helminthiasis [1, 2]. Various synthetic compounds have been proved to have anthelmintic activity. Some of the natural products have been proved to have anthelmintic activity but their safety profile is not favorable. So current research is focussed on natural products having an anthelmintic activity that may be useful in helminthiasis. *V. folia* Linn (Family: Verbenaceae) in English is named as three-leaved chase tree, called Nirnocci Sirunocci in Tamil. The leaves, roots, essential oils parts are mostly used as a dried whole plant are used in the traditional system of medicine. It is a stout aromatic found throughout the greater part of India, Western Ghats, and Himalaya southwards. It is a shrub or small tree with a growing height of about 1 to 4 meters. The leaves of the 3-foliolate of *Vitex negundo* closely resemble *V. trifolia* [3, 4]. These leaves are used for rheumatic pain and inflammation [5, 6] and have, anti-convulsant, sedatives-hypnotic activity [7] etc. This plant has already proven its free radical scavenging and *in vitro* antioxidant activity [8], hepatoprotective activity [9], wound healing activity [10] and anticancer activity [11]. The prime active constituents of the plant are isabietatriene-3-ol, beta-sitosterol, dihydrosolidagenone, friedelin, isovitexin, rotundiduran, vitetrifolin-A, vitetrifolin-B, vitetrifolin-C, vitexycarpin [12-14]. The nematodes plasma

membrane nematodes biosynthesized from phosphatidylcholine [2]. This phosphatidylcholine serves as a precursor for the production of glycol, reverted by the nematodes to avoid host immune response. In phosphatidylcholine biosynthesis, phosphoethanolamine methyltransferase enzyme is responsible for methylation of phosphatase [15, 16]. The *Plasmodium falciparum* of phosphoethanolamine methyltransferase (4FGZ) possesses a single methyl-transferase domain that methylates all three phosphatases [17, 18] and has been shown to be essential for the growth and sexual reproduction of the parasite [19]. *In silico* molecular docking technique plays an important role in the drug design and discovery to predict the conformations of each ligand molecule at the active site; hence, the *in silico* (molecular docking) studies of newly synthesized compounds. We have carried out the evaluation of phosphoethanolamine methyltransferase inhibitory activity of those phytoconstituents present in *V. trifolia* Linn and results are reported. The objective of the present study is an attempt to the *in vitro* anthelmintic and antibacterial studies of total methanolic leaf extract of *V. trifolia* Linn and examine its activities phosphoethanolamine methyltransferase inhibitory activity of its phytoconstituents by *in silico* docking studies using AutoDock 4.2.

MATERIALS AND METHODS

Collection and processing of plant material

The *V. folia* Linn leaves were collected in the month of December 2015, from our college botanical garden in Anaikuttam, Sivakasi, Tamilnadu. Then it's authenticated by Mr. V. Ganesan, M. Sc., Ph. D., Associate professor, and Head, a center for research and PG studies

in botany, Ayyanadar Janaki Ammal College, Sivakasi, Tamilnadu, India. A voucher specimen (number: 2015/12/COG012) has been maintained in our lab for future reference.

Preparation of crude extracts

The leaves of *V. folia* Linn after the collection was cleaned and removed of the adhering materials and allowed to shade dried, coarsely powdered and first extracted with hexane for removal of fatty and coloring material, then marc was dried and packed in the Soxhlet extractor. The packed material was extracted successively with methanol. After completion of the extraction, these extracts were allowed to undergo the distillation process for recovering the solvent and concentrate the extract. The concentrated extract was dried under vacuum in desiccators containing anhydrous calcium chloride. The dried products were weighed in order to determine the percentage of yield. The color and consistency were noted and the percentage yield was calculated [20].

Animal and instruments

In Indian adult earthworm (*Pheretima posthuma*) was chosen for this study, due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings [21]. Student's organ bath (it's consists of an outer jacket, made up of Perspex; the inner organ bath made up of glass with a capacity 40 ml, length 40 cm and width 6 cm; thermostatically controlled heating rod; stirrer, glass coil, and tissue holder), Sherrington rotating drum and frontal writing lever for the organ bath method.

Details of software

Python 2.7-language was downloaded from www.python.com, Cygwin (data storage) c: \program and Python 2.5 were simultaneously downloaded from www.cygwin.com, Molecular graphics laboratory (MGL) tools and Auto Dock 4.2 was downloaded from www.scripps.edu, Discovery studio visualizer 2.5.5 was downloaded from www.accelerys.com, Molecular orbital package (MOPAC), Chemscketch was downloaded from www.acdlabs.com. Online smiles translation was carried out using cactus.nci.nih.gov/translate/[22, 23].

Determination of extraction yield (% yield)

The yield (% w/w) from all the dried extracts was calculated as:

$$\text{Percentage yield (\%)} = (W_1 * 100) / W_2$$

Where W_1 is the weight of the extract obtained after evaporation of the solvent; and W_2 is the weight of the plant powder. The extracts were kept in airtight containers to avoid the loss of any volatile principles or/and activities until further use.

Antibacterial activity

Antibacterial screening was carried out in the total methanolic leaf extract of *V. trifolia* by Kirby-Bauer disc diffusion method in three different concentrations (4 µg, 8 µg, 16 µg/disc) against organisms such as *Shigella*, *staphylococcus*, *streptococci*, *Streptococcus pneumoniae* (gram-positive) *Haemophilus influenzae*, *klebsiella*, *Proteusvulgaris* and *Salmonella typhi* (gram-negative). Inoculation on Mueller-Hinton agar plate was done by the streaking technique method. The entire agar surface was streaked with the help of a sterile cotton swab and allowed for 5 min to dry the inoculum. The Sterile paper disc, impregnated with the total methanolic leaf extracts (200, 400 and 800µg/ml) was placed on the surface of the medium in each petri dish. Negative control discs with solvent (DMF) and standard discs with ciprofloxacin 5µg were also placed in each petri dish and incubated for 18 h at 37°C. After incubation, the diameter of the zone of inhibitions (in mm) was recorded. This zone of inhibition value was compared with standard ciprofloxacin 5µg/disc and solvent blank [24].

In vitro anthelmintic assay

The anthelmintic activity was evaluated in Indian adult earthworm (*Pheretima posthuma*) due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings [21, 25] by Devi *et al.* method [26]. Piperazine citrate served as a

standard drug (10 mg/ml), dimethylformamide used as a co-solvent for the extract, saline as a solvent blank, student's organ bath (it's consist of an outer jacket, made up of Perspex; the inner organ bath made up of glass with a capacity 40 ml, length 40 cm and width 6 cm; thermostatically controlled heating rod; stirrer, glass coil, and tissue holder) Sherrington rotating drum and Frontal writing lever were used.

Petri dish method

Pheretima posthuma was placed in a petri dish containing three different concentrations (25, 50, 100 mg/ml) of a total methanolic leaf extract of *V. trifolia* solutions; Piperazine citrate (10 mg/ml) was used as a standard reference and saline served as a solvent blank. Nearly equal-sized Indian earthworms were divided into five groups, each group consisting of four earthworms. These worms were released into 50 ml of sample and standard solution and observed the worms. The compound having anthelmintic property would produce paralysis (or) death. The time of paralysis of individual worms was noted. The paralyzed worms that not recover in normal saline were considered as dead [26, 27].

Organ bath method

Anthelmintic assay in the organ bath method was carried as per the method reported by Danquah *et al.* 2012 with minor modifications [28].

All the extracts and the standard drug solution were freshly prepared during the experiment. In this method, the experiment was carried out in the student's organ bath. Before starting the experiment the heating rod was switched off, the outer jacket of the student's organ bath was filled with cold water to sense the worm in humid condition. Assemble the Sherrington rotating drum was assembled; kymograph and adjust the frontal writing lever were adjusted to a magnification value below 5 for recording the response. The lower part of the worm was tied to the hook of the tissue holder using a piece of thread; the upper part was also tied to the recording lever using thread. Oxygen was bubbled through the aeration tube make sure aeration doesn't interrupt the response. With sufficient counterweight applied to the lever, the worm was kept upright in the organ bath. The responses were recorded in smoked papers fixed to the drum. The speed of the drum was adjusted to 0.12 mm/Sec by changing the gear in Sherrington rotating drum. Let replace the water using normal saline an organ bath, spontaneous movement of the worm was recorded in kymograph this response served as a control. Then the response of worms in the presence of standard drug Piperazine citrate (10 mg/ml) and various concentrations of total methanolic leaf extract of *V. trifolia* 25, 50, 100 mg/ml was recorded, the fresh worm was used for every procedure. Time for paralysis (seen as a decrease in spontaneous movement and no movement respectively) of the worm was recorded on a slow-moving Sherrington rotating drum. Declined response represented the termination of the experiment.

Molecular docking study

Molecular simulation based on docking was performed using Autodock 4.2 software package. For the docking studies, the structures of the compounds were generated from Chemscketch software. The known crystal structure of the enzyme (PDB ID: 4FGZ) was obtained from the Protein Data Bank. Autodock 4.2 suite of programs which utilizes the Lamarckian Genetic Algorithm was implemented for the docking studies of phosphoethanolamine methyltransferase inhibitor activity. In the initial stage of docking, all the water molecules were removed and the hydrogen atoms were added, followed by computing Gasterger charges, as required in the Lamarckian Genetic Algorithm. For the docking analysis, the grid size was set to 70 Å, 70 Å and 70 Å along X, Y and Z-axis with 0.375 Å grid spacing. The docking parameters used were as follows: GA population size = 100 and the maximum number of energy evaluation = 2,500,000, other parameters used were default values. The lowest binding energy conformation was searched out and used for further analysis [29-31].

Statistical analysis

The data obtained were expressed as mean±SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed

by Dunnett's t-test. At 95% confidence interval, p values < 0.001 were considered significant [31].

RESULTS

Extraction yield (% yield) of the various extracts

The percentage yield of the total methanolic extract of *V. trifolia* was 31.12%w/w. Its greenish-black color and sticky in nature. The extracts were kept in airtight containers to avoid the loss of any volatile principles or/and activities until further use.

Evaluation of antibacterial activity

The antibacterial screening was carried out in the total methanolic leaf extract of *V. trifolia* by Kirby-Bauer Disc Diffusion Method in three different concentrations (4 µg, 8 µg, 16 µg/disc) against various gram-positive and gram-negative bacteria, then the diameter (in mm) of the zone of inhibition was recorded. This zone of inhibition value was compared with standard ciprofloxacin

5µg/disc and solvent blank. All the selected concentration (4 µg, 8 µg, 16 µg/disc), standard drug ciprofloxacin (5 µg/disc) significantly inhibited the bacterial growth, but the solvent blank disc did not inhibit the bacterial growth against both gram-positive and gram-negative bacteria (fig. 1). Thus indicating that the extract had antibacterial activity. All the selected concentrations showed antibacterial efficacy, particularly 16 µg/disc of *V. trifolia* extract showed potent inhibition against all the eight strains when compared with 4, 8 µg/disc. From the observation, the zone of inhibition (ZOI) was measured and it has been tabulated (table 1) and it was found that the ZOI of the extract was found to be varying between 7-14 mm, with respect to most of the test bacteria. A comparison with solvent blank and standard antibiotic ciprofloxacin (5µg/disc) was recorded (fig. 2). From the results of ZOI values and their comparison to that of the standard ciprofloxacin, it is evidenced that the total methanolic extract of *V. trifolia* was effective against gram-positive and gram-negative bacteria. The phytoconstituents of the plant may be responsible for this antibacterial activity.

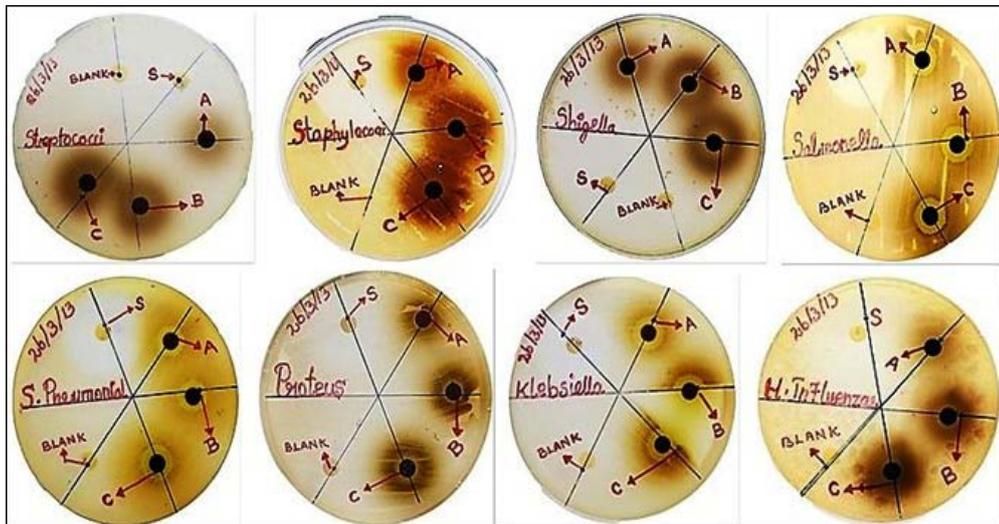


Fig. 1: Antibacterial activity of the total methanolic leaf extract of *Vitex trifolia* against various bacterial strains by kirby-bauer disc diffusion method, A-4 µg/disc, B-8µg/disc, C-16µg/disc, Blank-Solvent Control, S-ciprofloxacin 5µg/disc

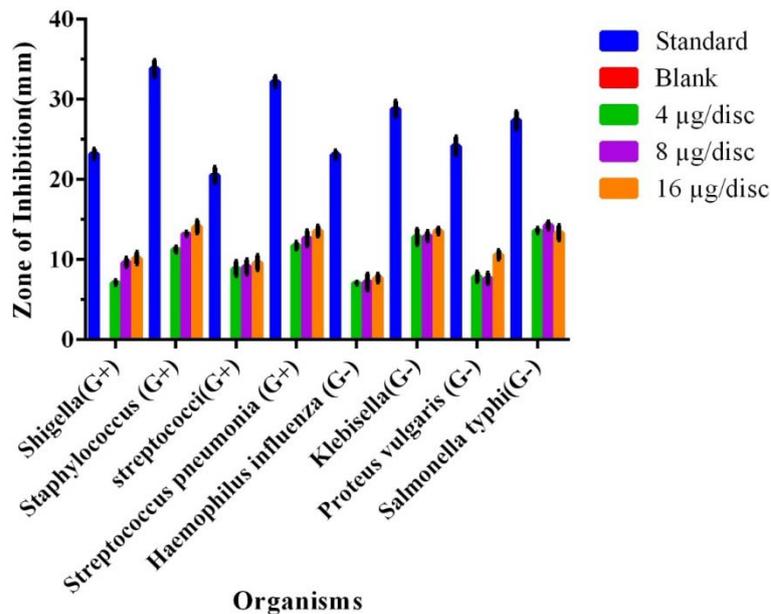


Fig. 2: Antibacterial activity of various concentrations of total methanolic leaf extract of *Vitex trifolia* against various bacterial strains by Kirby-bauer disc diffusion method

Table 1: Antibacterial activity of total methanolic leaf extract of *Vitex trifolia* against various bacterial strains by Kirby-Bauer disc diffusion method

| Organism | Zone of inhibition (in mm) | | | | |
|--|--------------------------------------|-------|---|------------|------------|
| | Standard (Ciprofloxacin 5µg/disc) | Blank | Total methanolic extract of <i>Vitex trifolia</i> | | |
| | | | 4 µg/disc | 8 µg/disc | 16 µg/disc |
| <i>Shigella</i> (G ⁺) | 23.22±0.61 | - | 7.12±0.25 | 9.65±0.54 | 10.20±0.72 |
| <i>Staphylococcus</i> (G ⁺) | 33.85±1.02 | - | 11.28±0.35 | 13.23±0.25 | 14.11±0.76 |
| <i>streptococci</i> (G ⁺) | 20.56±0.95 | - | 8.89±0.89 | 9.11±0.88 | 9.62±0.88 |
| <i>Streptococcus pneumonia</i> (G ⁺) | 32.23±0.67 | - | 11.75±0.45 | 12.64±0.92 | 13.57±0.64 |
| <i>Haemophilus influenza</i> (G ⁻) | 23.11±0.52 | - | 7.05±0.15 | 7.21±1.02 | 7.72±0.51 |
| <i>Klebisella</i> (G ⁻) | 28.83±0.97 | - | 12.86±0.97 | 12.94±0.58 | 13.59±0.37 |
| <i>Proteus vulgaris</i> (G ⁻) | 24.18±1.12 | - | 7.87±0.61 | 7.64±0.67 | 10.61±0.54 |
| <i>Salmonella typhi</i> (G ⁻) | 27.38±1.09 | - | 13.63±0.28 | 14.28±0.46 | 13.34±0.90 |

G+-Gram-Positive Bacteria, G--Gram-Negative Bacteria, Each value was represented as mean±SEM, n=6 independent experiments

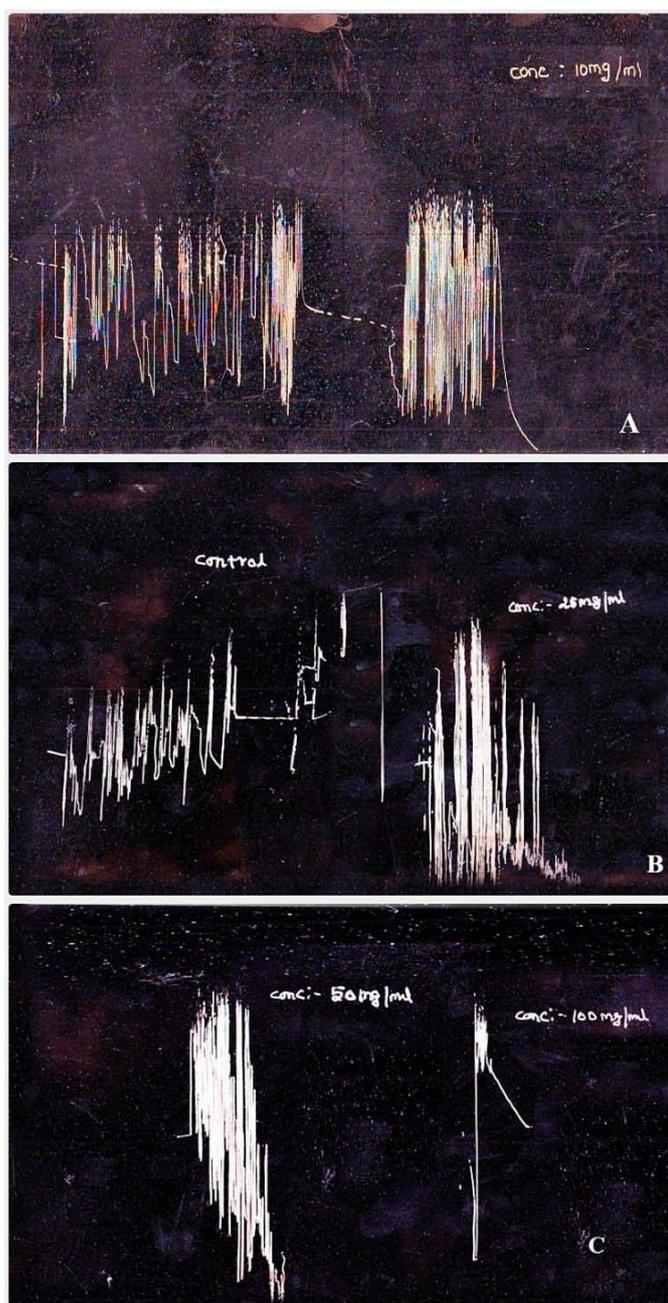


Fig. 3: Anthelmintic activity of the total methanolic leaf extract of *vitex trifolia* Linn against Indian adult worms (*Pheretima posthuma*) by organ bath method, A-Paralysing Response of *P. posthuma* against saline and Piperazine citrate (10 mg/ml), B and C-Paralysing Response of *P. posthuma* against saline and total methanolic leaf extract of *Vitex trifolia* at different concentrations (25, 50, 100 mg/ml)

In vitro anthelmintic assay**Anthelmintic activity by petri dish method**

Anthelmintic activity was carried out for three different concentrations (25, 50, 100 mg/ml) a total methanolic leaf extract of *V. trifolia* by petri dish method against adult Indian worms *Pheretimaposthuma*, Piperazine citrate (10 mg/ml) was used as a standard reference. Observations were made for the time of paralysis of individual worms against at various concentrations of the extract and standard. Paralysis was assumed to have occurred when the worms did not revive even in normal saline. The selected three concentrations (25, 50, 100 mg/ml) produced an anthelmintic activity (table 2). The anthelmintic activity increased in a dose-dependent manner.

Anthelmintic activity by organ bath method

Earthworms are invertebrates composed of many segments. It has a special layer that being slimy, enables the earthworm for spontaneous movement. This motility response was recorded in the

presence of standard drug Piperazine citrate (10 mg/ml) and various concentrations of total methanolic leaf extract of *V. trifolia* 25, 50, 100 mg/ml, the fresh worm was used for every sample. The worms were treated with standard drug Piperazine citrate (10 mg/ml) and various concentration of total methanolic leaf extract of *V. trifolia* 25, 50, 100 mg/ml. The motility of the worms was recorded in kymograph during the treatment (fresh worms were used for every sample).

As shown in fig. 3 the worms showed normal spontaneous movement in saline treatment. On treatment with a standard drug (Piperazine citrate (10 mg/ml) and various concentrations of methanolic extract of *V. trifolia* 25, 50, 100 mg/ml worms muscle started to paralyze which was represented in the response in kymograph, by a declined response from baseline graph (table 2). All the selected concentrations (25, 50, 100 mg/ml) significantly produced a paralyzing effect on worms. At the same time, worms showed quick paralysis time was very short in high concentration. So it produced the anthelmintic activity in a dose-dependent manner.

Table 2: Anthelmintic activities of the total methanolic leaf extract *vitex trifolia* against Indian adult worms (*Pheretima posthuma*) by petri dish method

| Groups | Worms | (In min) | |
|-----------------------------|-------|-----------------------------------|-----------------------------------|
| | | Time taken for paralysis (in min) | Time taken for paralysis mean±SEM |
| Solvent Blank | A | - | - |
| | B | | |
| | C | | |
| | D | | |
| Piperazine Citrate 10 mg/ml | A | 20.45 | 21.58±0.61 |
| | B | 21.03 | |
| | C | 21.54 | |
| | D | 23.32 | |
| 25 mg/ml | A | 14.11 | 13.78±0.68 |
| | B | 12.24 | |
| | C | 15.49 | |
| | D | 13.31 | |
| 50 mg/ml | A | 4.51 | 5.79±0.52 |
| | B | 6.09 | |
| | C | 7.01 | |
| | D | 5.58 | |
| 100 mg/ml | A | 5.01 | 4.57±0.27 |
| | B | 4.11 | |
| | C | 4.08 | |
| | D | 5.10 | |

Each value was represented as mean±SEM, n=6 independent experiments

In silico docking studies results

In silico docking studies of the 11 selected phytoconstituents of *V. trifolia* were carried out with phosphoethanolamine methyltransferase (4FGZ).

The selected phytoconstituents had various types of functional groups and most of the functional groups possessed good phosphoethanolamine methyltransferase inhibitor activity. Among

them, Abietatriene-3-ol showed higher phosphoethanolamine methyltransferase inhibitory activity. Moreover among the selected 11 compounds, two compounds showed binding energy value between-6 to-7 kcal/mol, three compounds showed values between-7 to-8 kcal/mol, four compounds showed values between-8 to-9 kcal/mol, a compound showed values between-9 to-10 kcal/mol and another compound showed values above-10 kcal/mol (table 3).

Table 3: Docking parameters of compounds in phosphoethanolamine methyltransferase (4FGZ)

| Ligands | Binding energy (kCal/mol) | Inhibitory constant (µM) | Intermolecular energy (kCal/mol) |
|---------------------|---------------------------|--------------------------|----------------------------------|
| Phosphoethanolamine | -6.03 | 38.29 µM | -7.82 |
| Abietatriene-3-ol | -10.25 | 30.91nM | -10.84 |
| Artemetin | -7.33 | 4.26µM | -9.42 |
| Beta-sitosterol | -8.19 | 984.3nM | -10.28 |
| Dihydrosolidagenone | -9.3 | 152.9 nm | -10.49 |
| Friedelin | -7.28 | 4.58µM | -9.37 |
| Isovitexin | -6.56 | 15.65µM | -9.54 |
| Rotundiduran | -6.87 | 9.14µM | -8.66 |
| Vitetrifolin-A | -8.13 | 1.1µM | -8.73 |
| Vitetrifolin-B | -8.93 | 284.06nM | -11.02 |
| Vitetrifolin-C | -7.99 | 1.4µM | -9.78 |
| Vitexycarpin | -8.14 | 1.07µM | -10.23 |

Table 4: Docking parameters of compounds and binding site in phosphoethanolamine methyltransferase

| Phosphoethanolamine | Abietatriene-3-ol | Artemetin | Beta-sitosterol | Dihydrosolidagenone | Friedelin | Isovitexin | Rotundiduran | Vitetrofolin-A | Vitetrofolin-B | Vitetrofolin-C | Vitexin | Vitexycarpin |
|---------------------|-------------------|-----------|-----------------|---------------------|-----------|------------|--------------|----------------|----------------|----------------|---------|--------------|
| 34 ASN | 14 LEU | 10 ASP | 19 TYR | 14 LEU | 14 LEU | 10 ASP | 10 ASP | 14 LEU | 10 ASP | 10 ASP | 14 LEU | 19 TYR |
| 35 TYR | 19 TYR | 14 LEU | 35 TYR | 63 GLY | 19 TYR | 19 TYR | 14 LEU | 19 TYR | 62 ILE | 14 LEU | 19 TYR | 31 PHE |
| 36 ILE | 36 ILE | 19 TYR | 36 ILE | 65 GLY | 37 SER | 36 ILE | 19 TYR | 63 GLY | 63 GLY | 19 TYR | 63 GLY | 34 ASN |
| 37 SER | 63 GLY | 36 ILE | 37 SER | 85 ASP | 62 ILE | 37 SER | 62 ILE | 85 ASP | 85 ASP | 62 ILE | 85 ASP | 35 TYR |
| 61 ASP | 85 ASP | 37 SER | 63 GLY | 86 ILE | 63 GLY | 85 ASP | 63 GLY | 86 ILE | 86 ILE | 63 GLY | 86 ILE | 36 ILE |
| 63 GLY | 86 ILE | 62 ILE | 64 SER | 111 ILE | 65 GLY | 86 ILE | 84 ILE | 87 CYS | 111 ILE | 85 ASP | 87 CYS | 37 SER |
| 64 SER | 90 ILE | 63 GLY | 66 LEU | 129 ALA | 85 ASP | 109ASN | 85 ASP | 90 ILE | 132 HIS | 86 ILE | 109ASN | 38 SER |
| 66 LEU | 109 ASN | 65 GLY | 85 ASP | 132 HIS | 86 ILE | 110 ASP | 86 ILE | 109 ASN | 133 LEU | 111 ILE | 110ASN | 61 ASP |
| 68 GLY | 110 ASP | 85 ASP | 86 ILE | 133 LEU | 90 ILE | 111 ILE | 110 ASP | 110 ASP | | 132 HIS | 111 ILE | 63 GLY |
| 69 GLY | 111 ILE | 86 ILE | ARG 127 | | 111 ILE | 128 ASP | 111 ILE | 111 ILE | | 133 LEU | 112LEU | 64 SER |
| | 129 ALA | 90 ILE | 128 ASP | | 128 ASP | 129ALA | 129ALA | 129 ALA | | | 128 ASP | 65 GLY |
| | 132 HIS | 128 ASP | 129 ALA | | 129ALA | 132 HIS | 132 HIS | 132 HIS | | | 129ALA | 66 LEU |
| | 133 LEU | 129 ALA | 132 HIS | | 132 HIS | | 133LEU | 133LEU | | | 132HIS | 68 GLY |
| | | 130 ILE | 133 LEU | | 133 LEU | | | | | | 133LEU | 69 GLY |
| | | | | | | | | | | | | 85 ASP |
| | | | | | | | | | | | | 128 ASP |
| | | | | | | | | | | | | 132 HIS |
| | | | | | | | | | | | | 160 TYR |
| | | | | | | | | | | | | 181 TYR |
| A | -6.03 | -10.25 | -7.33 | -8.19 | -9.3 | -7.28 | -6.56 | -6.87 | -8.13 | -8.93 | -7.99 | -0.33 |
| B | 38.29µM | 30.9 nm | 4.26µM | 984.3 nm | 152.9 nm | 4.58µM | 15.65µM | 9.14µM | 1.1µM | 284.06nM | 1.4µM | 570.75m |
| C | -7.82 | -10.84 | -9.42 | -10.28 | -10.49 | -9.37 | -9.54 | -8.66 | -8.73 | -11.02 | -9.78 | -3.32 |

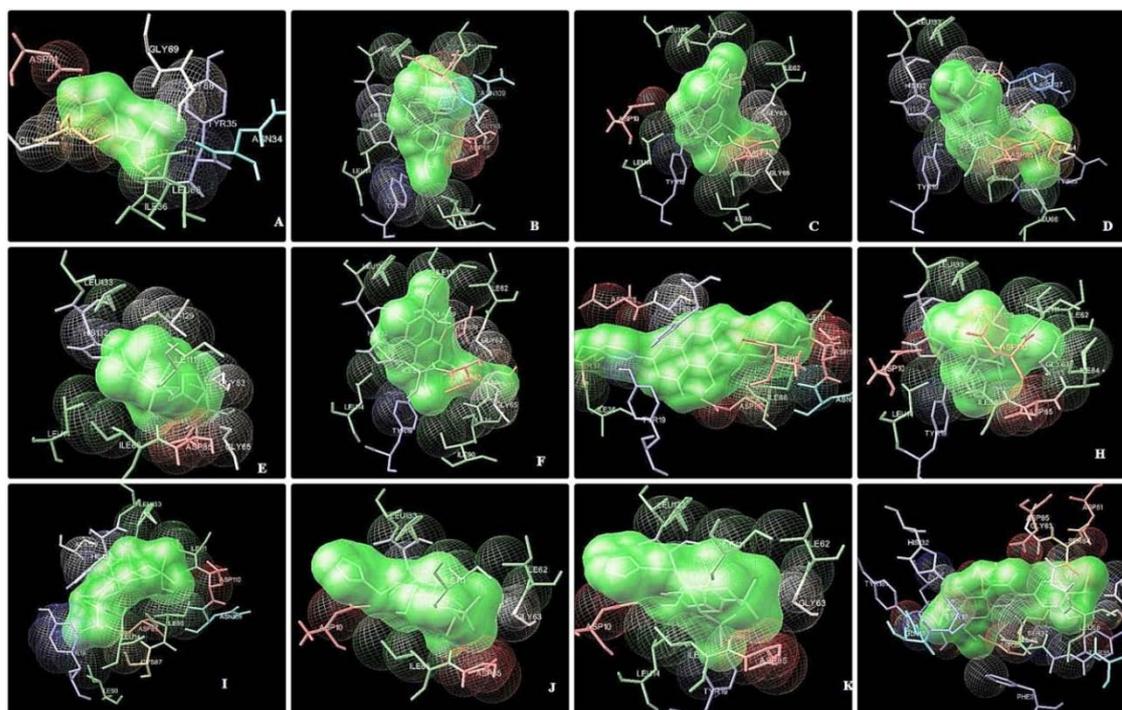


Fig. 4: Docked pose of phosphoethanolamine methyltransferase (4FGZ) with phosphoethanolamine and phytoconstituents of *Vitex trifolia* linn, A-Phosphoethanolamine, B-Abietatriene-3-ol, C-Artemetin, D-Beta-sitosterol, E-Dihydrosolidagenone, F-Friedelin, G-Isovitexin, H-Rotundiduran, I-Vitetrofolin-A, J-Vitetrofolin-B, K-Vitetrofolin-C, L-Vitexycarpin

$$\text{Binding energy} = A + B + C - D$$

where, A indicates the sum of intermolecular energy, Wandervalls energy (vdW), hydrogen bonds, desolvation energy and electrostatic energy (kcal/mol), final total internal energy (kcal/mol) denoted as a B, the torsional free energy (kcal/mol) mentioned as C, unbound system's energy (kcal/mol) marked as a D. In addition, various parameters like inhibitory constant (K_i), intermolecular energy, electrostatic energy, total internal energy, torsional energy, unbound external energy, cluster RMS and ref RMS were determined. Inhibition constant (K_i) is directly proportional to the binding energy. When the compounds inhibitory activity increased, there was a decrease in its binding energy. Similarly, intermolecular energy is directly proportional to the binding energy, i.e., lesser the intermolecular energy, lesser the binding energy. The other parameters like electrostatic energy, total internal energy, torsional energy, unbound external energy, cluster RMS and ref RMS is independent of the binding energy. The values of standard compound phosphoethanolamine (value of A-binding energy, B-inhibition constant, C-intermolecular energy).

Binding energy, inhibitory constant (K_i), inter molecular energy (-6.03 kcal/mol, 38.29 μ M, -7.82), were compared to abietatriene-3-ol (-10.25 kcal/mol, 30.91nM, -10.84), dihydrosolidagenone (-9.3 kcal/mol, 152.9nM, -10.43), vitetrifolin-B (-8.93 kcal/mol, 284.06nM, -11.02) (table 4). Among the selected compounds, abietatriene-3-ol, dihydrosolidagenone and vitetrifolin-B possess very potent phosphoethanolamine methyltransferase inhibitory activity.

DISCUSSION

Preliminary phytoconstituents of *V. trifolia* methanolic extract revealed the presence of alkaloids, coumarins, flavonoids, terpenoids polyphenols, saponins, and tannins. Also, this plant was proved to possess various active phytoconstituents [5, 6, 14].

In the antibacterial test, The total methanolic leaves extract of *V. trifolia* was screened for its antibacterial activity against organisms such as *Shigella*, *Staphylococcus*, *streptococci*, *Streptococcus pneumoniae* (gram-positive) *Haemophilus influenzae*, *klebisella*, *Proteus vulgaris* and *Salmonella typhi* (gram-negative) by Kirby-Bauer disc diffusion method. The antibacterial study revealed that (table 1) the total methanolic extract exhibited antibacterial activity, even in low concentration (4mcg/disc). At 16mcg/disc the extract showed a significant antibacterial effect, compared to blank and other concentrations. The antibacterial activity may be due to the presence of phytoconstituents such as alkaloids, flavonoids and phenolic compounds in this plan [20].

In the anthelmintic activity, the Indian earthworms (*Pheretima posthuma*) are invertebrates composed of many segments. A thin mucilaginous outer layer covered the earthworm, which is made up of complex polysaccharides. It is very essential for the free movement of earthworm. Any damage in the outer layer would restrict the movement due to paralysis [32, 33]. Anthelmintic drugs either kill or paralyze the worm. Normally, worms do not store energy, and energy is derived from the host by glucose uptake mechanism. Also, most of the drugs cause either flaccid paralysis through chloride ion channel by GABA mechanism or spastic paralysis through the cholinergic mechanism. When worms are paralyzed, they lose their ability to gripping power and are expelled through the gut with the help of the peristaltic movement. The drug may also affect the normal function of the worms by binding the glycoprotein present in the cuticle of the parasite and cause paralysis [34]. The earthworms moved normally, spontaneously this movement was recorded on kymograph. The mechanism of the most anthelmintic drug is paralysis of the worms. The paralyzed worms are expelled from the large intestine. Based upon this principle, worms were treated with saline, Piperazine citrate (10 mg/ml) and various concentrations of total methanolic leaf extract of *V. trifolia*. In the Petri dish method, the total methanolic extract of *V. trifolia* showed the mean paralyzing time of *Pheretima posthuma* with a dose of 25, 50 and 100 mg/ml were found to be 13.78, 5.79 and 4.57 min respectively. In the meantime of Piperazine citrate at a dose of 10 mg/ml causes paralysis in the above helminth in 21.58 min. In organ bath method time for paralysis (seen as a decrease in spontaneous

movement and no movement respectively) of the worm as recorded on a slow-moving Sherrington rotating drum. In saline treatment, worms are not paralyzed so it produced normal spontaneous movement for a long time, it serves as a control response, Piperazine citrate is a known anthelmintic drug, in this treatment worm are paralyzed for a short time, it serves as a standard reference. Then the time required to paralyze the worms in various concentrations of plant extract was noted. This time was compared to the standard and control-treated animal. From the above study, it was seen that the total methanolic leaf extract of *V. trifolia* showed a dose-dependent anthelmintic activity as compared to a standard drug Piperazine citrate. The worms paralyzing time was decreased at increasing concentration of the extract. Total methanolic leaf extract of *V. folia* produced a potent anthelmintic activity against the *Pheretima posthuma* when compared to reference standards.

In molecular docking studies, once compounds were successfully docked with an enzyme, the enzyme/ligand complex was analyzed. The analysis was based on various parameters such as hydrogen bond interactions, π - π interactions, binding energy, and RMSD. It gives information as to, whether the compounds were bound on active site or not [35, 36]. Normally, the thumb rule indicates that compounds bound in the active site of the enzyme by both hydrogen bond and π - π hydrophobic interactions, which means that compounds have significant biological activities. From the molecular docking studies, the potential binding sites of the phosphoethanolamine were clearly shown (table 4) and they were found to be 34 ASN, 35 TYR, 36 ILE, 37 SER, 61 ASP, 63 GLY, 64 SER, 66 LEU, 68 GLY, 69 GLY. This proves that the effective binding sites are bound with the selected phytoconstituents when compared with the phosphoethanolamine. It proves that the selected compounds have the ability to inhibit the phosphoethanolamine methyltransferase enzyme. The flavonoids displayed binding energy ranging between -6.56 kcal/mol to -10.25 kcal/mol. All the selected compounds showed less significant binding energy when compared to phosphoethanolamine (-6.03 kcal/mol). This proves that the phytoconstituents of *V. trifolia* Linn have a potential phosphoethanolamine methyltransferase inhibitory activity when compared to phosphoethanolamine. The molecular docking studies revealed (table 4) that most active compound abietatriene-3-ol was bound to the following sites 36 ILE, 90 ILE, 19 TYR, 14 LEU, 132 HIS, 85 ASP, 129 ALA, 86 ILE, 63 GLY, 133 LEU, 111 ILE, 110 ASP and 109 ASN. The potential binding sites of the phosphoethanolamine were clearly found to be, 34 ASN, 35 TYR, 36 ILE, 37 SER, 61 ASP, 63 GLY, 64 SER, 66 LEU, 68 GLY and 69 GLY (fig. 4). This proves that the effective binding sites are present in the compound abietatriene-3-ol compared with the phosphoethanolamine, in addition, selected compounds had the ability to inhibit phosphoethanolamine methyltransferase enzyme (table 3). Apart from that two more parameters such as inhibition constant (K_i) and intermolecular energy were determined. The phytoconstituents of *V. trifolia* Linn showed inhibition constant, ranging from 15.65 μ M to 30.91 nM. The chosen compounds had less inhibition constant when compared to the phosphoethanolamine (38.29 μ M). The inhibition constant is directly proportional to binding energy and observed results indicated that the inhibition constant is decreased simultaneously if binding energy decreased. Thus, the phosphoethanolamine methyltransferase inhibitory activity of the phytoconstituents was found to be higher compared to phosphoethanolamine. The phytoconstituents showed intermolecular energy ranging between -8.66 kcal/mol to -10.84 kcal/mol which was lesser when compared to the phosphoethanolamine (-7.82 kcal/mol). Intermolecular energy is also directly proportional to the binding energy. The observed results indicated that there was a decrease in the intermolecular energy of all the selected compounds with a simultaneous decrease in the binding energy. Based on the docking studies, the phosphoethanolamine methyltransferase inhibitory activity of the selected compounds was found to decrease in the order of Isovotixin, Rotundiduran, Friedelin, Artemetin, Vitetrifolin-C, Vitetrifolin-A, Vitexcarpin, Beta-sitosterol, Vitetrifolin-B dihydrosolidagenone, and abietatriene-3-ol. On the basis of the above study, abietatriene-3-ol and dihydrosolidagenone possess potential phosphoethanolamine methyltransferase inhibitor binding sites compared to that of the phosphoethanolamine. The results may

be attributed due to differences in the position of the functional groups in compounds.

V. trifolia has various active phytoconstituents, particularly the methanolic extract contains alkaloids, coumarins, flavonoids, terpenoids polyphenols, saponins, and tannin. The antibacterial activity was screened to explore that the extract may directly attack the parasite or kill the parasite by stimulating the host defense mechanism. The performed *in vitro* anthelmintic evaluation report confirmed that the total methanolic extract showed significant anthelmintic activity by paralyzing the worm, due to the expected mechanisms such as cholinergic mediated acetylcholine release or direct binding of acetylcholine into the muscarinic or nicotinic receptor. The activation of the cholinergic receptor alters the normal depolarisation and repolarisation process and it leads to spastic paralysis. In the organ bath method, it was noticed that the worms had lost their contractile power, maybe due to flaccid paralysis. The flaccid paralysis caused by GABA mediated chloride ion flow through Cl⁻ channel and as a result reduces the muscle tone and causes weakness of the muscle. The *in vitro* anthelmintic evaluation report clearly indicates that the extracts showed their anthelmintic property in a dose-dependent manner which is effective in the treatment of helminthiasis, particularly against adult worm. In addition, to strengthen the data to move forward towards *in vivo* study the drug design screening was performed. In this study, we targeted the phosphoethanolamine methyltransferase enzyme, because the enzyme plays a vital role in the parasite life cycle and enzyme-catalyzed methylation of phosphatases during the process of phosphatidylcholine biosynthesis. Phosphatidylcholine is essential for the formation of the plasma membrane in nematodes, thus phosphoethanolamine was used as a standard ligand, and all the selected compounds were docked with an enzyme. The observed results indicated that compounds had less binding energy was compared to phosphoethanolamine. It was so interesting that the docking studies also prove that the total methanolic extracts of *V. trifolia* and its active constituents showed a significant anthelmintic property.

CONCLUSION

Based on the study report, the authors revealed that the extract showed an anthelmintic effect and proved that the active constituents of *V. folia* have good phosphoethanolamine methyltransferase inhibitory activity. The report of antibacterial, *in vitro* and *in silico* anthelmintic studies confirmed that the total methanolic leaves extract of *V. trifolia* and its active constituents were having a significant anthelmintic property with the valuable information about its mechanisms of action. Thus *V. trifolia* active constituents have high significance to be considered as a potential drug candidate in the treatment of helminthiasis, furthermore, it has a great scope to be investigated on different animal models.

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AUTHORS CONTRIBUTIONS

This research work has been designed by Soundararajan Muthukrishnan and he act as a Principal investigator, Ragunath and Blessy Susan Varghese were executed this research work under the guidance of principal investigator.

CONFLICT OF INTERESTS

Declared none

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