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Spectral characterization, docking and *in-vivo* anti-inflammatory activity of Isoivangustin, a constituent isolated from methanol extract of *Cyathocline purpurea*

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ABSTRACT

A sesquiterpene lactone, isoivangustin was isolated from the whole plant Cyathocline purpurea (Buch-Ham ex D. Don.) Kuntze (Fam. Asteraceae). The structure of this compound was elucidated and established by standard spectroscopic methods (IR, ^{1}H-NMR, ^{13}C-NMR, DEPT and MS data). Carrageenan induced paw edema model was used for evaluation of anti-inflammatory activity. Isoivangustin was subjected to molecular docking study to find out the binding interactions with the active site of TNF-alpha converting enzyme (TACE). Isoivangustin was found to be active in reducing inflammation (29.00 %) which was comparable to diclofenac (34.57 %) at 3rd hr. The docking score of isoivangustin and diclofenac with TACE were -5.341 and -7.358, respectively. In conclusion the result suggested that isoivangustin, a sesquiterpene lactone isolated from Cyathocline purpurea showed good anti-inflammatory activity which may be mediated by inhibition of tumor necrosis factor (TNF)-α.

Keywords: *Cyathocline purpurea*, Isoivangustin, Sesquiterpene lactone, Anti-inflammatory activity.

INTRODUCTION

The inflammatory response is a defense mechanism evoked by body tissues in response to injury or microbial invasion [1]. Inflammation protects the body against infection and injury but it can produce deleterious consequences to the host. The inflammatory response can lead to different diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis [2]. Inflammation develops in the classical forms of redness, swelling, heat and hyperalgesia. These symptoms result from the action of inflammatory agents such as bradykinin, serotonin, histamine, prostaglandins, leukotrienes and nitric oxide, which can originate locally or from cells that infiltrate in the site of insult [3]. Pain is a common symptom of various inflammatory diseases and an unpleasant sensory experience associated with actual or potential tissue damage [4]. The clinical treatment of inflammatory diseases is dependent on drugs which belong either to the non-steroidal or steroidal chemical therapeutics. The non-steroidal anti-

inflammatory drugs (NSAIDs) inhibit early steps in the biosynthesis pathway of prostaglandins by inhibition of cyclooxygenase (COX) enzymes and are the main drugs used to reduce the untoward consequences of inflammation [5]. However, the side effects of the currently available anti-inflammatory drugs pose a major problem in their clinical use. For instance, NSAIDs cause several serious adverse effects like gastric injury and ulceration, renal damage, and bronchospasm due to their non-selective inhibition of both isoforms of the COX enzyme [6]. Now a day the use of steroidal drugs as anti-inflammatory agents is also becoming highly controversial due to their multiple side effects [7]. As an alternative, plant based medicines are getting an increased therapeutics demand due to their less side effects and good activity.

Cyathocline purpurea (Buch-Ham ex D. Don.) Kuntze. Fam. Asteraceae is a seasonal Indian medicinal plant commonly found in moist habitats such as along watercourses and in rice fields throughout most of peninsular and northern India at an elevation of 1300 m [8]. Traditionally *Cyathocline purpurea* has anticancer [9], antimicrobial, anthelmintic, and hypotensive properties. The roots of this plant are also used to relieve stomach pains [8]. Literature survey revealed the presence of chemical constituents guaianolide, eudesmanolide, sesquiterpene lactones, isoivangustin and guaianolide, 6 α-hydroxy-4(14), 10(15)- guainadien-8α-, 12-olide [10] cythoclol [11]. The presence of sesquiterpene lactones containing alpha-methylene-gamma-lactone moiety like santamarin, 9 β-acetoxycostunolide and 9 β-acetoxyparthenolide have been reported to be present in *Cyathocline purpurea* [12]. Sesquiterpene lactones containing alpha-methylene-gamma-lactone moiety is also reported to possess anti-inflammatory activity [13].

In the earlier study, the methanol extract of *Cyathocline purpurea* (MECP) was reported for its ability to dose dependently inhibit carrageenan induced paw edema and cotton pellet induced granuloma in rats [14]. However, adequate research on a medicinal plant should be beyond screening for biological activity, aim at systematic standardization and develop into natural products or dosage forms which would effectively complement or supplement existing conventional therapies [15]. Therefore the objective of the present study was to isolate the active constituent(s) and evaluate its anti-inflammatory activity in rats.

MATERIALS AND METHODS

Procurement and authentification of plant

The whole plant *Cyathocline purpurea* (Buch-Ham ex D. Don.) Kuntze. Fam. Asteraceae was collected in the month of January 2013 from Pune, Maharashtra. The plant was then identified and authenticated by J. Jayanthi, Scientist C, Botanical Survey of India, Pune, India and voucher specimen (No. BSI/WRC/Tech/2013/1094) was deposited at that institute.

Chemicals

Carrageenan (Sigma-Aldrich, St. Louis, MO, USA), petroleum ether, acetone, ethyl acetate (Merck), methanol (Molychem, India), silica gel, 60-120 mesh (Merck), borosil glass column (height 60cm, diameter 3cm) purchased from local vendors, and diclofenac (gift sample from Emcure pharmaceuticals Ltd., Pune).

Extraction and isolation

The whole plant was shade dried and powdered. Dried powder (500 g) was extracted with methanol by maceration. The extract was then filtered and concentrated on rotary evaporator (Medica Instrument, India) under vacuum at 45°C and stored in desiccator (yield 6.9 % w/w). The extract (15 g) was further fractioned by liquid-solid separation chromatographic technique with pet. ether (F – 1), 10% acetone in pet. ether (F – 2), 20% acetone in pet. ether (F – 3), 30% acetone in pet. ether (F – 4), 50% acetone in pet. ether (F – 5), and methanol fraction (F – 6). Each fraction was concentrated on rotary evaporator (Yield: F – 1: 0.9 g, F – 2: 1.2 g, F – 3: 1.8 g, F – 4: 2.9 g, F – 5: 3.1 g, F – 6: 3.5 g). These fractions were evaluated for anti-inflammatory activity by carrageenan induced paw edema method in Wistar rats.

The most active fraction 30% acetone in pet. ether (F – 4), was subjected to column chromatography. The F – 4 (2.5 g) was dissolved in minimum amount of acetone and was adsorbed on silica gel (particle size: 60-120 mesh). This silica gel was loaded on column. Mobile phase used was pet ether: ethyl acetate in order of increasing polarity. Eluted sub-fractions were evaluated by Thin layer chromatography (TLC) plates (Merck, Germany) using mobile phase, pet. ether: ethyl acetate (7:3). All sub-fractions were monitored by TLC and sub-fractions showing similar bands were pooled together and labeled as P – 1 to P – 10. These pools were then concentrated on rotary evaporator

and tested for anti-inflammatory activity by carrageenan induced paw edema method. Pool P – 8 showed maximum anti-inflammatory activity. The impurities of pool P – 8 were removed by preparative TLC using pet. ether: ethyl acetate (7:3) as mobile phase to obtain pure compound 1 (42 mg). The structure of compound 1 was characterized by spectral analysis using IR, MS, ^{1}H-NMR, ^{13}C-NMR and DEPT.

Structure elucidation of compound 1

Purity and homogeneity of isolated compound 1 was determined using thin layer chromatography on silica gel plates activated with ethanol-sulphuric acid. IR spectra were recorded using KBr pellets on JASCO FT-IR 5300 spectrophotometer. Mass spectra were obtained on a Thermo Finigen Surveyor MSQ spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on 200 MHz and 50 MHz spectrometer, respectively (Bruker, Germany). Deuterated chloroform ($CDCl_3$) was used for recording NMR and tetramethylsilane (TMS) was used as an internal standard. Chemical shifts were reported as δ (ppm). The coupling constants (*J*) were reported as Hz.

Experimental animals and approval

Female Wistar rats weighing (180-220 g) were purchased from National Toxicology Centre, Pune, India. The rats were housed in the animal house at a ambient temperature of 25 ± 1 °C and relative humidity of 45 to 55% under 12-h light : 12-h dark cycle. The animals had free access to food pellets (Manufactured by Pranav Agro Industries Ltd., Sangli India) and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India (Approval No. CPCSEA/PCL/07/2014-2015)

Anti-inflammatory activity

Carrageenan induced rat paw edema

Female Wistar rats were divided into following groups,

A) Groups for fractions (F – 1 to F – 6) were as follows,

Group 1: - Vehicle control.
Group 2: - Standard, diclofenac 10 mg/kg, p.o.
Group 3: - Pet. ether fraction (F – 1), 100 mg/kg, p.o.
Group 4: - 10% acetone in pet. ether fraction (F – 2), 100 mg/kg, p.o.
Group 5: - 20% acetone in pet. ether fraction (F – 3), 100 mg/kg, p.o.
Group 6: - 30% acetone in pet. ether fraction (F – 4), 100 mg/kg, p.o.
Group 7: - 50% acetone in pet. ether fraction (F – 5), 100 mg/kg, p.o.
Group 8: - Methanol fraction (F – 6), 100 mg/kg, p.o.

B) Groups for pools (P – 1 to P – 10) were as follows,

Group 1: - Vehicle control.
Group 2: - Standard, diclofenac 10 mg/kg, p.o.
Group 3 to 12: - P – 1 to P – 10, respectively (10 mg/kg, p.o)

Inflammation was produced by injecting 0.1ml of 1% lambda carrageenan (Sigma Chemical Co., USA) in sterile normal saline into the sub plantar region of the right hind paw of the rat [18]. Rats were pretreated by test substance orally 1 hr before the carrageenan injection. The paw volume was measured from 0-6 hr, at an hourly interval using plethysmometer (Ugo Basile, Italy, Model No. 7140). The mean changes in injected paw volume with respect to initial paw volume were calculated. Percentage inhibition of paw volume between treated and control group was calculated by the following formula,

% Inhibition = (1-VT / VC *100)

Where, VT and VC are the mean increase in paw volume in treated and control groups, respectively.

Docking studies

Glide was used for docking study to examine the binding mode of isolated compound, isoivangustin with TNF-alpha converting enzyme (TACE) (PDB: 1ZXC). The ligands were prepared using LigPrep. The protein was refined using the protein preparation wizard present in Maestro 9.0. All the water molecules were deleted. Hydrogen atoms were added to the protein, including the protons necessary to define the correct ionization and tautomeric states of the amino acid residues. Prime interface module incorporated in Maestro 9.0 was used to add the missing residues of the

side chain. Each structure minimization was carried out with the impact refinement module, using the OPLS-2005 force field to alleviate steric clashes potentially existing in the structures. Minimization was terminated when the energy converged or the root mean square deviation reached a maximum cutoff of 0.30 Å. To find out active site grid was prepared using grid generation panel of glide with the default settings. Grid is prepared for defining the binding site of native ligand on the receptor. The ligand was selected to define the position and size of the active site [19-21]. Glide XP docking was used for docking purposes.

Statistical analysis

Data was expressed as mean ± SEM and statistical analysis was carried out by using Graph Pad 5.0 software (Graph Pad, San Diego, USA) by applying two-way ANOVA with Bonferroni test. $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Inflammation induced by carrageenan is an acute and highly reproducible inflammatory model [4]. The carrageenan-induced rat paw edema model has frequently been used to evaluate the anti-inflammatory agents [2]. The induction of edema by using carrageenan is believed to be biphasic in nature. The first phase involved within 1 hr of carrageenan administration is associated with the release of histamine and serotonin from mast cells. The second phase starts after 1 hr and is characterized by an increased release of prostaglandins (PGs) in the inflammatory area. During the second phase, the macrophages are known to release the large amounts of interleukin (IL) -1 which led to the increased accumulation of polymorphic nuclear cells (PMNs) to the site of inflammation. The activated PMNs then release the lysosomal enzymes and active oxygen species to destroy connective tissue and induce paw swelling [16]. In the fraction study (F – 1 to F – 6), the paw edema of the rats increased progressively after carrageenan injection. Fractions F – 4 and F – 5 reduced carrageenan induced inflammation significantly ($p<0.001$) at 3rd and 5th hr. Fractions F – 2 and F – 3 also significantly ($p<0.001$) reduced paw edema at 5th hr as compared to control group. Fraction F – 4 was found to exert the highest anti-inflammatory activity, i.e. 47.49 % inhibition of inflammation at 5th hr as compared to control group. Therefore it may be assumed that effect of fraction F – 4 is associated during the second phase, where the effect of PGs is prominent. On treatment with fraction F – 1 there was no significant inhibition while fraction F – 6 showed significant ($p<0.001$) anti-inflammatory activity at 5th hr. All the fractions produced lower effects than that of standard drug diclofenac (Table 1).

Table 1: Anti-inflammatory effect of fractions (F – 1 to F – 6)

Treatment Groups	Dose (mg/kg, p.o.)	Change in paw volume (ml)		
		1 hr	3 hr	5 hr
Vehicle (carrageenan) control	-	1.31 ± 0.10	2.30 ± 0.10	2.69 ± 0.09
Diclofenac	10	1.19 ± 0.04 (9.29)	1.35 ± 0.04*** (41.15)	1.25 ± 0.05*** (53.68)
F – 1 (pet. ether fraction)	100	1.29 ± 0.04 (1.53)	2.22 ± 0.07 (3.34)	2.56 ± 0.05 (5.02)
F – 2 (10 % acetone in pet. ether fraction)	100	1.27 ± 0.05 (3.18)	2.06 ± 0.07* (10.45)	2.28 ± 0.03*** (15.36)
F – 3 (20 % acetone in pet. ether fraction)	100	1.25 ± 0.04 (4.45)	2.01 ± 0.07** (12.41)	2.17 ± 0.08*** (19.57)
F – 4 (30 % acetone in pet. ether fraction)	100	1.22 ± 0.04 (6.62)	1.59 ± 0.09*** (30.77)	1.41 ± 0.07*** (47.49)
F – 5 (50 % acetone in pet. ether fraction)	100	1.25 ± 0.03 (4.58)	1.78 ± 0.06*** (22.57)	1.62 ± 0.06*** (39.81)
F – 6 (methanol fraction)	100	1.24 ± 0.03 (5.34)	1.99 ± 0.07** (13.21)	2.11 ± 0.07*** (21.73)

*Values are expressed as mean ± SEM for six animals and analysed by Two way ANOVA followed by Bonferroni post-hoc test, *p<0.05, **p<0.01 and ***p<0.001 when compared to carrageenan control. The figures in parenthesis indicate the percent inhibition.*

The anti-inflammatory effect of pools P – 1 to P – 10 obtained from column of fraction F – 4 (30 % acetone in pet. ether) revealed that pool P – 8 was found to be the most active in reducing the inflammation (29.00 % reduction at 3rd hr). It significantly ($p<0.001$) reduced the paw edema at 3rd and 5th hr. Pools P – 1 and P – 2 were also found to be active in reducing the inflammation but less active than pool P – 8. Pools P – 1 and P – 2 significantly ($p<0.001$) reduced paw edema at 5th hr. Pools P – 7 and P – 9 significantly ($p<0.001$) reduced paw edema at 5th hr. Treatment with other pools (P – 3, P – 4, P – 5, P – 6, P – 10) did not significantly inhibited paw edema (Table 2). Therefore the compound was isolated from the most active pool P – 8 by preparative TLC.

Table 2: Anti-inflammatory effect of pools (P – 1 to P – 10) collected from active fraction F – 4

Treatment Groups	Dose (mg/kg, p.o.)	Change in paw volume (ml)		
		1 hr	3 hr	5 hr
Vehicle (carrageenan) control	-	1.29 ± 0.03	2.16 ± 0.04	2.53 ± 0.04
Diclofenac	10	1.20 ± 0.03 (7.60)	1.41 ± 0.04*** (34.57)	1.22 ± 0.03*** (51.84)
P – 1	10	1.25 ± 0.04 (3.22)	1.91 ± 0.07* (11.45)	1.81 ± 0.05*** (28.46)
P – 2	10	1.25 ± 0.04 (3.35)	1.88 ± 0.06** (12.68)	1.73 ± 0.07*** (31.62)
P – 3	10	1.25 ± 0.01 (3.74)	2.02 ± 0.04 (6.26)	2.33 ± 0.04 (7.91)
P – 4	10	1.27 ± 0.05 (1.80)	2.11 ± 0.05 (2.24)	2.46 ± 0.04 (2.77)
P – 5	10	1.27 ± 0.04 (1.55)	2.09 ± 0.09 (3.09)	2.45 ± 0.08 (3.10)
P – 6	10	1.25 ± 0.05 (3.35)	2.07 ± 0.11 (3.87)	2.39 ± 0.09 (5.47)
P – 7	10	1.27 ± 0.03 (2.19)	1.91 ± 0.09* (11.45)	1.76 ± 0.07*** (30.30)
P – 8	10	1.24 ± 0.03 (4.25)	1.53 ± 0.04*** (29.00)	1.33 ± 0.05*** (47.56)
P – 9	10	1.28 ± 0.06 (1.03)	1.97 ± 0.05 (8.74)	1.90 ± 0.04*** (25.10)
P – 10	10	1.27 ± 0.02 (1.93)	2.11 ± 0.06 (2.32)	2.45 ± 0.06 (3.10)

*Values are expressed as mean ± SEM for six animals and analysed by Two way ANOVA followed by Bonferroni post-hoc test, *p<0.05, **p<0.01 and ***p<0.001 when compared to carrageenan control. The figures in parenthesis indicate the percent inhibition.*

Isolated Compound 1 possessed a molecular formula $C_{15}H_{20}O_3$, mp 139-140°C. IR (KBr) ν_{max} 3630 (-OH stretching), 2996 (Aliphatic), 1772 (-C=O stretching), 1153 (C-O stretching) cm^{-1}. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 3.46 (1H, dd, *J*=6.0, 10.0 Hz, H-1), 1.96 (1H, m, H-2α), 2.25 (1H, m, H-2β), 5.34 (1H, br.s, H-3), 1.98 (1H, m, H-5), 1.32 (1H, m, H-6α), 1.96 (1H, m, H-6β), 3.03 (1H, m, H-7), 4.62 (1H, ddd, *J*=5.0, 1.5, 10.0 Hz, H-8), 1.47 (1H, dd, *J*=6.0, 1.5Hz, H-9α), 2.59 (1H, dd, *J*=16.0, 1.5 Hz, H-9β), 6.15 (1H, br.d, *J*=1.5 Hz, H-13a), 5.67 (1H, br.d, *J*=1.0 Hz, H-13b), 0.85 (3H, s, H-14), and 1.62 (3H, d, *J*=1.5 Hz, H-15). ^{13}C-NMR and DEPT ($CDCl_3$, 50 MHz) δ 75.31 (d, C-1), 36.19 (t, C-2), 120.45 (d, C-3), 132.97 (s, C-4), 40.19 (d, C-5), 26.49 (t, C-6), 43.54 (d, C-7), 76.93 (d, C-8), 31.04 (t, C-9), 35.83 (s, C-10), 141.59 (s, C-11), 171.12 (s, C-12), 120.56 (t, C-13), 20.34 (q, C-14), and 10.46 (q, C-15). MS m/z 271.03 $[M-23]^-$

The structure and stereochemistry was established on the basis of above spectral data and comparison with the reported spectral data in the literature (Figure 1) [10].

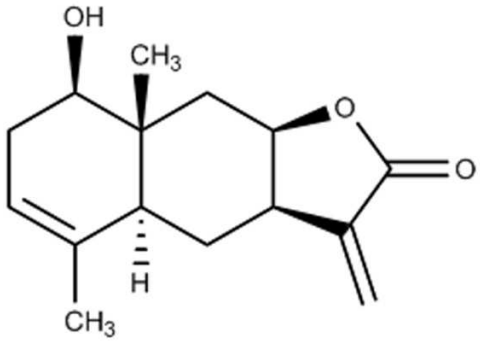

Figure 1: Structure of isolated compound 1 (Isoivangustin)

Docking was carried out to study the binding mode of the isolated compound, isoivangustin on the active site of TACE. Inflammation induced by carrageenan involves cellular migration, exudation of plasma and production of mediators such as IL-1β, IL-6 and tumor necrosis factor (TNF)-α [17]. The docking score of native ligand IH6, diclofenac and isoivangustin was found to be -7.432, -7.358 and -5.341 respectively. TACE converts membrane bound pro-TNF-α to mature and soluble TNF-α. The native ligand IH6 was successfully docked into the active site of TACE. Hydroxamate group of compound IH6 forms van der Waals interaction with zinc, the co-catalytic metal ion in the active site of the enzyme (Figure 2).

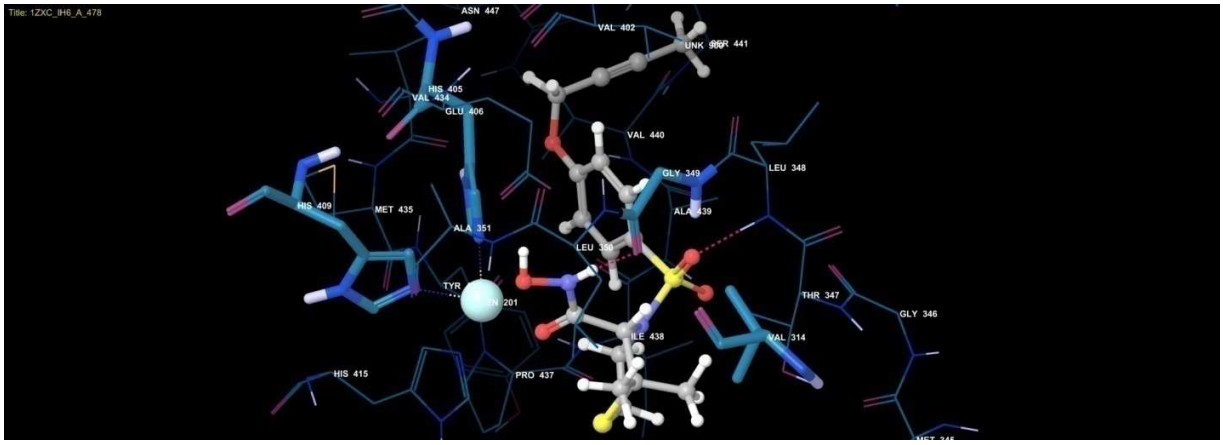

Figure 2: Binding of native ligand IH6 in the active site of TNF-α converting enzyme (TACE)

The compound IH6 actively takes part in forming hydrogen bond interaction with the key amino acids Gly349 and Leu348 in the enzyme protein. The phenyl ring forms an interaction with amino acid His405 by Π-Π stacking. Furthermore, the compound is surrounded with residues, such as Ala439, Leu348, Val434, Tyr436, His415, Ilu438 and Pro437 in the enzyme and makes contacts through van der Waals interactions with these amino acids and the docking score of compound IH6 with TACE was -7.432. Also, the binding studies of diclofenac with TNF-α were studied and it was found that it forms the van der Waals interaction with zinc and show Π-Π stacking with amino acid HIS405. The docking score for diclofenac with TACE was -7.358 (Figure 3).

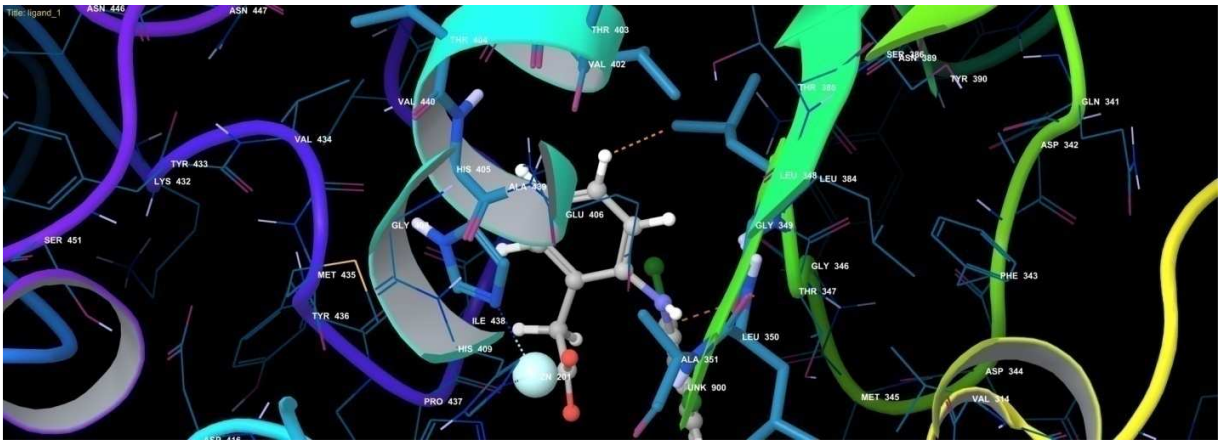

Figure 3: Binding of diclofenac in the active site of TNF-α converting enzyme (TACE)

Docking analysis of isoivangustin at the active site of TACE showed hydrogen binding with amino acid Gly349 and Leu348 like in native ligand IH6 which showed hydrogen bonding with same amino acids. Also, the octahydronapthyl ring fits into hydrophobic pocket formed by amino acid His405 in the enzyme. The docking score of isoivangustin with TACE enzyme was -5.341, which shows that it has good binding interaction with active site of TACE (Figure 4).

Validation of docking procedure:
In order to validate our docking procedure, we eliminated the co-crystallized ligand IH6 from the active site, and redocked within the inhibitor binding cavity of TACE enzyme. In this study, the root mean square deviation value was below 2Å, showing that our docking method is valid for the inhibitors studied.

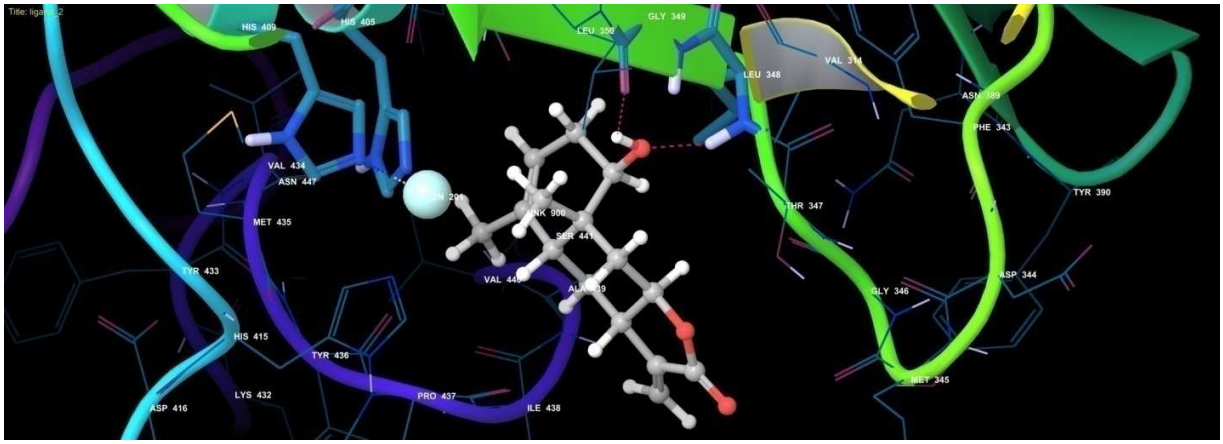

Figure 4: Binding of isoivangustin in the active site of TNF-α converting enzyme (TACE)

CONCLUSION

The results of the present study suggested that various fractions of methanol extract of *Cyathocline purpurea* has anti-inflammatory activity and isoivangustin, a sesquiterpene lactone isolated was the most active constituent contributing to its anti-inflammatory effect and the mechanism for its anti-inflammatory effect may be via inhibition of TNF-α. Further studies are underway to evaluate antiarthritic activity of isoivangustin in Freund's complete adjuvant induced arthritis model and find out its exact mechanism of action.

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