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# Pharmacological Evaluation of Polyphenols for Multiple Sclerosis by *In silico* and *In vivo* Methods

**SilpaVijayan*, Dr. Merlin N J, Dr. Shaiju S Dharan**
*Department of Pharmacology*
*Ezhuthachan College of Pharmaceutical Sciences*
*Marayamuttom, Neyyattinkara, Trivandrum-695124*

**Abstract**

**Aim**: MS is a chronic neuroinflammatory disease of the Central Nervous System characterized by neurodegeneration, demyelination, and astroglial proliferation. Polyphenols natural, synthetic or semisynthetic, organic chemicals characterized by multiples of phenol structural units. Flavonoids are colorful antioxidants found in plants and and are categorized into flavonols, flavones, flavanones, etc. The Flavonoids aroused considerable interest recently because of their beneficial effects on human health. They play an important role in the management of neurodegenerative diseases. The present study examined the activity of polyphenolic compounds for Multiple sclerosis and thereby suggesting the feasibility of its possible promise as natural neuroprotective agent with safety and efficacy.

**Methods**: *Insilico* molecular docking studies of lead compounds Vitexin, Isovitexin and Luteolin were carried out using various computer simulation tools like ACD Chemsketch 12.0, Molinspiration chemiinformatics, Swiss ADMETec. Phytochemical screening and *invitro* anti-inflammatory studies were carried out using various reagents and Raw 264 cell lines. Acute toxicity studies were performed on the basis of OECD guidelines 423 in wistar rats. Neuroprotective activity were isolated by behavioral tests and *invivo* biochemical analysis.

**Results and conclusion**: *Insilico* molecular docking studies of lead compounds revealed their drug like properties and also molecular docking performed and they performed better gliding score and interaction with the receptors. Phytochemical screening of the plant revealed presence of flavonoids and *invitro, in vivo* studies for their neuroprotective activity revealed their potency against Multiple sclerosis and suggested to be used as safe and effective natural agents for multiple sclerosis.

**Keywords**: Central Nervous System, *Insilico* molecular docking studies, Multiple sclerosis, Neuroinflammatory disease, Polyphenols.

## INTRODUCTION

Central nervous system id the part of nervous system consists of the brain and spinal cord. Myelin is a multilayered lipid rich structure that enwrap the axonal terminals in the Central nervous system which serves as the insulator to facilitate conduction of electrical nerve impulses. Myelin sheath play a crucial role in the fastest impulse conduction in the brain terminals with the use of minimal energy which is responsible for the higher and complex mammalian brain functioning. Loss of myelin is referred as demyelination which is found to be the main factor in the pathogenesis of neurodegenerative diseases which include Multiple sclerosis. Multiple sclerosis (MS) is a clinically important heterogeneous demyelinating disorder and also referred as an autoimmune condition which involves a complex interaction between immune system and neural cells. Multiple sclerosis is the disease that affecting over 2.5 million people worldwide.

### Genetics of multiple sclerosis

The first genetic factor related to the disease is human leukocyte antigen (HLA) locus that located in the major histocompatibility complex (MHC). There are certain genomic tools confirmed the association of the HLA class II haplotype DRB1*15:01–DQA1*01:02–DQB1*06:02 with MS. HLA DRB*1501 have the strongest association with MS and also there are 14 other regions associated with the disease containing several genes. Multiple sclerosis-associated allele directs the expression of a novel form of the tumor necrosis factor (TNF)-R1 protein, which can block TNF and mimics the effect of TNF-blocking drugs.

### Polyphenols and flavanoids

Polyphenols are natural, synthetic, and semisynthetic organic chemicals derived from plants characterized by the presence of large multiples of phenol structural units. Flavonoids possess potential benefit effects on human health and can be used as antiviral, anti-allergic, anti-platelet, anti-tumor, antioxidant and anti-inflammatory agents. On the basis of insilico and molecular docking studies the compounds that possess better drug like properties were selected for the study. Among the various neuroprotective flavonoid compounds, Vitexin (figure 1), isovitexin ( Figure 2) and luteolin ( Figure 3) were selected for the present study.

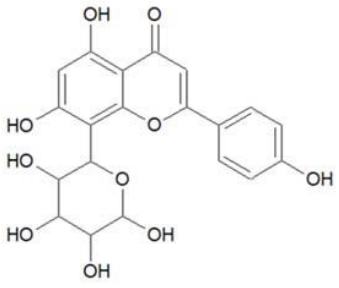


**Figure 1: Structure of Vitexin**

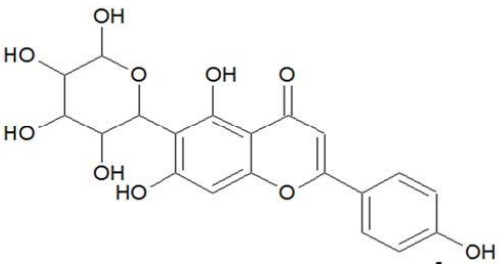


**Figure 2: Structure of Isovitexin**

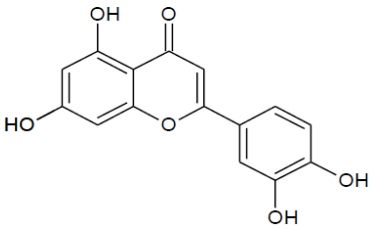


**Figure 3: Structure of Luteolin**

**Receptors**

Human Leukocyte antigen (HLA) is a gene complex which are expressed in the antigen presenting cells and are the gene complex present in chromosome 6 of Major Histocompatability complex. (MHC). HLA genes play a critical role in the immune system to clear certain extracellular and intracellular bactrial infections and certain viral infections on the nervous system. There are certain haplotype forms such as HLA DR2-DQ6, HLA DR4-DQ8, HLA DR3-DG2) are present in HLA regions that are associated with neuroinflammation. HLA DR2 receptor (Figure 4) is a family proteins involved in the cellular processes in the release of proinflammatory mediators.

TLR4 is a protein belongs to the family of Pattern Recognization Receptors. It plays an important role in the innate immune system. On activation of TLR4, it leads to the intracellular signaling pathway NF-κB and inflammatory cytokine production that causes innate immune system activation. The three dimensional structure of TLR-4 receptor is shown in figure 5.

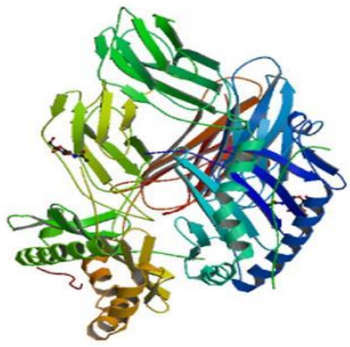

**Figure 4: Three Dimensional structure of HLA DR 2 receptor**

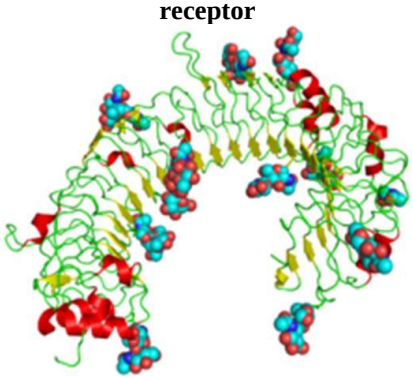

**Figure 5: Three dimensional structure of TLR-4 receptor**

**Passiflora edulis**

Passifloraedulis is commonly known as passion fruit in English kodikkai or poonakkai in Malayalam is belongs to the family Passifloraceae. Traditionally the fruit pulp is used as sedative, antiasthmatic and emetic. The plant leaves are used in the treatment of insomnia and traditionally known to produce a restful sleep without any narcotic hangover. The leaves contain wide varieties of chemical constituents like polyphenolic compounds like flavonoids, carbohydrates and glucosides. The vitamins and minarals present in the plant such as Phosphorus,niacin, and vitamin B-6.

The present research focused on the pharmacological evaluation of Flavanoids of P. edulis leaf extract for Multiple sclerosis using in silico and in vivo methods.

## MATERIALS AND METHODS

All the chemicals (reagents, solvents and neurocell lines) were purchased from ECPS laboratory and from commercial suppliers Sigma-Aldrich, US, Merk, Germany Nice chemicals Ltd, India, Chemdyes corporation, Gujarat, Yarrow chemicals, Mumbai, National Centre for Cell Sciences (NCCS), Pune, India, and they were used further without purification.

**Softwares used**

Chemical structure of compounds were drawn by ACDchemsketch 12.0, in silicostudies like biological activity prediction was carried out using molinspirationchemiinformatics, Swiss ADMET,molsoft, /Pre ADMET, admet SAR and PASS online softwares and docking was carried out using AutodockPyRxVina software. The statistical analysis was carried out by Graphpad prism softwares v7.0.

***Insilico* studies**

The bioactivity score of selected compounds were evaluated using Molinspiration chemiinformatics server. The toxicity of selected neuroprotective agents and their drug likeness scores were evaluated by computational method using Swiss ADMET software. PASS values was predicted using PASS online computer program and docking was performed using Autodock vina PyRx software [44].

**Molecular docking studies of vitexin, isovitexin and luteolin**

Docking studies of the lead molecules were performed using AutodockvinaProgram in PyRxsoftwares. The binding affinity of the lead compounds Vitexin, Isovitexin and luteolin with 3D3L- Humanarachidonatelipoxygenase, 2Z64 , The mouse TLR4 and MD-2 complex receptor [47].

**Plant collection and Preparation of crude hydroalcoholic extract of leaves of P. edulis by soxhlet extraction**

Fresh leaves of Passifloraedulis of the family Passifloraceae used for the study were collected and authentication of medicinal plant were carried out by Dr. Boby. The dried leaf powder of Passifloraedulis was subjected to extraction using suitable solvents for the separation of medicinally active portions. Soxhlet extraction or hot continuous extraction method was used

for the separation of suitable concentration of active constituents.

**Phytoconstituent screening of hydroalcoholic leaf extract by qualitative and quantitative estimation**

The hydro alcoholic leaf extract of p.edulis was subjected to phytochemical analysis by qualitative and quantitative estimation for the detection of the major chemical constituents.

***In vitro* anti-inflammatory activity**

The anti- inflammatory potential of flavonoids in vitro involve the inhibition of the synthesis and activities of different proinflammatory mediators such as eicosanoids, cytokines and adhesion molecules etc. Anti- inflammatory agents are responsible for the treatment of various neurological disorders in which inflammation plays the crucial pathological role. Thus plant derived anti-inflammatory agent are involved in the prevention of neuroinflammatory and neuroimmune diseases [51].

***In vivo* pharmacological studies**

The experiments were performed after Institutional Animal Ethics Committee had approved the project on and the approval number was ECPS/IAEC-2-2018-4/22 for Research foe education purpose on small animals.

**Acute toxicity studies of hydroethanolic extract of p. Edulis**

Acute toxicity of hydroalcoholic extract of leaf extract of *P.edulis* were carried out as per the OECD guideline 423 after animal ethical clearance from institutional animal ethics committee. Male wistar rats were fasted overnight for 24 hr prior to dosing. [60-61]. Following the periods of fasting, the body weight of each rat was determined and the dosing of the extract and control group were calculated according to the body weight. The concentration of the extracts used were calculated using the formula;

Volume given=dose rate×weight of the animal(kg) Concentration(mg/kg)

**Evaluation of neuroprotective activity of hydroalcoholic extract of P. edulis (HEPE) for multiple sclerosis**

Chemically induced MS in wistar rats were used in the present study to evaluate the neuroprotective activity.

Cuprizone (CPZ) is an oxalic acid bis (cyclohexylidenehydrazide) copper chelating agent induces high demyelination in different regions of brain including corpus callosum. Animals were fed with powder diet mixed with cuprizone (0.2%) that causes cell death of oligodentrocytes that produce progressive demyelination. The demyelination is estimated by several behavioural tests such as rota rod tests, elevated plus maze test etc [66].

**Experimental design**

30 Male wistar rats were divided into 5 groups of 6 animals each. Group I serves normal control, group II serves as Negative control (with MS untreated). Group III serves as standard which receives resveratrol (250mg/kg) administered orally by dissolving in CMC. Group IV and V receives hydroalcoholic extract (200mg/kg and 400mg/kg) respectively. All rats except from the normal control group were orally administered with CPZ that mixed with the powdered chow simultaneously with the treatment.

**Assessment of Multiple Sclerosis**

Rats have to be weighed 3 times a week. The behavioral tests were inducted at 5 days interval. Rats that were moribund were sacrificed and the date of sacrifice was recorded for calculating the survival time. At the end of experiment, brain weight is measured and brain will be harvested and sections shall prepare for histopathological analysis. Blood samples was collected from all group of animals by cardiac puncture and the estimation of anticholinesterase activity was carried out.

**Assessment of hind limb paralysis**

The hind limb paralysis of rats that induced with MS can be measured visually.[70]

**Behavioral tests**

The behavioral studies on animals give the visible manifestation of activity of the central nervous system. The behavior of animals can be correlated with measurement of brain electric or chemical activity to evaluate the disease condition. In the present study the disease progression and its statuses were evaluated with various behavioral tests of animals such as maze apparatus testing, Rota rod tests, actophotometer etc. [75]

**Maze studies**

**T-maze**

The T- Maze is the apparatus used in the study of fear and anxiety. The test is based on the natural aversion of rats for open and elevated areas. The number of entries into the open arms and the time spent in the open arms are used as indices of open-space induced anxiety in rat. [75-78]

**Morris Water Maze**

In the present study each group of experimental animals were subjected for acquisition training with the invisible platform. There are two basic trials, one utilizing a visible and the other hidden platform. On the first experimental day rats were trained to swim in the maze (in the absence of platform) in 60 seconds. In five subsequent days the special memory of the experimental animals were assessed on the basis of the escape latency period of animals in the morris water maze. [75-78].

**Actophotometer**

Before kept the animal in the apparatus, turned the equipment on and checked that all the photocells are working for accurate recording. Then the experimental animals were placed in the activity box for 10 minutes. The basal activity score was also recorded.[75-78]

**Rota rod test**

The apparatus was turned on and the animals were placed on the rotating cylinder. The fall of different groups of animals from the cylinder was recorded and correlated with neurological functioning. [75-78].

**Biochemical analysis**

Blood collection was carried out in Day 22. Prior to blood collection, the rats were anesthetized with diethylether. The blood samples were collected by cardiac puncture and centrifuged at 14000 r/min for 10 min to separate the serum. Biochemical parameter like cholinesterase activity, Protein estimation, Estimation of Reduced Glutathione,

Catalase activity, Estimation of Nitrite Levels and Super oxide dismutase level were evaluated.

**Histopathological investigations**

Following blood sampling, the mice were sacrificed by anesthetized with diethyl ether. Brain were harvested and fixed in 10% formalin. After fixation for at least 24 h, the tissues were processed for 16 h by an automated tissue processer. Processes tissues were then embedded in paraffin. Four micrometers thick sections were cut from the paraffin block and stained with hematoxylin and eosin (H&E). Each slide was examined under a light microscope with assistance of a pathologist. At least 10 fields from each slide of each group were examined to evaluate the histological changes. Sections were evaluated for tumour cell cytology, mitotic rate, growth pattern, necrosis and metastatic tumor nodules present on these tissue [83-85].

**Statistical analysis**

The data's were expressed as mean standard error mean (SEM). The data's were analysed by using Graph pad software version 7.03 and Graph pad insat 3.01 demo version by Unpaired t test and One way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls test.

## RESULTS AND DISCUSSIONS

***In silico* studies**

**Prediction of molecular descriptors**

The molecular descriptor values of compounds Vitexin, Isovitexin and Luteolin was obtained using Molinspirationchemiinformatics online soft wares. The Lipinski rule of five is evaluated. The molecular descriptors related to the Lipinski rule of 5 were summarized in table 1.

**Table 1: Molecular descriptors of compounds**

| Compounds | MW g/mol | Log P | NHA | NHD |
|---|---|---|---|---|
| Vitexin | 432.4 | 0.29 | 10 | 7 |
| Isovitexin | 432.4 | 0.29 | 10 | 7 |
| Luteolin | 286.23 | 1.4 | 4 | 6 |

**Drug likeness evaluation**

The drug likeness properties of the compounds were evaluated using pre ADMET software. The drug likeness values of compounds were summarized in table 2.

**Table 2: Drug likeness properties**

| Compounds | Drug likeness score |
|---|---|
| Vitexin | - 1 |
| Isovitexin | - 1 |
| Luteolin | - 0.86 |

**Toxicity evaluation**

Toxicity evaluation of Vitexin, Isovitexin and Luteolin were evaluated using pre ADMET software. The toxicity parameters of compounds were summarized in table.3.

**Table 3: Toxicity evaluation**

| Toxicity | Vitexin | Isovitexin | Luteolin |
|---|---|---|---|
| Amies toxicity | Non mutagen | Non mutagen | Non mutagen |
| Carcinogenicity | Non carcinogen | Non carcinogen | Non carcinogen |
| HERG inhibition | Medium Risk | Medium Risk | Medium Risk |
| Acute Oral Toxicity | Class III | Class III | Class II |

**Biological activity prediction**

The prediction of different biological activities of lead compounds were evaluated using PASS (Prediction of Activity Spectra for Substances) online software. Vitexin, Isovitexin and Luteolin were evaluated for their anti-inflammatory, MAO inhibition, antioxidant and freeradical scavenging properties using the software. The bioactivity scores of the compounds were summarized in table 5

**Table 4: ADME evaluation**

| Parameters | Vitexin | Isovitexin | Luteolin |
|---|---|---|---|
| BBB | 0.31 | 0.36 | 0.16 |
| HIA | 31.42 | 31.37 | 63.49 |
| Cyp p450 inhibition | Inhibitor | Inhibitor | Inhibitor |
| All subunit | Non substrate | Non substrate | Non substrate |
| Proten binding | 95.09 | 99.71 | 99.62 |

**Table 5: Biological activity of compounds**

| Activity | Vitexin | Isovitexin | Luteolin |
|---|---|---|---|
| Anti-inflammatory | 0.680 | 0.647 | 0.670 |
| Antioxidant | 0.858 | 0.717 | 0.782 |
| Free radical scavenger | 0.766 | 0.693 | 0.755 |
| MAO inhibition | 0.544 | 0.534 | 0.584 |

Insilico computer simulation studies and docking studies are proved to be the best tool used to investigate the complementarity and level of interaction at the molecular level between the compounds of natural or synthetic origin and a potential target. The bioactivity score of selected compounds were evaluated using Molinspiration chemiinformatics server. The toxicity of selected neuroprotective agents and their drug likeness scores were evaluated by computational method using Swiss ADMET software. PASS (Prediction of Bioactivity Spectrs for Substances) has been employed as a strong potential tool to predict the biological activity spectrum of lead compounds. In the present study, screening of 3 lead compounds were done using molinspiration. From the results of the study, Vitexin, isovitexin and luteolin have the molecular weight of 432.4,432.4 and 286.23 which are in the range that referred by rule of five. Vitexin and

isovitexin have the partition coefficient (log P) values of 0.29 and luteolin has value of 1.4 which was found to be less than the standard range. Vitexin and isovitexin posess 10 Hydrogen bond acceptor and 7 hydrogen bond donor groups. Both the compounds have high Hydrogen bond donor groups. In the case of luteolin the values were 4 and 6 respectively so it was with in the limit. From the molinspiration studies Vitexin and isovitexin were found failed in one rule, they possess high hydrogen bond acceptor groups. As the rule says that the one violation of the rule is acceptable, the compounds are considered for the next step of studies. The toxicity evaluation studies were carried out using pre ADMET software version 2.0. Pre ADMET was used for the prediction of drug likeness, ADME properties and also were used for the toxicity evaluation studies. The results of the study showed the compounds did not possess carcinogenic property so are safe to be used. The lead compound showed medium risk of HERG inhibition. Acute oral toxicity evaluation showed both Vitexin and Isovitexin were at class III category and Luteolin was in class II category. From the results of present study, both vitexin and Isovitexin are in Class III category and Luteolin is in Class II category. The lead compounds were found to possess no toxicity according to the in silico studies. ADME properties were evaluated for the lead compounds. As the study is dealing with neuroprotective activity of the selected flavonoids, it has been found that to be important to check the BBB (Blood Brain Barrier) penetration capacity of the compounds. From the results, Vitexin, Isovitexin and Luteolin possess the values 0.31, 0.36 and 0.16 so they will absorbed to CNS. Luteolin possess the value that less than the range of highly absorbed drugs. It has the HIA value of 63.49% and is was in the category of moderately absorbed drugs. All the compounds have strong binding capacity with plasma protein which was not beneficial. PASS online softwares were used for the prediction of biological properties of the lead compounds. The Pa values of Vitexin , the main constituent present in Passiflora eduliswas evaluated for PASS using PASS online software From the above results of PASS prediction shows Vitexin has significant anti-inflammatory, antioxidant and Freeradical scavenging activities when compared to Isovitexin and Luteolin. The MAO inhibition activity is high for Luteolin. Comparing the PASS values, Vitexin will posess significant anti-inflammatory activity thus best activity against Multiple sclerosis.

**Molecular docking studies of Vitexin, Isovitexin and Luteolin with Human leukocyte antigen (HLA DR-2) receptor and Toll like receptor TLR-4 for neuroprotective activity against Multiple sclerosis**

Molecular docking studies of Vitexin, Isovitexin and Luteolin with Human leukocyte antigen (HLA DR2) receptor and Toll like receptor were carried out. In this study, the virtual screening and molecular docking methods for Vitexin, Isovitexin and Luteolin against 2 target proteins 1BX2 (Figure 6) and 2Z64(figure 7).with Autodock scoring functions by PyRx Virtual screening Program for neuroprotective activity were studied. The Human leukocyte antigen (HLA) receptor, HLA DR2 is the one which showed better docking score with all the lead compounds. The free energy associated with the docking of HLA receptor with Vitexin, Isovitexin and Luteolin were found to be -9.2, -8.8 and -9.1 Kcal/mol. So the binding affinity of the compounds with the HLA DR2 receptor was in the order of Vitexin>Luteolin>Isovitexin. Standard compound showed -7.9 Kcal/mol as the binding energy. From the result Vitexin, Isovitexin and Luteolin has better docking score with the receptor than that of standard. In case of 1BX2 receptor, Vitexin is the compound which has better docking score and proposed to posess better activity against neuroinflammation and thus against Multiple sclerosis. 1BX2 is the receptor of HLA that have crucial role in genetic pathology of the disease. With the HLA receptor in docking all the lead compounds have more than 3 hydrogen bonds and have amino acid residues Aspargine, Tyrosine, Tryptophan, Glycine etc. Toll like receptor (TLR 4) play an important role in the neuroimmune system. The docking score of compounds with the receptor was found to be lesser than that obtained with HLA receptors. The gliding scores of Vitexin, Isovitexin and Luteolin were found to be -8.2, -7.3 and -7.9 K cal/mol. With that receptor Vitexin was found to be better docked with the receptor. The results of docking of Vitexin, Isovitexin and luteolin with the receptors are shown in the table 6. The docking details such as number of Hydrogen bond interactions and its length, Aminoacid residues involved in the docking are summarized in table 7.

**Table 6: ΔG values of docking of compounds for neuroprotective activity**
**Molecular docking studies of Vitexin, Isovitexin and Luteolin with Human leukocyte antigen (HLA DR-2) receptor and Toll like receptor TLR-4 for neuroprotective activity against Multiple sclerosis.**

| Sl No. | Docking | Autodock score |
|---|---|---|
| 1 | Vitexin- 1BX2 | -9.2 K cal/mol |
| 2 | Vitexin – 2Z64 | -8.2 K cal/mol |
| 3 | Isovitexin – 1BX2 | -8.8 K cal/mol |
| 4 | Isovitexin – 2Z64 | -7.3 K cal/mol |
| 5 | Luteolin – 1BX2 | -9.1 K cal/mol |
| 6 | Luteolin – 2Z64 | -7.9 K cal/mol |
| 7 | Standard – 1BX2 | -7.9 K cal/mol |

**Table 7: Docking details**

| Docking | No. of Hydrogen bond interactions | Lengths of Hydrogen bond interactionsA0 | Aminoacid residues involved |
|---|---|---|---|
| Vitexin- 1BX2 | 5 | 456,432,496,423,389 | ASP,TYR,GLY |
| Vitexin – 2Z64 | 1 | 356 | ASP |
| Isovitexin – 1BX2 | 5 | 496,506,456,378,435 | TYR,GLY |
| Isovitexin – 2Z64 | 4 | 500,496,435,456 | ASP, GLY,TYR |
| Luteolin – 1BX2 | 7 | 499,367,342,287,345,421,433 | TYR,GLY,ASP,ARG |
| Luteolin – 2Z64 | 6 | 500,455,487,476,543,435 | ASP,TYR,GLY |
| Resveratrol -1BX2 | 2 | 487,476 | ASP,GLY |

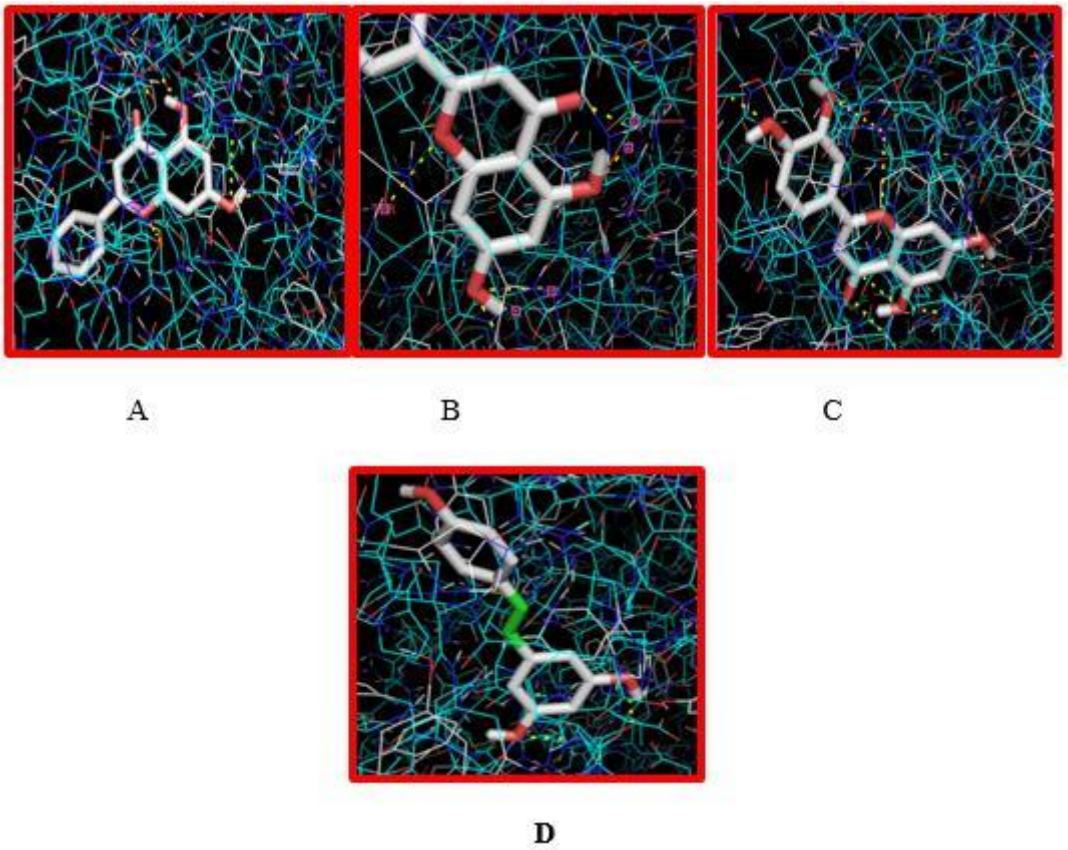


**Figure 6: Docked image of A- Vitexin, B- Isovitexin, C- Luteolin, D- Standard with 1BX2**

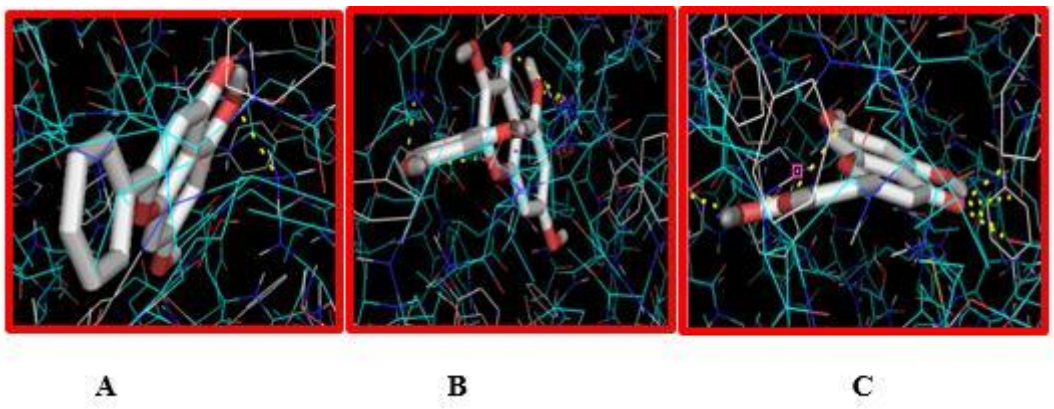


**Figure 7: Docked images of A- Vitexin, B- Isovitexin, C- Luteolin with 2Z64**

**Preliminary phytochemical screening**

Qualitative phytochemical analysis of the extract has been designed to evaluate the presence of flavonoids, alkaloids, steroids and tannins. Quantitative analysis showed the presence of high amount of flavonoid contents in the hydroethanolic extracts of P. edulis. Flavonoid content was found to be higher than the other phytoconstituents. In this quantitative study estimation of crude polyphenols from hydroalcoholic extracts of P.edulis showed the presence of a significantly high amount of phytoconstituents mainly responsible for their neuroprotective activity.

***In vitro* anti-inflammatory studies**

The anti-inflammatory assays of the extract were based on the assessment of ability of the extract to inhibit the enzymes which catalyses the oxidative stress in neural tissue and also assess the level of certain proinflammatory mediators in cell lysate. The extracts were found to be good inhibitor of both the enzymes therefore must possess better anti-inflammatory properties. The reduction in the cyclooxygenase and lipoxygenase levels were found to be in a dose dependent manner. The total hydroethanolic extract exhibited good inhibitory activity against the arachdonic acid metabolism by inhibiting the pathways there by inhibit the production of potentially active proinflammatory mediators thus must exhibit better anti-inflammatory activity. The hydroethanolic extract also reduced the cellular nitrite levels, inducible nitric oxide synthase and myeloperoxidase significantly. The results of various anti-inflammatory assays were summarized in the table 8.

**Acute toxicity studies**

On the basis of literature reviews, it has been reported that P. edulis seems to be safe at a dose level of 2000 mg/kg, and the LD50 is considered be 2000mg/kg. Therefore with reference to reviews of P. edulis, the extract was administered directly at the dose level of 2000mg/kg.

**Effect of HEPE on hind limb paralysis**

Effect of HEPE on hind limb paralysis were observed in cuprizone induced MS in wistar rats were recorded in 8th, 16th and 21st days. The animals on cuprizone treatment were observed to posess paralysis on the hind limbs. The observations were made at 8 day interval and the results shows that there were a progressive reduction in the functioning of limbs in the group of wistar rats that received omnly cuprizone and not treated. The groups received the standard drug, HEPE at 200, 400mg/kg showed a gradual reduction in the hind limb paralysis. The higher dose of HEPE (400mg/kg) was found to be more effective and the results were almost near to the results obtained from the group of animals received standard. From the results of assessment of the hind limb pparalysis in the experimental animals, it was clear that the hydroethanolic extract of the plant which was found to contain considerable amound of lead compounds Vitexin, Isovitexin and Luteolin are effective against the hind limb paralysis and loss of function. The effects of animals that treated with higher dose of extract was found to be comparable with that of standard dose treated animals. Thus have neuroprotective activity.

**Table 8: Results of *In vitro* anti-inflammatory assays**

| Concentration (µg/ml) | COX Inhibition (%) | LOX Inhibition (%) | MPO activity) (U/ml) | NO Synthase Inhibition (%) | Concentration of cellular nitrite (µg) |
|---|---|---|---|---|---|
| Standard (Diclofenac) | 100±0.001*** | 100±0.001*** | 0.000721±0.003*** | 100±0.005*** | 471,35±0.006*** |
| HEPE @ 25µg/ml | 13.6±0.0577 | 13.44±0.005 | 0.0028±0.031 | 41.95±0.03 | 380.16±0.001 |
| HEPE @ 50µg/ml | 32.63±0.012*** | 25.02±0.0033*** | 0.0012±0.005*** | 67.23±0.005*** | 328.19±0.002*** |
| HEPE @ 100µg/ml | 42.88±0.005*** | 27.87±0.0057*** | 0.0007±0.136*** | 93.73±0.136*** | 297.99±0.003*** |

**Table 9: Effect of HEPE on behavior of animals on Morris water maze.**

The values are expressed as mean ± S.E.M, n= 5. The statistical analysis were carried out using one way ANOVA followed by Student-Newman-Keuls test, Where ***P<0.001

| Treatment group | Days | | | |
|---|---|---|---|---|
| | Day 5 | Day10 | Day15 | Day 20 |
| **Normal control** | 4.4±0.509 | 4.5±0.812 | 4.12±0.812 | 3.90±0.812 |
| **MS control-CPZ (0.5%)** | 15.6±0.812 | 16.02±0.509 | 16.10±0.509 | 20±0.509 |
| **CPZ + RV (250mg/kg)** | 6.8±0.374 | 6±0.374*** | 6.5±0.509** | 6.8±0.374 |
| **CPZ + HEPE (200mg/kg)** | 14.2±0.302 | 14.5±0.509 | 13.92±0.707** | 13.82±0.201 |
| **CPZ + HEPE (400 mg/kg)** | 9±0.707** | 9.2±0.707 | 8.94±0.374** | 7.94±0.707*** |

**Table 10: Effect of HEPE on behavior of animals on rotarod.**

The values are expressed as mean ± S.E.M, n= 5. The statistical analysis were carried out using one way ANOVA followed by Student-Newman-Keuls test, Where ***P<0.001.

| Treatment group | Days | | | |
|---|---|---|---|---|
| | Day5 | Day10 | Day15 | Day20 |
| **Normal control** | 9±0.2449 | 9.4±0.2889 | 9.3±0.2108 | 9.5±0.3164 |
| **MS control-CPZ (0.5%)** | 2.4±0.3612 | 2.4±0.3152 | 2.5±0.2889 | 2±0.807 |
| **CPZ + RV (250mg/kg)** | 5.7±0.2449*** | 5.6±0.2449 | 5.5±0.2449 | 5.4±0.2108 |
| **CPZ + HEPE (200mg/kg)** | 3±0.3163 | 3.3±0.2449 | 2.9±0.3162 | 3.10±0.707** |
| **CPZ + HEPE (400 mg/kg)** | 4.5±0.4213** | 4.4±0.319 | 4±0.2108 | 4.3±0.302** |

**Table 11: Effect of HEPE on the behavior of animals on actophotometer.**

The values are expressed as mean ± S.E.M, n= 5. The statistical analysis were carried out using one way ANOVA followed by Student-Newman-Keuls test, Where ***P<0.001

| Treatment groups | Days | | | |
|---|---|---|---|---|
| | Day5 | Day10 | Day15 | Day20 |
| **Normal control** | 1.052±0.015 | 1.046±0.0176 | 1.056±0.0087 | 1.065±0.0067 |
| **MS control-CPZ (0.5%)** | 0.332±0.0174 | 0.312±0.016 | 0.341±0.0115 | 0.339±0.0534 |
| **CPZ + RV (250mg/kg)** | 0.58±0.0089*** | 0.56±0.0098 | 0.55±0.0079 | 0.557±0.0065 |
| **CPZ + HEPE (200mg/kg)** | 0.454±0.0116 | 0.449±0.0116 | 0.458±0.0332** | 0.453±0.018 |
| **CPZ + HEPE (400 mg/kg)** | 0.528±0.00969 | 0.526±0.0089** | 0.529±0.0442 | 0.530±0.0221** |

**Effect of HEPE on behavioral studies**

The behavioral tests were carried out on experimental animals to determine the cognitive functions of rats including learning and memory. The tests were designed also to evaluate the locomotor activity and muscle strength of exprimental animals. In the present study performed 5 behavioral tests on Cuprizone induced MS rats ata an interval of 5 days. For the behavioral study of the animals different apparatus were used such as Rota rod, Actophotometer, Morris ater maze and T- maze. Morris water maze test was performed to evaluate spacial learning, memory and cognitive mapping of experimental animals. The escape latency, the time taken by the animal to find the platform was measured and then coorrelated with the spacial memory and learning of the animal. The results obtained from the morriz water maze(figure 9) test showed that CPZ- treated animals presented with significantly higher escape latency period in the Morriz water maze relative to the control and HEPE treated groups (table 9). There was a significant difference in the escape latency period of groups treated with CPZ and HEPE. The escape latency period of groups of CPZ+RV was significanly reduced in the Day 10 (***P<0.001). Groups treated with HEPE 2 00 mg/kg showed that lesser time to locate the platform and had significantly (**P<0.01) reduced period of escape latency. Treatment with higher dose of HEPE (400mg/kg) showed significantly reduced period of escape latency on days Day 15 and Day 20. They showed significantly reduced escape period on day 15 (**P<0.01) and on day 20 (***P<0.001). From the results of the study it has been cleared that the HEPE at different doses increased the spacial memory and cognitive mapping capacity of the animal effectively.

Rota rod test was performed to evaluate the balance, grip strength and motor cordination of the experimental animals. The length of the time the animal stays on rotating rod is measure of their motor functions. The results of the rota rod test showed Oral administration of CPZ significantly affected the muscle grip performance of the rats. The groups treated with CPZ showed impairment in the gripstrength performances. Treatment with HEPE at both the doses significantly (**P<0.01) the muscle strength (dalayed falling of time) (table 9) (figure 8).

Actophotometer was setted for masuring the locomotor activity of the experimental animals. From the results of the study(Figure 9), it has been shown that Treatment with higher dose of HEPE (400mg/kg) showed a dignificantly improved locomotion activity significantly (** P<0.01 ) on the Day 10 and Day 20. The animals received the 200mg/kg of HEPE showed incresed locomotion significantly (**P<0.01 ) on Day 15. The group which received CPZ and standard resveratrol of 250mg/kg showed a significant (***P<0.001) improvement in locomotor activity fom the day Day 5 ( table 10)The group of animals which received only Cuprizone showed gradual decrease in locomotor activity from Day 5 to Day 20. Increased locomotor activity was indicated by increase in the reading of digital counter. From the results it has been clear that treatment with HEPE at 200mg/kg and 400mg/kg increased the locomotor activity of the experimental animals as compared to negative control animals.

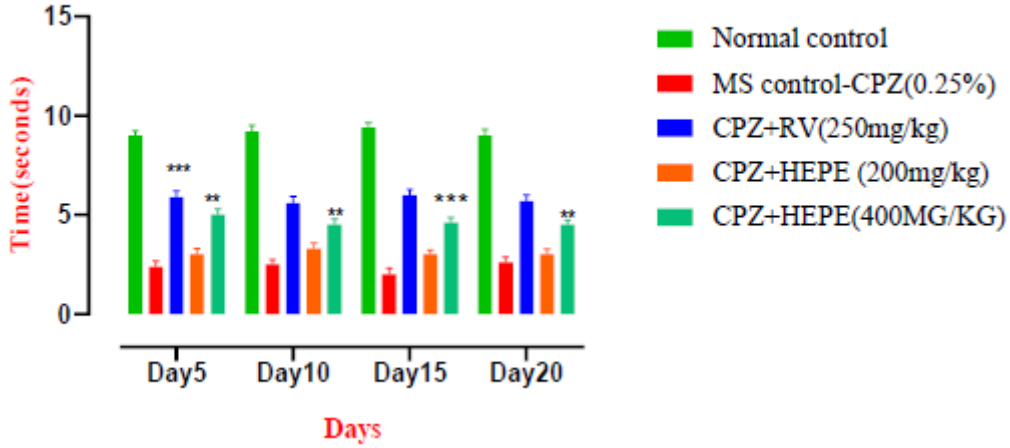


**Figure 8: Effect of HEPE on behavior of animals on rota rod.**

The values are expressed as mean ± S.E.M, n= 5. The statistical analysis were carried out using one way ANOVA followed by Student-Newman-Keuls test, Where ***P<0.001.

**Effect of HEPE on behavior of animals in Actophotometer.**

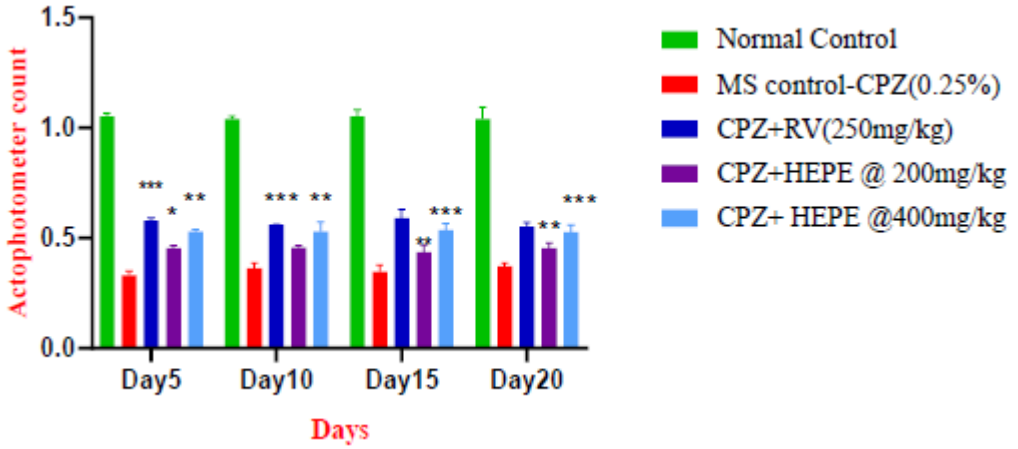


**Figure 9: Effects of HEPE on behavior of animals on Actophotometer.**

The values are expressed as mean ± S.E.M, n= 5. The statistical analysis were carried out using one way ANOVA followed by Student-Newman-Keuls test, Where ***P<0.001.

**Estimation of Reduced Glutathione**

Estimation of reduced Glutathione (GSH) provided relevant measure of the cellular oxidative stress. The CPZ induced rats showed a significant decrease in the level of GSH. And the level of GSH was found to be increased significantly (**P<0.01) in dose of 200mg/kg and (***P<0.001) dose of 400mg/kg as compared to CPZ induced animals.GSH is an important non-enzymic antioxidant and its levels were correlated with the antioxidant and free radical scavenging property of HEPE. From the results it was found that HEPE has better antioxidant and free radical scavenging properties.

**Estimation of cellular nitrite levels**

The cellular nitrite levels were measured and it indicate the level of oxidative stress. The nitrite levels were found to be increased significantly in CPZ treated animals. From the results obtained, the treatment of HEPE at 200 mg/kg reduced the level of cellular nitrite significantly (***P<0.001) The nitrite levels decreased significantly at a dose of HEPE at 400mg/kg (**P<0.01) as compared to the group of experimental animals treated with only Cuprizone.

**Estimation of Superoxide dismutase (SOD)**

SOD is the enzyme that catalyses the dismutation of superoxide into oxygen,an important antioxidant mechanism. The level of the antioxidant enzyme SOD was found to be dicreased in the CPZ treated animals. The results of the study shows that the HEPE at 200 mg/kg and 400mg/kg increased the level of SOD significantly.(*P<0.05) and (**P<0.01) respectively.

**Estimation of Catalase activity**

Catalase is an important enzyme that protect the cell from oxidative damage. The level of catalase was found to be decreased in the CPZ treated group as compared to the

control. The treatment with the extract shows better catalase activity compared to CPZ treated groups. HEPE at 200 mg/kg increased catalase activity significantly (*P<0.05) and HEPE at 400 mg/kg increased catalase level at high significance (**P<0.01) as compared by group of animals treated with CPZ only. The results of the present study showed that the HEPE possess better antioxidant property that was conformed for their neuroprotective activity.Therefore the lead compounds in HEPE, Vitexin, Isovitexin and Luteolin was proved to be neuroprotective against CPZ induced MS which was proved to possess anti-inflammatory and immunomodulatory effects.

**Effect of HEPE on histopathology of brain tissue.**

The histopathological studies of brain was done by Haematoxylin & Eosin stains. Group of experimental animals induced with CPZ exhibited a prominent vacuole formation. Rats treated with HEPE at 200mg/kg and 400mg/kg exhibited a presence of healthy granular cells and possess few damaged cells respectively. Animals received higher dose of HEPE (400mg/kg) exhibited healthy granular cells which rescembles the control group.

## CONCLUSION

For the present study, number of polyphenols, flavonoids are screened by in silico methods to find out better lead compounds. After evaluating number of flavonoids, 3 lead compounds were selected for the further study. In silico molecular docking studies of selected lead compounds, Vitexin, Isovitexin and Luteolin were carried out with 2 receptors involved in the MS pathogenesis. Based on the results of in silico screening and molecular docking assessment all the three lead compounds are confirmed to possess better neuroprotective activity and were suggested for the treatment of a neuroimmune disorder, Multiple sclerosis. The hydroethanolic extracts of leaves of P.edulis have been reported to possess high amount of vitexin, isovitexin and luteolin. In vitro anti-inflammatory studies were carried out for the HEPE and shows better anti-inflammatory activity as compared to the standard. The anti-inflammatory activity of the plant extracts were proposed to be due to the presence of the lead compounds Vitexin, Isovitexin and Luteolin. Thus, the lead compounds can be used a potent anti-inflammatory agent to protect the health of humans against kinds of ailments due to inflammation. In vivo behavioral studies showed that the anti-inflammatory and neuroprotective activity of HEPE increased the cognitive functions of experimental animals. It has been found that HEPE improved the muscle strength, memory, learning and locomotor activities of animals. Administration of HEPE recovered the normal levels of biochemical parameters like reduced glutathione, Catalse, Superoxide dismutase, and level of cellular nitrites. The studies indicated that the Vietxin, Isovitexin and Luteolin present in the plant extract possess beter anti-inflammatory, anti-oxidant and free radical scavenging properties. The histopathological examination of corpus callosum region showed that the vacuoles and neurodegeneration were decreased by administration of HEPE. The present study suggests that the compounds Vitexin, Isovitexin and Luteolin present in the HEPE possess better neuroprotective, anti-inflammatory, anti-oxidant and immunomodulatory action to be suggested for safe use against Multiple sclerosis.

From the study, it has under stood that the lead compounds mediate the overexpression of HLA haplotypes HLA DR2-DQ6, HLA D4- DQ8 and HLA DR3-DQ2 that play critical role in autoimmunity. Major histocompatibility complex (MHC) code for protein found in the surfaces of immune cells. HLA is the gene that code for immune system in the nervous system which contain different haplotypes. It can activate the CD4 T cells in response to viral/bacterial infections and to produce proinflammatory mediators. In the case of Multiple sclerosis as it is a neuro autoimmune disorder, mutation of the haplotypes produce self-peptides/ mimics to activate the T cells and generate auto immune responses of MS. Overexpression of HLA haplotypes in immune cells leads to over activation of the T cells that will leads to tissue damage in nervous tissues and leas to neuroinflammatory auto immune Multiple sclerosis. The main function of MHC is to clear any type of bacterial (extracellular/intracellular) or viral infections. HLA is normally a gene complex containing 3 regions, Class I, Class II and Class III. Class II area is most polymorphic region of HLA and contain 4 subunits of α1, α2, β1, and β2. β1 and β2 are present in the antigen presenting cells. The class II region has DP, DQ and DR as haplotypes which are dealing with Multiple sclerosis. The lead compounds, Vitexin, Isovitexin and Luteolin are docked to the HLA receptor that possess genes that code for myelin basic protein. Over expression of the polymorphic DP, DQ and DR alleles results in increased activation of immune cells which mistakenly recognizes myelin protein as the foreign material and will start the immune responses that ends with neuroinflammation and formation of lesions in the brain. As the compounds docked with the HLA receptor more effectively and were proved to be effective for the treatment of lesions and inflammations in the Multiple sclerosis.

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