

## VIRTUAL SCREENING OF POTENT ANTI-INFLAMMATORY MOLECULES FROM BOERHAAVIA DIFFUSA.LINN AGAINST NEUROINFLAMMATION FOR P38A INHIBITOR(S)

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### **Abstract:**

Inflammation in the central nervous system is a common feature in Neurodegenerative diseases like Amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, and Multiple Sclerosis. Experimental evidences have shown the extensive increase of pro-inflammatory cytokines (PC) acts as a major contributor in disease progression of chronic neurodegenerative disorders. Major PC, such as IL-6 and TNF $\alpha$ , are produced by cells involved in these disease progressions by involving p38 $\alpha$  MAPK pathway at cellular level. Hence p38 MAPK family is being investigated as a potential therapeutic target for many CNS disorders. Since a variety of approved drugs for the treatment of neurodegenerative diseases have reported potentially serious side effects. Hence there is a massive need for these therapies from natural sources.

In the present study, we have chosen a medically important ayurvedic plant Boerhaavia diffusa.linn (BD). Based on the research review on BD, Eupalitin, Eupalitin-3-O- $\beta$ -D-galactopyranoside,  $\beta$ -sitosterol, Liriodendrin, Boeravinone B, Punarnavine; potent anti-inflammatory phytochemicals were selected to explore its pharmaceutical properties. The initiation of early ADME screening studies of these phytochemicals also showed impressive pharmacokinetic properties, to be a potent drug molecule. Among them, Eupalitin,  $\beta$ -sitosterol, Boeravinone B, Punarnavine were qualified for druglikeness. Further, docking experiments were used to screen the selected phytochemicals for p38 $\alpha$  inhibitors. The studies indicated Eupalitin and Boeravinone B as potent p38 $\alpha$  inhibitors as it showed the compact binding to the biologically active sites. Additionally, these phytochemicals were evaluated for its anti-neuroinflammatory effects using in-vitro cell line studies on microglial cells in comparison with the whole plant extract.

Our pilot studies suggests that these natural phytochemicals from potent anti-inflammatory valued plant has potency in targeting all the neurodegenerative disease in common from being a potent p38 $\alpha$  inhibitor along with a good druggable property. In future these phytochemicals can be experimented to explore the mechanism of action at molecular level.

**Keywords:** Boerhavia diffusa.linn, Docking, p38 $\alpha$  MAPK, neuroinflammation, Docking

**Abbreviation:** Boerhavia diffusa(BD), mitogen-activated protein kinase (MAPK), Absorption, Distribution, Metabolism, and Excretion (ADME), Quantitative Structure–Activity Relationship (QSAR), Central nervous system (CNS)

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### **Introduction:**

Neuroinflammation is a defensive mechanism of central nervous system (CNS) against injury and infection (1). It's a complex process involving many biochemical parameters and different type of residential cells on the CNS. Among all the cells; microglia have a major role in neuroinflammation. The activated microglia secretes many prominent pro-inflammatory cytokines namely tumor necrosis factor $\alpha$ (TNF- $\alpha$ ) and interleukins-6(IL-6) (2) in response to stimulus from the endogenous or the exogenous source. These cytokines are involved in defensive mechanism of inflammation by promoting the migration of microglia to the site of trigger in CNS (3). These biochemical reactions regulate to repair and recover from the damage in the CNS, a trigger of neuroinflammation. Whereas the uncontrolled activation of microglia by these inflammatory mediators leads to a chronic neuroinflammation underlying many neurodegenerative disorders including Parkinson's, Alzheimer's and multiple sclerosis(4).

As these pro-inflammatory cytokines are involved in leading the neuroinflammation, many drugs are mainly targeted to the pathway involving in the secretion of pro-inflammatory cytokines (5). One such major pathway is p38 $\alpha$  MAPK. Many research studies on the neurodegenerative disorders have revealed the involvement of p38 $\alpha$  MAPK in microglial cells in disease aberration (6). Hence targeting drugs for p38 $\alpha$  MAPK will be a very effective for amelioration of neuroinflammation. Moreover there is a raising interest in inhibitors from plants or plant derived phytochemicals because of its less adverse effects.

Hence in the present study;Boerhaavia diffusa.linn, a medicinally important plant has been chosen(7). Research studies have shown the potency of derived phyto-compounds from Boerhaavia diffusa plant against inflammation (8). On this background on BD for treatment of brain related disorders(9), the phyto-compounds are screened for p38 $\alpha$  MAPK inhibitor in the microglial cell lines using docking and in-vitro method. By the end of the study we successfully revealed the potent p38 $\alpha$  MAPK inhibitor contributing for inhibition of neuroinflammation.

### **Materials and Methods:**

#### **Materials:**

Eupalitin and Boerhavinone B was purchased from natural remedies and was prepared in DMSO (Sigma) and stored in small aliquots at 20<sup>0</sup>C. The following reagents were used: RPMI (Gibco), fetal bovine serum (Sigma), sodium pyruvate (Sigma), glutamine (Sigma), streptomycin/penicillin (Sigma), LPS (Sigma) derived from Salmonella enterica serotype typhimurium SL1181. Accessories used for cell culture were of cell culture grade from Nunc.

#### **Methods:**

#### **Listing out the potent anti-inflammatory compounds from Boerhaavia diffusa.linn:**

All the potent phytochemicals were chosen from the literature review on the Boerhaavia diffusa plant on pubmed (<https://pubmed.ncbi.nlm.nih.gov/?term=boerhavia+diffusa>). The research articles mentioning the anti-inflammatory phytochemicals from Boerhaavia diffusa.linn were short listed with its structural details using pubchem.

#### **QSAR study of the phytochemicals:**

Quantitative Structure–Activity Relationship of the phytochemicals were screened to calculate the molecular properties and bioactivity score of the phytocompounds. Using the Molinspiration

cheminformatics server the listed compounds were investigated for its druglikeness, pharmacokinetic properties to predict its biological activity.

### **Docking of phytochemicals to p38 MAPK:**

Ligand preparation and protein preparation was done according to the VaradarajuKR et.al, (10). In brief, Eupalitin ,  $\beta$ sitarosterol, Boerhavinone B and Punarnavine/Lunamrine were chosen as ligands and drawn in chemsketch and were optimized for further docking studies.

As a targeted receptor protein, 3QUE PDB file was selected from Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank(PDB). Then, water molecules, metal ions, cofactors, and ligands were removed and used for docking.

The binding sites for docking of ligands were selected from literature review (11,12) and using Autodock 4.2 (<http://autodock.scripps.edu/wiki/AutoDock4/>), a novel and robust automated docking method docking of the ligands to the receptor protein was done.

### **Cell culture:**

BV2 microglia cell line cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 2mM L-glutamine, streptomycin 40 $\mu$ g/mL, penicillin 40U/mL. Once confluent, cells were split 1:10 using trypsin/EDTA solution and cultured at 37 °C in 5% CO<sub>2</sub>.

### **In-vitro cytotoxicity assay:**

BV2 cells were seeded in 96 well plates ( $2 \times 10^5$  cells /ml), cultured for 24h. Followed by treatment with Eupalitin and Boerhavinone B for 24h. Then the culture medium was replaced with MTT solution (5 mg/ml) and incubated for 4 h at 37 °C in 5% CO<sub>2</sub>. Thereafter 150 $\mu$ l of MTT solution was replaced with DMSO and mixed thoroughly on a plate shaker and read at 540 nm.

### **Anti-inflammatory assay:**

BV2 cells were seeded in 96 well plates ( $2 \times 10^5$  cells /ml), cultured for 24h. Thereafter, cells were pre-treated with Eupalitin and Boerhavinone B for 1hr prior to stimulation with LPS (100 ng/ml). Twenty-four hours after stimulation, cell supernatants were collected and centrifuged. Concentrations of TNF $\alpha$  and IL-6 in cell supernatants were measured with a commercially available ELISA kit (eBioscience), followed by measurements in a plate reader at a wavelength of 450 nm.

### **Statistical analysis:**

In-vitro experiments were performed at least three times and in triplicates and the Data are expressed as mean  $\pm$  SEM. Statistical analysis was performed using one way ANOVA using Graphpad prism 8.0. Statistical significance was considered at  $p < 0.05$ .

### **Results:**

#### **1. Listing out the potent anti-inflammatory compounds from Boerhaavia diffusa.linn:**

From review of research articles, Eupalitin, Eupalitin-3-O- $\beta$ -D-galactopyranoside,  $\beta$ -sitosterol, liriiodendrin, Boerhavinone B and punarnavine were chosen and short listed based on the experimentally proven reported data.

#### **2. ADME property of potent anti-inflammatory phytochemicals to be an efficient drug molecule:**

As the short listed phytochemicals were screening for neuroinflammation, its pharmacokinetic properties are of being drug able will be an important parameter to target for p38 MAPK. The short listed phytochemicals were undergone, to screen for druglikeness. The data shown,

Eupalitin,  $\beta$ -sitosterol, Boerhavinone B and punarnavine were found to be satisfying all the drug able parameters (Table-2).

### **3. Binding of potent molecules to the biologically active site of the p38 MAPK:**

Drug able phytochemicals were virtually screened for its ability to bind to the p38 MAPK to act as an enzyme inhibitor. Our docking results showed the binding of Eupalitin to both the active and kinase activity site though strong hydrogen bonding (figure 1a, figure 2, Table-3) and Boerhavinone B binding only to the kinase activity site through strong hydrogen bond (figure 1b, Table-3).

### **4. In-vitro analysis of selected phytochemicals against neuroinflammation:**

As potent anti-inflammatory and good having a druglikeness and p38 MAPK binding phytochemicals from BD; Eupalitin and Boerhavinone B were further tested against neuroinflammation in BV2 microglial cell lines.

Hence Eupalitin and Boerhavinone B were undergone cytotoxicity assay to find the optimum concentration for further analysis. From the experimental data, 25 $\mu$ M and 50 $\mu$ M concentrations of Eupalitin; and 50 $\mu$ M and 100 $\mu$ M concentrations of Boerhavinone B (Figure-3) was selected for further anti-inflammatory studies as they were above the IC<sub>50</sub> value and evne the cells were morphologically intact at those concentrations.

### **Discussion:**

Neuroinflammation is a critical and important feature of many neurodegenerative disorders (4). Inflammation in the CNS involves the activation of major CNS resident cell called microglia (2). The endogenous and exogenous stimuli activates the microglia for secretion of pro-inflammatory cytokines. But under the normal conditions, microglia helps in maintaining the homeostasis of the CNS (13). Whereas the uncontrolled stimuli or chronic inflammation leads to neurodegenerative condition in the brain. Henceforth reduction in the neuroinflammation by reduction the microglial activation has promised a target for treating the neurodegeneration.

Mitogen-activated protein kinases (MAPKs) are serine/ threonine protein kinases that regulate cellular properties in response to several extracellular stimuli such as growth factors, inflammatory cytokines, and G protein-coupled receptors (12). In inflammation condition, it's been shown that the p38 $\alpha$  MAPK has prominent role. Research studies have even shown the inhibition of p38 $\alpha$  MAPK alleviating the inflammation in the disease condition (6). Hence in the present study, a traditionally important plant with known anti-inflammatory phyto-compounds were chosen to target p38 MAPK in neuroinflammation.

Eupalitin, Eupalitin-3-O- $\beta$ -D-galactopyranoside,  $\beta$ -sitosterol, liri dendrin, Boerhavinone B and punarnavine were chosen for the studies on neuroinflammation. These phyto-compounds have proven potent anti-inflammatory activity (14-18) (Table-1). As these compounds are targeted for neuroinflammation, analyzing the druglikeness and ADME property is essential for further consideration. By using Molinspiration cheminformatics server all the short listed compounds were analysed for its druglikeness. Lipinski's rule of five states a norms for drug to be orally active (19).

According to that, the orally active drug should have 1) Molecular mass less than 500 Dalton 2) High lipophilicity (expressed as LogP less than 5) 3) Less than 5 hydrogen bond donors 4) Less than 10 hydrogen bond acceptors 5) Molar refractivity should be between 40-130. Accordingly; Eupalitin,  $\beta$ -sitosterol, Boerhavinone B and punarnavine satisfied the Lipinski's rule and had high pharmacokinetic values with great gastrointestinal absorption (Table-2).

As the compounds mentioned in table-2 satisfied basic rules of druglikeness, it was further screened for p38 $\alpha$  MAPK inhibitor. Among all the p38 MAPK's, p38 $\alpha$  is involved in inflammatory pathway (12). Hence for ligand-protein docking 3QUE PDB file was selected. Major biologically active site amino acids of p38 $\alpha$  MAPK were considered for docking. Among all the phyto-compounds (Table-3) Eupalitin and Boerhavinone B showed the strongest hydrogen bond with the biological active sites of the p38 $\alpha$  (Figure-1 & 2) which was similar with the other ligands on p38 $\alpha$  (20,21).

Henceforth Eupalitin and Boerhavinone B were further considered for in-vitro studies using microglia cell lines against neuroinflammation. Before the assay, compounds were tested for its cytotoxicity using microglial cell line. Based on the cytotoxicity studies 25 $\mu$ M and 50 $\mu$ M of Eupalitin ; 50 $\mu$ M and 100 $\mu$ M of Boerhavinone B was considered for further analyses against neuroinflammation (Figure-3).

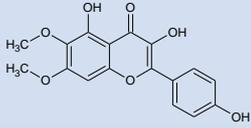
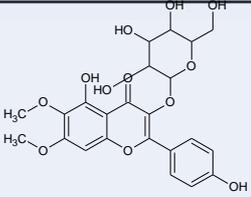
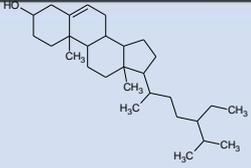
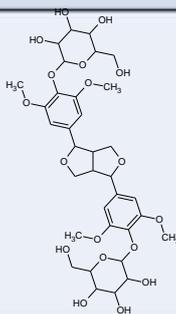
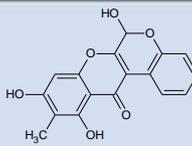
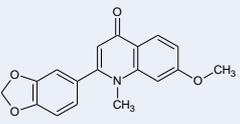
To analyze the compounds efficacy against neuroinflammation, compounds were pretreated with microglial cells for 24hrs followed by treatment with LPS (100ng/ml) to trigger the inflammation in the cells. After 8hrs of incubation, the cell supernatant was analyzed for pro-inflammatory cytokines. The ELISA analysis of pro-inflammatory cytokines TNF- $\alpha$  and IL-6 showed, both Eupalitin and Boerhavinone B had very good effect against neuroinflammation. Comparatively, 50 $\mu$ M Eupalitin showed prominent inhibition of neuroinflammation (Figure 4-5).

On a whole, the present study we have predicted the possible way of inhibition neuroinflammation by docking and confirmed by in-vitro studies. The study even confirms the neuroprotective effect of BD and the derived phyto-compounds of BD. Our data further suggests to confirm the p38 MAPK inhibition using more biochemical experimentation and to explore the molecules ability to be a natural drug against neuroinflammatory disorders.

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Anti-inflammatory phytochemicals of BD	Chemical formula	Structure of phytochemicals	Chemical Group of phytochemical
Eupalitin	$C_{17}H_{14}O_7$		Flavonoid
Eupalitin-3-O-β-D-galactopyranoside	$C_{23}H_{24}O_{12}$		Glycosidic Flavonoid
β-sitosterol	$C_{29}H_{50}O$		Sterol
Liriodendrin	$C_{34}H_{46}O_{18}$		Lignin
Boeravinone B	$C_{17}H_{12}O_6$		Isoflavone (Flavonoid)
Punamavine/lunamarine	$C_{18}H_{15}NO_{14}$		Alkaloid

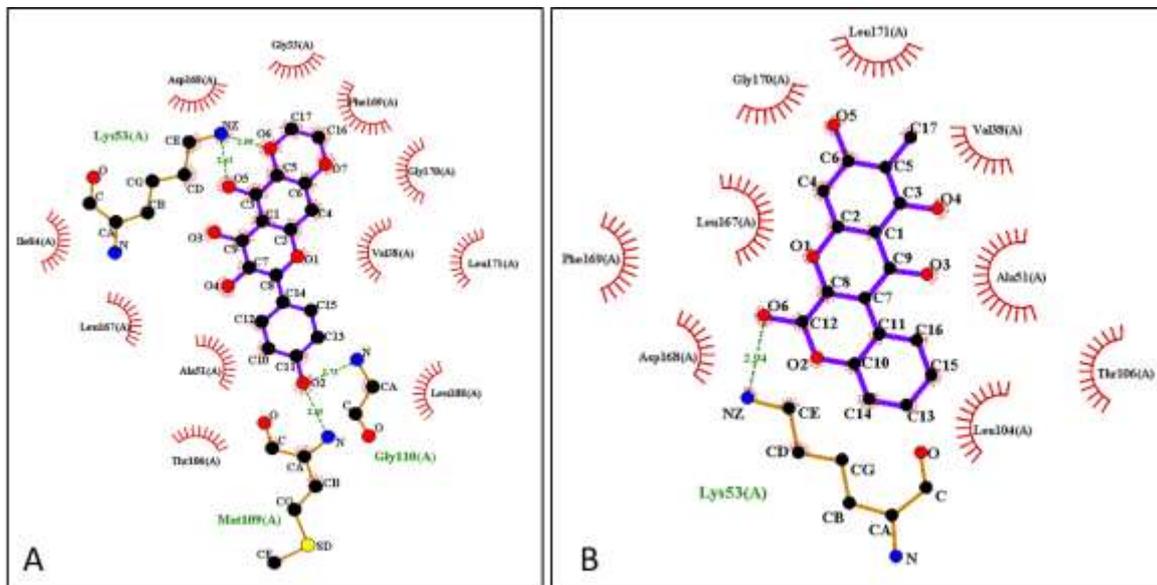
**Table 1:** Details of short listed potent anti-inflammatory phytochemicals of Boerhaavia diffusa.linn (BD)

Anti-inflammatory compounds of BD	Molecular weight	Druglikeness (Lipinski rule Violation)	Pharmakinetics (GI absorption)
Eupalitin	330.29g/mol	Yes, 0 violation	High
Eupalitin-3-O-β-D-galactopyranoside	492.4 g/mol	No, 2 violations	Low
β-sitosterol	414.7 g/mol	Yes, 1 violation	Low
Liriodendrin	742.7 g/mol	No, 3 violation	Low
Boeravinone B	312.27g/mol	Yes, 0 violation	High
Punarnavine/lunamarine	309.3 g/mol	Yes, 0 violation	High

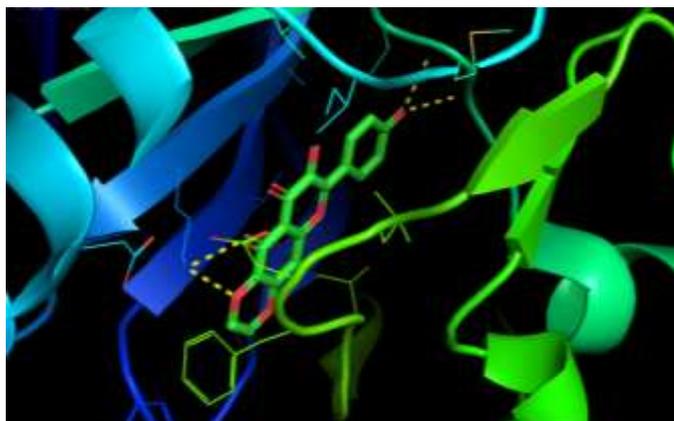
**Table 2:** Pharmacokinetics and druglikeness of the potent anti-inflammatory phytochemicals of BD

BIOLOGICALLY ACTIVE SITES OF P38 MAPK	RESIDUE	Anti-inflammatory phytochemicals of Boerhaavia diffusa.linn			
		Eupalitin	β-sitosterol	Boeravinone B	Punarnavine/lunamarine
Active site	Leu 108	-----	-----	-----	-----
	Met 109	H-Bond	-----	-----	-----
DFG- out site (Allosteric site)	Asp 168	Hydro-pho	Hydro-pho	-----	-----
	Phe 169	Hydro-pho	Hydro-pho	Hydro-pho	Hydro-pho
	Gly 170	Hydro-pho	Hydro-pho	Hydro-pho	Hydro-pho
Kinase activity site	Lys 53	H-Bond	Hydro-pho	H-Bond	Hydro-pho
	Asp 168	-----	Hydro-pho	Hydro-pho	Hydro-pho
	Glu 71	-----	Hydro-pho	-----	-----
Phosphorylation motif	Thr 180	-----	-----	-----	-----
	Tyr 182	-----	-----	-----	-----

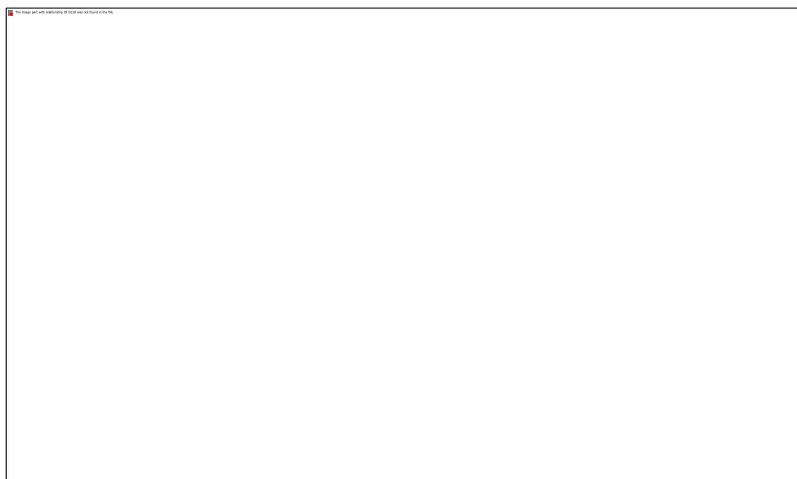
**Table 3:** Chemical interaction details of potent anti-inflammatory phytochemicals on docking with p38 $\alpha$  MAPK.



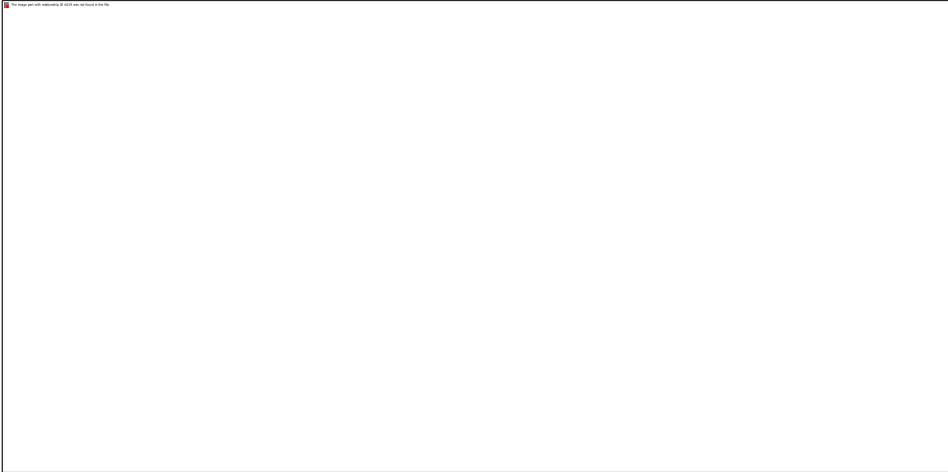
**Figure 1:** Docking of listed ligands with the p38 $\alpha$  MAPK biologically active site. A) Eupalitin B) Boerhavinone B



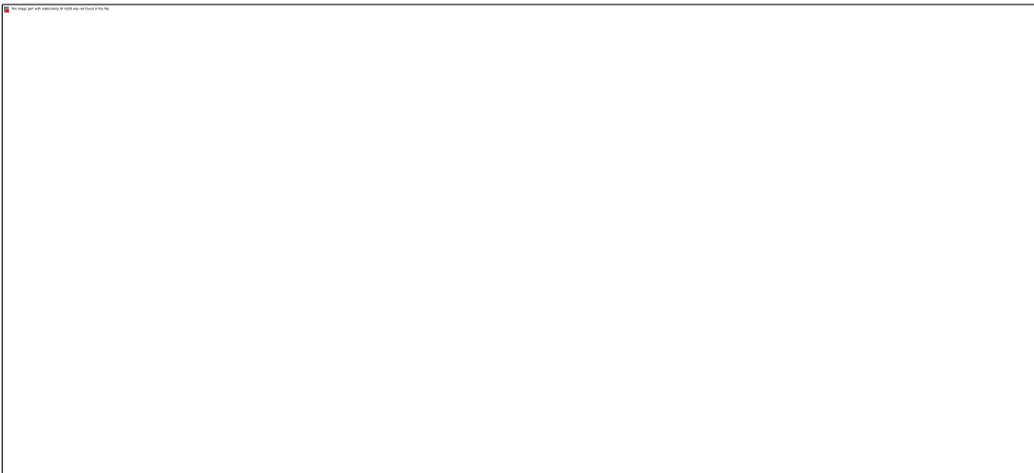
**Figure 2:** 3D view of Eupalitin binding to p38 $\alpha$  MAPK biologically active site



**Figure 3:** Cytotoxicity of predicted p38 $\alpha$  MAPK inhibitor; Eupalitin and boerhavinone B in BV2 microglial cell lines.



**Figure 4:** Inhibition of IL-6 secretion on treatment with predicted p38 $\alpha$  MAPK inhibitor; Eupalitin and boerhavinone B in BV2 microglial cells on LPS induction.



**Figure 5:** Inhibition of TNF- $\alpha$  secretion on treatment with predicted p38 $\alpha$  MAPK inhibitor; Eupalitin and boerhavinone B in BV2 microglial cells on LPS induction.