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Novel inhibitor of brain indoleamine 2,3 dioxygenase, docking and experimental studies

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ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is a haem-containing monomeric enzyme that catalyze the conversion of tryptophan (L-TRP) to N-formyl kynurenine. IDO activity is regulated by cytokines. Pro inflammatory cytokines are potent inducers of IDO, whereas anti-inflammatory cytokines are IDO inhibitors. Prostaglandin E2 induces IDO activity. In inflammation the relationship between immune system and the kynurenine pathway play an important role. IDO is an important therapeutic target for the treatment of inflammation. Present study evaluates the binding of *Hypericum perforatum* (HP) against IDO enzyme using MVD software acute and chronic effects of HP on IDO enzyme activity. Docking results show that HP fit well in the allosteric site of IDO. Energy scores for HP -158.687 Kcal/mol. Administration of HP (500mg/kg/3ml) shows that serum IDO activity was significantly increased (171%, P<0.01) and (114%, P<0.01) respectively after acute and chronic treatment. Brain IDO activity was decreased by 42%, (P<0.01) after acute and 43% (P<0.01) chronic treatment. It is concluded from the present study that HP is noncompetitive inhibitor of IDO as proofs by docking further its inhibitory effects on brain IDO reveals its anti-inflammatory effect.

Key Words: Indoleamine 2,3-dioxygenase, tryptophan, Molegro Virtual Docker, *Hypericum perforatum*, cytokines, N-formyl kynurenine.

INTRODUCTION

The first rate limiting step of kynurenine pathway is the catabolism of tryptophan (L-TRP) to N-formyl kynurenine which is take place in the presence of haem containing enzyme indoleamine 2,3-dioxygenase (IDO) (Sono *et al*., 1996). IDO is usually found in the tissues, such as neuroglia, macrophages of the central nervous system in mammals (Botting, 1995). The activity of IDO is regulated by cytokines. Pro inflammatory cytokines such as Interferon-gamma, interlukin-1 or tumor necrosis factor –α are potent inducers of IDO, however anti-inflammatory cytokines such as interlukin-4 and interlukin-10 are IDO inhibitors (Weiss *et al*., 1999). Besides interferon-gamma and tumor necrosis factor –α, other pro inflammatory molecules such as prostaglandin E2 (PGE2) synergistically induce an increase of IDO activity (Braun *et al*., 2005, Kwidzinski *et al*., 2005 and Robinson *et al*., 2005). In inflammation the link between immune system and kynurenine pathway play an important role. An infectious microbe such as bacteria depends on TRP for their survival and the ingestion of tryptophan play a crucial role during infectious diseases (Carlin *et al*., 1989 and Grohmann *et al*., 2003). In serotonin synthesis pathway the availability of TRP is the rate limiting factor. So, the induction of IDO by cytokines decreases the availability of TRP for serotonin synthesis (Mellor and Munn ,1999; Munn *et al*., 1999). Mostly IDO is expressed in many cancers like gastric, pancreatic, colorectal and prostate cancers (Uyttenhove, 2003), ovarian cancer (Tanazaki *et al*, 2014). The most common complication of cancer is cancer related fatigue (CRF) and its treatment that can significantly impair quality of life. However, the actual mechanisms remain understood, now the inflammation is supposed to be an important factor of CRF in addition to effects of depression, anxiety, insomnia, and other factors. One key biological pathway that may link inflammation and CRF is indoleamine 2,3-dioxygenase (Kim *et al*, 2015). Since 1990s *Hypericum perforatum* (HP), is also referred to as St. John's wort, has been clinically approved herbal medicine (Roder *et al*., 2004). As yet species of hypericum family plant have potential medicinal value. HP has anti-depression, anti-viral and anti-inflammatory activities. Previous studies by Hammer *et al*. (2007) showed that different doses of HP extracts inhibit prostaglandin E2 production through lipopolysaccharide (LPS)-induction. Currently SJW is used in treating eczema, inflammation related disorders, cancer, burns, viral and bacterial disease and also researcher proved its effect as an antioxidant and neuroprotective agent (Klemow, 2011).

IDO is an important therapeutic target for the treatment of above mention disease. We can overcome this problem by finding the inhibitors of this enzyme. Until, the best known IDO inhibitors are 1MT (Sono and Cady *et al*., 1991), ß-Carboline (Eguchi *et al*., 1984). In 2006, effective nano molar inhibitors were isolated from marine invertebrates extract (Brastiano *et al*., 2006 and Pereria *et al*., 2006). Simultaneously new brassinin based IDO inhibitors were published (Banerjee *et al*., 2008, Gaspari 2006). In this work we have selected one structure hits of protein from protein data bank (PDB) overly expressing in disease. The docking of HP with IDO has not yet been done. Therefore, we performed docking against single crystal structure of IDO further we analyzed the acute and chronic effects of HP on IDO enzyme activity after administration.

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MATERIALS AND METHODS

Computational studies

Molegro Virtual Docker was used to perform docking. HP was selected for this study. The selected ligands structure was built using ChemDraw (Figure 1) software and imported to MVD workspace in 'sdf' format. All necessary valency checks and H atom addition were thus performed using the utilities provided in MVD.
The crystal structure selected for this study is IDO complex with phenyl imidizol (PIM) (Figure 2), from the available 2 crystal structures (July 2014). This crystal structure of IDO (PDB, ID, 2D0T) downloaded to MVD workspace from protein data bank (http://www.rcsb. org/pdb), under the criteria that they had a reasonable resolution (≤ 2.8 A^0) and involved the non-mutated IDO enzyme in complex with ligands. The steps involved in docking were:

1. Importing the molecules and ligands
2. Preparing the molecules
3. Creating template
4. Docking

Drugs and chemicals

L-tryptophan and Kynurenine from Sigma chemical Co (St, Louis, Mo), HP was, gift from pharmaceuticals (Medics Pharma, Pakistan). All others chemicals used were of highest analytical grade.

Drug preparation

500 mg/kg/3 ml of HP were dissolved in vehicle (1 ml ethanol: 2 ml saline). Drug was administered according to the body weight of rats.

Animals and treatment

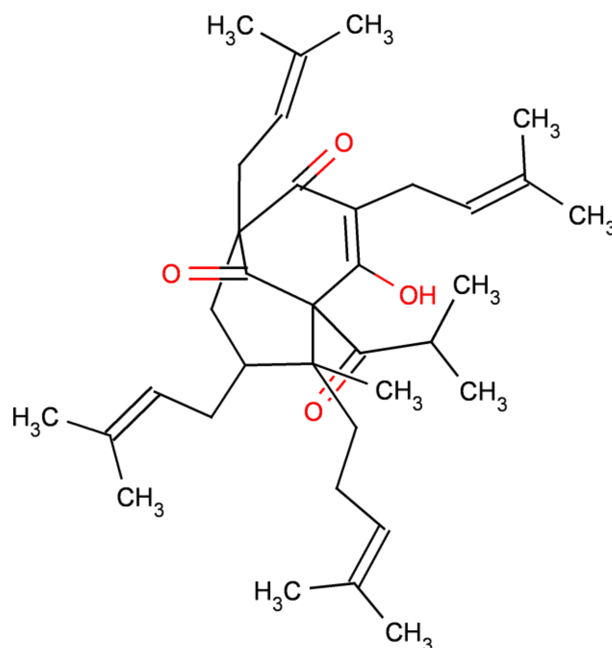
Locally bred male Albino Wistar rats (150-250 gm body weight) were used in this investigation. Animals were kept in plastic cages under natural light dark cycle at room temperature 25±2°C. The animals had free access of food and water and were acclimatized to their environment before any treatment of experiment. Animals were divided into different groups. Each groups had six rats. Test animals received HP (500 mg/kg/3ml) and control rats received 3 ml vehicle (ethanol: saline) (1:3 v/v) by oral gavage and were decapitated after 3.5 hrs and after 7 days treatment respectively.

Enzymatic determination

Frozen brain samples were weighted and homogenized for one minute using Ultra-Turrax homogenizer in ice-cold water and 12% (w/v) $HClO_4$. Volumes of water and $HClO_4$ were used as I gm tissue add 1 ml water and 2 ml $HClO_4$. The homogenates were kept in ice-cold tubes for 10 min then centrifuged at 6000 rpm for 10 min at 4°C. The supernatants were poured and volume was adjusted to 4 ml with 6 % (w/v) $HClO_4$. A 0.5 ml portion of each homogenate was filtered and used or stored. IDO activity was determined by calculating KYN/TRP ratio in serum and brain as described by (Badawy and Morgan, 2010).

Preparation of serum extract

To each ice-cold 1 ml Eppendorf tube, add 0.5 ml (500μL) of the plasma or serum sample and 0.45 ml (450μL) of HPLC-grade water. Add 50 μL of 60% $HClO_4$ and vortex for 5-10 seconds. Allow to stand at room temperature (still in the ice-cooled rack) for 5 minutes. Spin in a high-speed centrifuge at 8000 rpm at 4°C for 10 minutes. Decant the supernatant carefully into another Eppendorf tube and make it up to the 1 ml mark with 6% $HClO_4$. Store in the freezer, or, if to be used freshly, then; filter 0.5 ml in a Vectaspin tube at 8000 rpm in a high-speed refrigerated centrifuge at 4°C for 10 min. Transfer the filtrate into an HPLC vial using a pasteure pipette. Serum TRP and KYN concentration were determined by HPLC method of Badawy and Morgan (2010).



(1R,5S,6R,7S)-4-hydroxy-6-methyl-1,3,7-tris(3-methylbut-2-en-1-yl)-6-(4-methylpent-3-en-1-yl)-5-(2-methylpropanoyl)bicyclo[3.3.1]non-3-ene-2,9-dionemethylpropanoyl)bicyclo[3.3.1]non-3-ene-2,9-dione

Figure 1: Structure of HP selected for docking against IDO

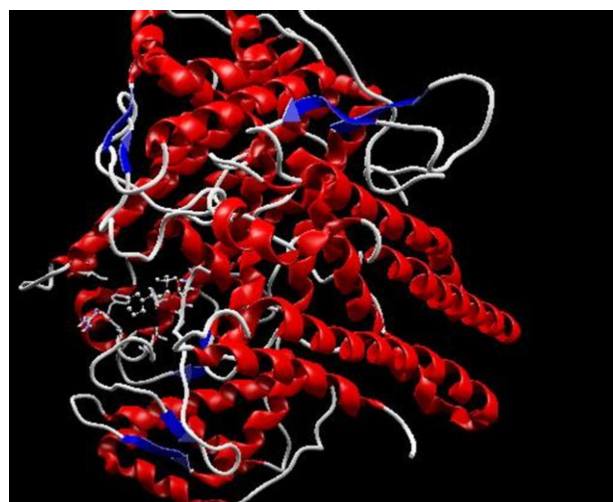


Figure 2: Structural cartoon of Indole amine 2,3-dioxygenase (PDB code 2D0T). The α helices and β strands are represented as coils (red) and arrows (white), respectively. PIM is represented in ball and stick. Model prepared using MVD.

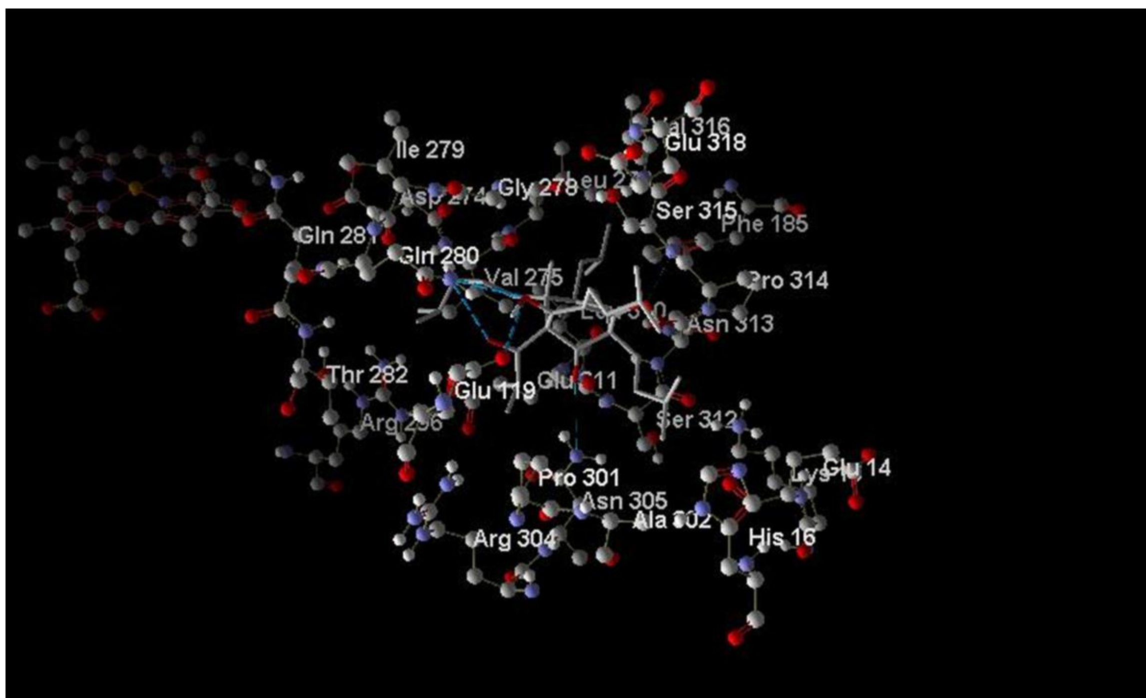


Figure 3: The best scored docking solution of IDO with HP. The cofactor haem and amino acids in the active site are presented in ball and stick with element color and ligand is presented in thick lines with element color (where carbon is grey, oxygen is red, nitrogen is blue and sulpher is yellow and hydrogen in white). Blue lines represented the hydrogen bonds in between the ligand and IDO.

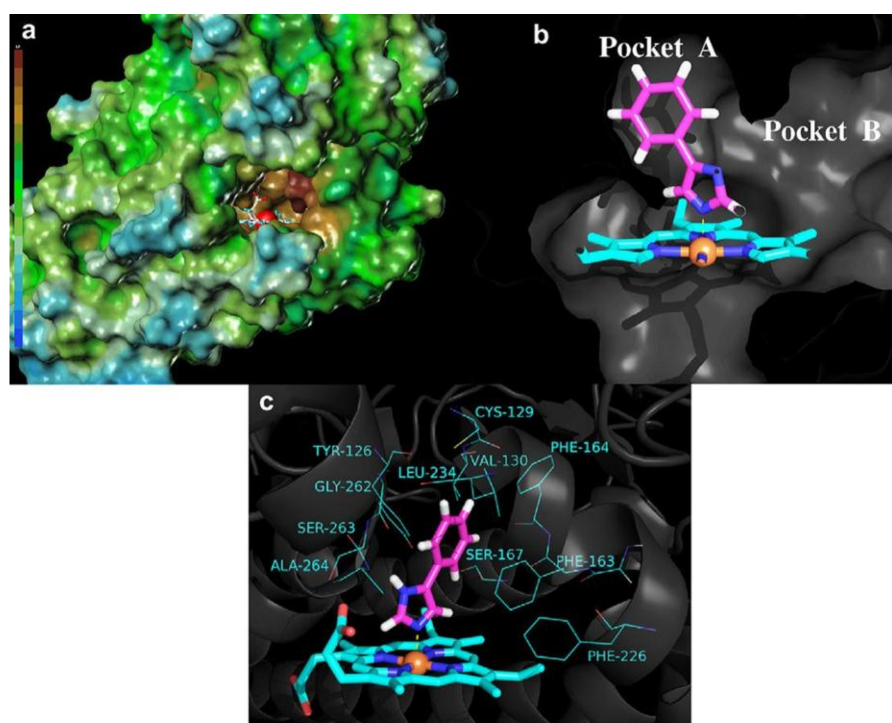


Figure 4: Lipophilicity mapped of IDO (color code: brown = high lipophilicity, green=medium lipophilicity and blue=high hydrophilicity; picture made with MOLCAD (30) and (b and c) active site of IDO with PIM as absorbed in the X-ray crystal structure (PDB ID 2D0T). (Adapted from Dolusic *et al.*, 2011).

Table 1: MolDock score; Rerank score and the hydrogen bond energy of the docked compounds.

Ligand	MolDock Score	Rerank Score	Log P	MW	Torsion	HBa	HBd	HB
Hypericum perforatum	-158.687	-119.329	9.67	543	11	4	1	-7.48

HBa= hydrogen bond acceptor, HBd= hydrogen bond donor, MW= molecular weight

Table 2: Amino acids residue around active site docked against IDO.

Ligand name	Amino acid residues	Ligand binding amino acid
Hypericum Perforatum	Glu 119,Arg 256,Thr 282,Val 275,Gln 280,281	Glu 119,Gln 282, Asn305, Ser 315,Val 275, Asn 313

Arg=arginine; Gly= glycine; Glu= glutamate; His= histidine; Leu= leucine; Phe= phenylalanine Ser=serine; Thr= threonine; Tyr=tyrosine; Val= valine

Table 3: Acute and Chronic effects of HP on serum (500m g/kg) indoleamine 2,3-dioxygenase activity.

Parameter	Control	Acute 3.5 hours	7-days	One way- ANOVA Df(1,15)
Serum total TRP μg/ml	14.1±0.69	7.6±0.32* -46%	7.9±0.36* -43.60%	F=57.46 (P<0.01)
Serum Kynurenine ng/ml	620±4.9	927±3.4* 49.50%	803±1.7* 29.50%	F=1927 (P<0.01)
KYN/TRP	44.5±2.7	121±5.0* 171%	95.4±2.2* 114%	F=125.3 (P<0.01)

*Experimental details are given in material and methods section. All values are mean±SEM of six rats, in each group. Treated groups were administered HP (500mg/kg/3ml) by oral gavage and were killed after 3.5 hour and 7 days. Control animals received equal volume of saline at dose of 3ml/kg. Statistical analysis was performed using one way ANOVA. The significance of difference between HP and control is indicated by*P<0.01.*

Table 4: Acute and chronic effects of HP (500mg/kg) on brain indoleamine 2,3-dioxygenase activity.

Parameter	Control	Acute 3.5-hours	7-days	One way- ANOVA Df(1,15)
Brain TRP μg/g	3.2±0.07	4.2±0.34* 31.20%	5.5±0.14** 71.80%	F=27.6 (P<0.01)
Brain Kynurenine ng/g	80.2±2.5	55.7±0.97** -30.50%	76.6±1.0 N.S.	F=53.9 (P<0.01)
KYN/TRP	24.4±0.51	14.1±0.72** -42%	13.8±0.45** -43%	F=113.4 (P<0.01)

*Experimental details are given in material and methods section. All values are mean±SEM of six rats, in each group. Treated groups were administered HP (500mg/kg/3ml) by oral gavage and were killed after 3.5 hour and 7 days. Control animals received equal volume of saline at dose of 3ml/kg. Statistical analysis was performed using one way ANOVA. The significance of difference between HP and control is indicated by *P<0.05, **P<0.01.*

Statistical analysis

Data are expressed as mean ± standard error of mean. Analysis was performed by using one- way ANOVA followed by Tukey's test for stepwise comparison between the individual values. Difference between the two groups were considered significant when P<0.05.

RESULTS

Mol Dock score and H-bond energies when HP were dock against IDO

Table 1 shows Mol Dock score, re- rank score and the hydrogen bond energy of selected ligand. Table 2 shows amino acid residues present in the active site of IDO and also ligand binding amino acids. Figure 1 shows selected ligand structures were built using ChemDraw software. Figure 2 shows crystal structure of IDO (PDB, ID, 2D0T) complex with ligand PIM is represented in ball and stick model prepared by using MVD software. Figure 3 shows dock structure of IDO with (HP) selected ligands. Ligand (*Hypericum perforatum*) bind into the allosteric site with mol dock score -158.687 k cal/mol and binding site consist of amino acid residues like Val 275, Gln 280, Glu 119, Ser 312, Asn 313.

Experimental studies

Acute and chronic effects of HP on serum indoleamine 2,3-dioxygenase activity

Table 3 shows effects of HP (500mg/kg/3ml) on serum total TRP, serum KYN and KYN/TRP. Data analyzed by one-way ANOVA shows significant effect of drug (F=57.46, P<0.01, F= 1927,P<0.01,F=125.3,P<0.01) on serum total TRP, KYN and ratio respectively. Individual comparison by Tukey's test shows that there were significantly decreased serum total TRP (46%, P<0.01) and (43.6%, P<0.01) in acute treated rats and chronic treated rats respectively when compared with saline control. Table also shows there were significantly increased in serum KYN (49.5%, P<0.01) and (29.5%, P<0.01) respectively in acute treated rats and

chronic treated rats when compared with saline control. Table also shows there were significantly increased serum KYN/TRP ratio (171%, P<0.01) and (114%, P<0.01) in acute and chronic rats when compared with saline control.

Acute and chronic effects of HP (500mg/kg) on brain indoleamine 2,3-dioxygenase activity

Table 4 shows effects of HP (500mg/kg/3ml) on TRP and KYN. Data analyzed by one-way ANOVA shows significant effect of drug (F=27.6, P<0.01, F=53.95 F=113.4,P<0.01) on brain TRP, KYN and KYN/TRP ratio. Individual comparison by Tukey's test shows that there were significantly increased brain TRP (31.2%, P<0.01) and (71.8%, P<0.01) respectively in acute and chronic treated rats when compared with saline treated rats. Table also shows there were significantly increased brain KYN (30.5%, P<0.01) in acute rats while no significant effect was observed in chronic treated rats when compared with saline injected control rats. Table also shows that there were significantly decreased (42%, P<0.01 and 43%, P<0.01) brain KYN/TRP ratio respectively in acute and chronic treated rats when compared saline control rats.

DISCUSSION

The enzyme IDO plays a physiologically important role in the human body as it can control the levels of essential amino acid (L-Tryptophan), and a number of its neurotoxic metabolites. IDO is frequently expressed in many cancers such as gastric, pancreatic, colorectal and prostate cancers (Uyttenhove, 2003), ovarian cancer (Tanazaki *et al*, 2014) and inflammation (Kim, 2015). To identify IDO inhibitors for therapeutic purpose we have selected IDO protein. For this purpose, IDO was docked with HP. In each docking run, the best poses were selected on the basis of their MVD score.

As previously described, ligand was automated dock within the defined grid representation after docking a score was assigned according to the quality of fit and top ten candidate were selected on the basis of exhibiting lowest predicted binding energy. This ligand was further optimized according to their energy minimization active side chain residue.

More negative the energy score (Kcal/mol) more is the binding affinity. Energy scores of HP is -158.687 K cal/mol this data also indicates that by additionally optimization these possible ligands can make a strong therapeutic inhibitor of IDO enzyme. In the reported crystal structure of IDO, PIM interacts with the haem iron, PIM binding site consist of amino acid residues Tyr126, Cys 129, Val 130, Phe 163, Phe 164, Ser 167, Leu234, Gly 262, Ser 263, Ala 264 and the haem ring (Figure 4C). Additional hydrogen bond is possible due to side chain of Arg 231. Haem ring and Phe 163, Phe 226, Leu 234, Ile 354 formed hydrophobic pocket (pocket B, Figure 4b) (Dolusic *et al*., 2011). HP binding site consist of amino acid residue Glu 119, Arg 256, Thr 282, Val 275, Gln 280,281, Ser 315. HP binds with IDO at allosteric site.

In *vivo* IDO activity can be measured by calculating the proportion of product:substrate (KYN/TRP). Therefore, an increase in the ratio will enhance the enzyme activity and a decrease in ratio will decline the enzyme activity. In the present study acute and chronic treatment with HP decreased serum total TRP while increased serum KYN and KYN/TRP ratio which reflect the induction of IDO. In contrast brain TRP level significantly increased and decreased level of KYN and KYN/TRP ratio which reflect the inhibition of brain IDO following single and chronic dose of HP.

In the present study, HP induce serum IDO while inhibit the brain IDO which shows that mode of action of HP is different in different tissue. Previously it has been reported by (Wesam *et al*., 2014) that HP decreased the levels of hippocampal TNF-α, and corticosterone in the serum. We have also found increased level of brain TRP and decreased level of brain KYN and KYN/TRP ratio which is consistent with (Wesam *et al*., 2014) who has found significant decreases in TRP and KYN level but insignificant effect on hippocampus KYN/TRP in response to acute treatment following different doses. In another experiment Calapai and coworker, (2001) have showed that higher doses of (250-500 mg/kg) SJW given orally to the rats has significantly increased the levels of serotonin, norepinephrine and dopamine content in brain which decreases immobility time in forced swim stress. Previously we (Bano and Dawood, 2008) have also reported anxiolytic property of SJW, that increases serotonin metabolism in neuron but reduce its release under adverse conditions and serotonergic variations in swim stressed rats (Ara and Bano, 2009). It has been also reported that 10 mg SJW inhibit TDO increased serum free TRP and serum corticosterone and enhance brain TRP and serotonin level (Bano *et al*., 2010).

CONCLUSION

It is concluded that HP is possible lead molecules having their highest docking score. Present results also proof HP is noncompetitive inhibitor of IDO. It is concluded that administration of HP inhibit brain IDO activity could contribute in its anti-inflammatory effect.

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