ORIGINAL ARTICLE

Docking studies of artemether and curcumin with pro-inflammatory inhibitory proteins and their *in-vivo* simulation on level of lysosomal acid hydrolyses

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Abstract

Context: The present work is to perform a docking analysis by AutoDock version 4.2 for artemether (ARM) and curcumin (CUR) with few validated protein targets (cyclooxygenase [COX-2] and tumor necrosis factor [TNF-α]) for anticancer therapy. **Aims:** The object of this work was to analyze inhibitory action of one artemisinin derivative ARM and a natural coloring pigment CUR against pro-inflammatory (a chemical substance that became inflammatory only after it is altered by metabolic process) by computational docking parameters and simulate these effects on the adjuvant arthritic rats were compared with that of drug combination and indomethacin. **Materials and Methods:** Two-dimensional (2-D) Structure of ARM and CUR was drawn using ChemSketch, the 2-D structure of ligands as well as receptor were converted into the three-dimensional (3-D) structure and optimized with 3-D geometry. The regions of interest used by AUTODOCK version 4.2 were defined by considering grid area by making a grid box around the active sites. The anti-inflammatory activity of ARM and CUR was assessed by measuring paws swelling and lysosomal enzyme activity in control and experimental rates. **Results:** The binding energy of the ARM and CUR with the human COX and TNF-α protein is found accordingly. That is the indication of two molecular combinations and their efficacy explain against the pro-inflammatory protein scaffolds. Increased paw diameter and lysosomal enzyme activity in the arthritic animals were significantly suppressed to near normal levels in rats treated with an equimolar concentration of drug combination and 3 mg/kg indomethacin. **Conclusions:** A good correlation was observed in binding affinity of ARM and CUR against the selected targets for pro-inflammatory enzyme inhibition. These results indicate that combination of the drug has promising anti-arthritic activity as a result of its stabilizing action on lysosomal enzyme activity.

Key words: Pro-inflammatory, artemether, curcumin, docking, lysosomal enzyme activity, anti-arthritic activity

INTRODUCTION

Inflammation is a sequential molecular event which could take some time for initiation to progression. Increasing evidence from population's pathological survey suggest that inflammation associated diseases could be prevented during progression.[1] With this research, our aim is to investigate the inhibitory effect of curcumin (CUR) and artemether (ARM) by applying the bioinformatics techniques and its *in-vivo* simulation on the level of lysosomal acid hydrolyses. ARM and CUR is a functionally labile molecule with the potential to transform the biological activity of a number of the diverse target molecule through different binding affinity and interaction [Figures 1 and 2].[2] One of the reasons for this combination study is including cost consideration, pharmacokinetic mismatch, drug and molecule resistance, cross-resistance and side effects so it may efforts are investigating to the discover new partner drug.[3]

CUR is a diferuloylmethane molecule (1,7-bis(4-hydroxy-3 methoxyphenol)-1,6- heptadiene- 3,5-dione) containing two residues of ferulic acid that is connected by a methylene

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Received: 24-10-2016
Revised: 19-11-2016
Accepted: 27-11-2016

bridge. CUR has two hydrophobic phenyl domains that are joined by a flexible linker, and molecular docking studies have found that CUR can adopt many confirmations suitable for maximizing hydrophobic contacts with the protein to which it is bound. The hydrophobic structure of CUR, that is, the phenolic and carbonyl functional groups located on the ends and in the center of the molecule, it can participate in hydrogen bonding with a target macromolecule. This structure provides a powerful and direct electrostatic interaction to increase complimentary free energies of association. Due to its β-diketone moiety, CUR undergoes keto-enol tautomerism and exist entirely in the enol from both in solution and in a solid phase, it gives extra chemical function in CUR.[4,5] The prime enol form allows the midsection of the molecule for contributing and allow hydrogen bonds. The enol form also makes a perfect chelator of positively charged metals, which are frequently found in the active sites of specific proteins.[6] The amalgamation of hydrophobic interaction including p-p interaction, wide hydrogen bonding, meta chelation and covalent bonding, covering such a huge exterior gives CUR many possible mechanisms to interact with the target molecule.

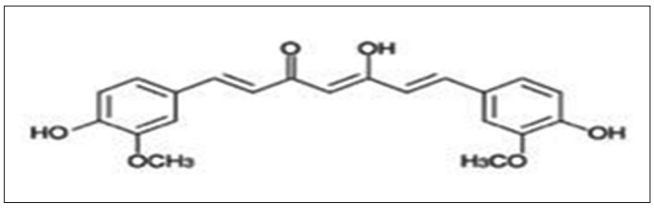

Figure 1: Curcumin

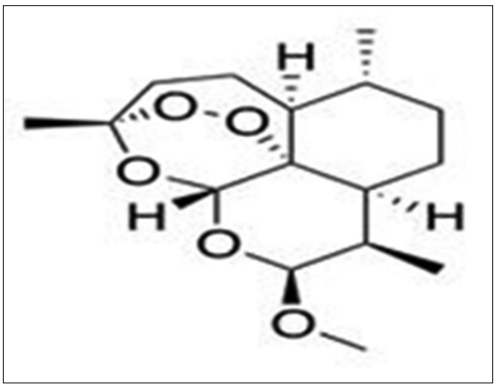

Figure 2: Artemether

Artemisinin is a hydrophobic molecule capable of permeating the cellular membrane to elicit anti-oxidative functions. Like anti-malarial approach, artemisinin act *via.* the endoperoxide components present in its configuration, and generation of reactive oxygen species such as superoxide anion and hydroxyl radicals, to induce cellular damage. In the presence of free iron, artemisinin can convert itself into cytotoxic carbon-centered radical, it is an intoxicating alkylating agent, to induce direct oxidative kill to cancer cells. The role of heme or iron in artemisinin-mediated anti-cancer activity was more established by the use of cobalt protoporphyrin, a heme oxygenase activator, resulting in the degradation of heme and failing of artemisinin anticancer activity [Figure 3].[7]

Interaction of Biochemical markers like cyclooxygenase (COX) and lipooxygenase (LOX) inhibitors are being investigated as potential anti-inflammatory drug targets. COX-2 is indispensable to progress in the design of the new drug, more in particular directed on chemoprevention or chemotherapy of human being cancer.[8]

COX-1: Inhibited the enzyme activity by direct binding through Ser530.[9]

COX-2: Inhibited the enzyme activity by direct binding through Val523, Val116, Ala516, and Tyr355; interacted by hydrogen bonds with Ala562 and inhibited PGE-2 production.[10]

Tumor necrosis factor (TNF-α): Interacted with TNF- α by hydrophobic, van der Waals forces, and H-bond.

Cys129 in TNF-α was found as the binding site for CUR.[11]

Experimental molecular docking shows the various interactions between the COX enzyme and Artemisinin and CUR also showed interaction among Val 523, Val 116, Ala516, and Tyr 355 of COX-2. In agreement with these explanations, CUR was

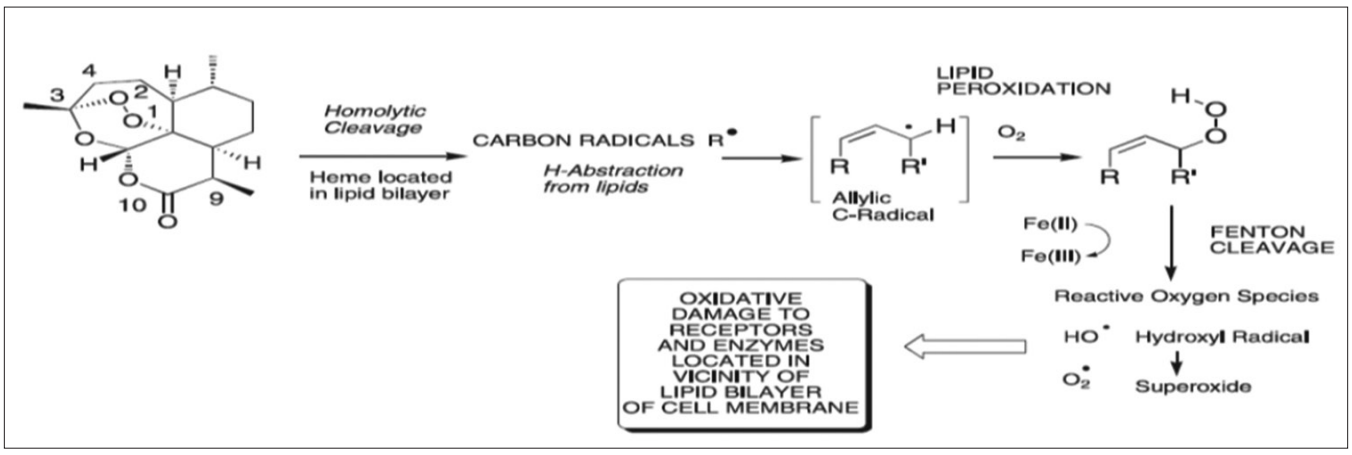

Figure 3: Proposed chemical mechanism for artemisinin medicated lipid peroxidation[7]

found to possess antioxidant activity. CUR interacts directly with COX-2 by forming H-bonds with Ala 562 with an estimated binding energy of-5.71 kcal/mol. CUR was found to dock at the receptor binding sites of TNF-α by molecular docking. Several residues, including Leu 89 Asn 90 Asp 105, Asn 106, and Cys 129 on TNF-α were requisite for binding to CUR. CUR exhibited direct interaction with TNF-α by noncovalent interactions (such as hydrophobic and H-bonds) and also by covalent interaction (such as π-π aromatic interaction at Tyr 201 and π-cation interaction at Lys 126). These findings propose that CUR may manipulate or even interrupt the signal transduction between TNF-α and its receptor by direct binding and thereby may suppress inflammation-induced by this cytokine.[12] The clinical manifestation of arthritis related to both sites of affected vessel and severity of inflammation and the acute stage is represented by fever, weight loss, and elevated C-reactive protein levels.[13] With this refine study we have found that the COX-2 and TNF-α showing a correlation with ARM and CUR activity in terms of pro-inflammatory protein inhibitions and suppression of lysosomal acid hydrolyses.

SUBJECTS AND METHODS

Preparation of Ligands

Two-dimensional (2-D) structure of ARM and CUR was drawn using ChemSketch, the 2-D structure of ligands were converted into the three-dimensional (3-D) structure using PyRx tool to generate atomic coordinates and optimized with 3-D geometry. The optimized structure was saved in PDB format for AutoDock 4.2 compatibility. The structure of both ligands was given in Figure 4.

The human COX and TNF-α Receptor was searched in the protein data bank and downloaded. PDB id 6 COX is downloaded from PDB portal [Figures 5-7].[13]

Preparation of Receptor

Several experimentally determined 3-D structures of COX-2 and TNF-α co-crystallized with inhibitors which provide the basics for using structured based approaches for the discovery of new inhibitors. Chain A was selected for docking by parsing the protein structure in Chimera (UCSF, CA, USA). This protein structure was refined by removing all waters of crystallization. Hydrogen was added to the receptor and finally gasteiger charges were assigned. Non-integer charges on six residues were dispersed on the whole residues.[14]

Preparation of Docking File

All the calculations were carried out by using Autodock 4.2 as docking tool. The visualization and other programs necessary for docking studies were performed out by means of Pymol, Chimera, DS visualizer, MMP plus.

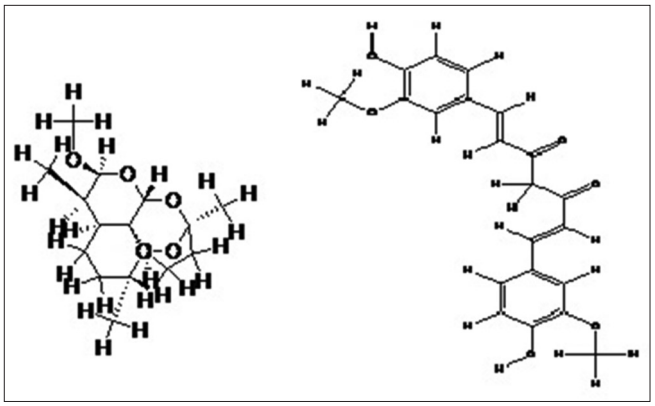

Figure 4: Two-dimensional structure of ligand Artemether and curcumin[12]

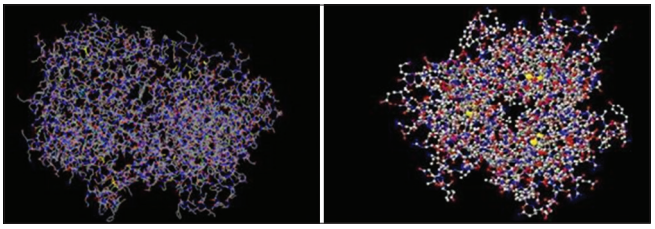

Figure 5: The crystal structure of the human cyclooxygenase protein (PDB id 6 cyclooxygenase)

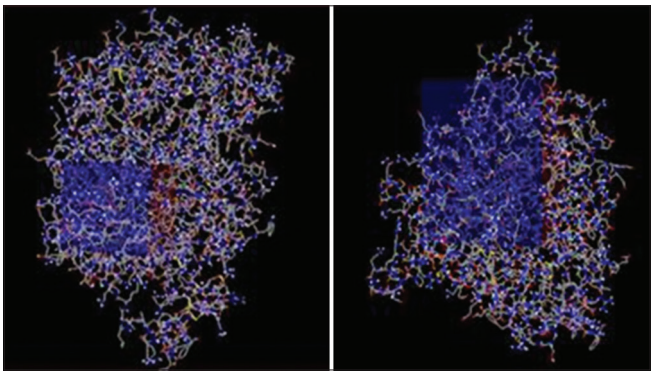

Figure 6: The crystal structure of the human tumor necrosis factor-α protein (PDB id 1- tumor necrosis factor)

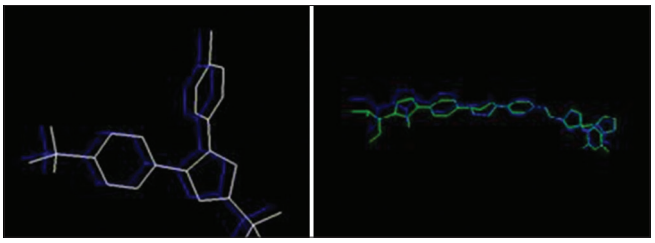

Figure 7: Grid box covering all active sites in cyclooxygenase, and tumor necrosis factor-α receptor

Ligands - Recepter Interaction using Molecular Docking

The regions of interest used by AUTODOCK version 4.2 were defined by considering grid area by making a grid box around

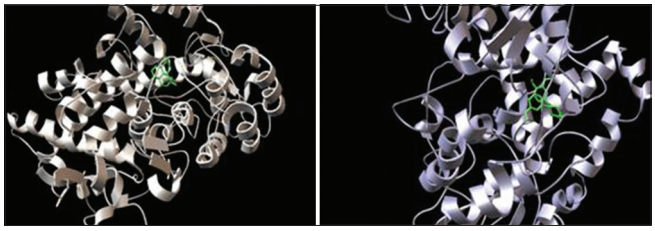

Figure 8: Perfectly overlayed structure of docked conformation of ligand with reference to its crystallized conformation

the active sites. Grid box plays a central role in the process of docking as it is made to cover all the amino acids present in active sites necessary for binding other than those present in the receptor. Grid box has 3 thumbwheel widgets which let us change the number of points in the x, y, and z dimensions. The spacing between grid points can be adjusted with another thumbwheel, the value in the study taken is 0.436 Å and number of points considered are 40, 40, and 40 points in the x, y, and z dimensions and −29.049, 23.526, and 46.984 as x, y, z centers. Molecular docking assists in studying drug/ligand or receptor/protein interactions by identifying the suitable active sites in the protein, obtaining the best geometry of ligand - receptor complex and calculating the energy of interaction for a different ligand to design more effective ligand. Ligand structure and the receptor structure were docked together by setting required values of some important parameters like cavity number and rotation angle [Figures 10 and 11].

In-vivo Simulation Study

The experimental study was carried out using adult female albino rat of Wistar strain weighing between 140 and 150 g purchased from DRDO, Gwalior. The animals were maintained under standard laboratory condition at temperature 25 ± 2°C and a 12 h natural light period. Commercial pellet diet and demineralized water were provided ad libitum. The institute Animal Ethical Committee accepted all the procedure and protocols.

Drug combination preparation

Equimolar concentration (1 mg/ml) of ARM and CUR was prepared by dissolving accurately weighed drug in 98% ethanol solution.

Experimental design

Rats were divided into six groups each comprising six animals. Group I served as control. In group II, arthritis was induced by intradermal injection of complete Freund's adjuvant (0.1 ml) into the right hind paw. Groups III and IV were treated with drug combination 1 mg/kg body weight and indomethacin, respectively, from day 11 to 18 after treatment with complete Freund's adjuvant.

Assessment of arthritis

Arthritis was assessed by means of physical and biochemical measurement. Paw volume was assessed by measurement of the right ipsilateral hind paw using a Vernier scale. Rates were sacrificed by cervical decapitation on day 19 of the experiment. Plasma has separated from blood collected into EDTA-coated tubes, and the liver and spleen were immediately dissected out and homogenized in ice-cold 0.01 M Tris-HCl buffer, pH 7.4 to give a 10% homogenate.

Lysosomal enzyme activity was determined in serum, liver, and spleen. Acid phosphatase was assayed by the method of King,[15] cathepsin D,[16] β- glucuronidase,[17] protein,[18] and N- acetyl glucosaminidase was determined by the method of Marhun.[19]

RESULT AND DISCUSSION

Results were expressed as means ± standard deviation and statistical analysis using student's t- test were performed to determine significant differences between groups.

Docking Result

Validation of docking

To ensure that the ligand orientations and positions obtained from the docking studies represent valid and reasonable potential binding modes of the inhibitors, the docking methods and parameters used were validated by redocking the crystallized receptor and ligand, and overlaying the docked and crystallized ligand chemical structures.

Binding energy

The molecular docking simulation method is validated as the binding energy of the substrate ligand S58, and ADAM 10 with the human COX protein and human TNF-α of binding energies are −10.25 kcal/mol and −9.27 kcal/mol, respectively, which lies within the predefined range of −5 to −15 kcal/mol.

The binding energy of the ARM and CUR with the human COX protein is found to be −8.67 Kcal/Mol and −7.01 Kcal/Mol, respectively.

The binding energy of the ARM and CUR with the human TNF-α protein is found to be −9.40 Kcal/Mol and −9.54 Kcal/Mol, respectively.

Overlay crystal structure

The docked conformation of ligand should be perfectly overlayed with the crystal structure of the downloaded protein. This testing of the Autodock docking algorithm with COX (already present within receptor as a complex) is completed successfully and the docked confirmation of the COX is perfectly superimposed with reference structure of COX, that is, its crystal structure. The re-docking of this ligand was successfully achieved to get finer results [Figure 8].

Interaction Studies

Similar interactions between the docked ligand and the receptor should be observed after docking, to that of the interactions present in the crystallized structure of protein.

Molecular Docking Simulation Studies

Docking of ARM and CUR Ligand on COX and TNF-α is done. All the bonds of ligand were kept flexible, whereas no residues in receptor were made flexible. In the estimation by Autodock 4.2, CUR shows better affinity with all pro-inflammatory targets than ARM. Interaction of CUR and ARM has represented the docking energy in given Figure 9. All molecule were docked this study was validated by checking chemical resemblance, alignment, and binding energy.

In-vivo effects

Measurements of the paw volume of rates with adjuvant-induced arthritis revealed an increase in ankle diameter, from day 4 to 19. The increase in ankle diameter of arthritis control rates was found to be 81%, the paw diameter was reduced by 38% and 35% in arthritic rates treated with drug combination and indomethacin, respectively. The activity of lysosomal and various antioxidant enzyme in control and experimental group is presented in Table 1. Levels of acid phosphatase, cathepsin-D, β- glucuronidase, and N- acetyl glucosaminidase increased significantly ($P < 0.001$) in rates with adjuvants - induced arthritis (Group II) Treatment with drug combination significantly reduced the level of enzyme (Group V), the effect being almost the same that of indomethacin (Group VI). The soft swelling seen around the ankle joints was found to be a result of edema of periarticular tissue. An increase in granulocyte and monocyte has been found to be associated with changes in ankle diameter.[20] Paw swelling was significantly reduced in arthritic rates treated with drug combination. A characteristic feature of adjuvant-induced arthritis rates is the correlation between the development of inflammation and the release of the lysosomal enzyme into the extra cellular compartment.[21] It is now generally believed that lysosomal instability results in the release of the lysosomal enzyme in the tissue of rheumatoid joints which play an important role in the rheumatoid processes. Extensive infiltrations of leukocytes in the adjuvant paw lead to an increase in the level of lysosomal enzymes,[22] which in turn initiate the synthesis of inflammatory mediators such as thromboxanes, prostaglandins, and leukotrienes cycloxygenase and tissue necrosis factor. It is likely that reduction of the release of such enzyme would prove beneficial, and this indirectly confirms the protective effects of herbal drugs. Drugs capable of stabilizing the lysosomal membrane can reduce inflammation.[23] Drug combination might exert its effect by modifying the lysosomal membrane in such a way that its capable of fusing with the plasma membrane and thereby preventing the release of the lysosomal enzyme.[24] An important mechanism of anti- inflammatory activity is the membrane stability-modulating effect.[25,26]

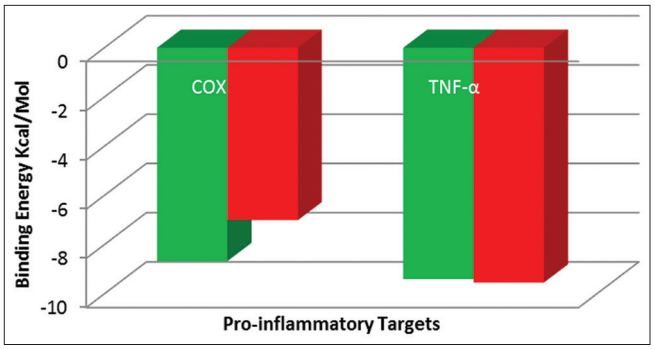

Figure 9: Comparison in binding energy of pro-inflammatory targets and ligands

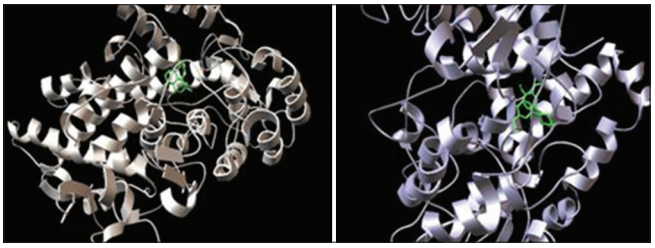

Figure 10: Bound conformation of artemether and curcumin with human cyclooxygenase protein

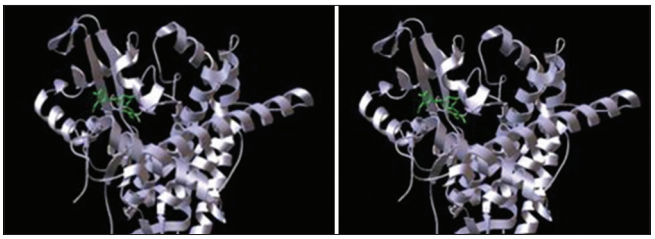

Figure 11: Bound conformation of artemether and curcumin with human tumor necrosis factor-α

CONCLUSION

From the above molecular docking simulation and molecular modeling studies, it is concluded that the CUR and ARM act as potent inhibitors of pro-inflammatory human protein and may act as a potent drug for the treatment of various types of inflammation associated diseases since it shows a good binding affinity with the macromolecules. Our docking studies confirm that the main interaction of COX and TNF-α with ARM and CUR is hydrogen bond and Vander wall interaction. The CUR and ARM also strictly follow Lipinski's Rule of five, thus having very good drug score as well as drug-likeness (ADME) score. The six-membered phenyl ring plays a vital role in holding the molecule at the place (binding) at the active site by three important H- bonds. Hopefully, this

Table 1: Molecular interaction of ARM and CUR with inflammatory protein

Docked molecule	Number of hydrogen bond	Length of hydrogen bond (Å)	Lowest binding free energy (Kcal/mol)
ARM-COX-2	2	1.128	−8.67
ARM-TNF-α	2	1.542	−9.40
CUR-COX-2	2	1.236	−7.01
CUR-TNF-α	2	1.478	−9.54

COX: Cyclooxygenase, TNF: Tumor necrosis factor, ARM: Artemether, CUR: Curcumin

study will be use full for the design and development of novel pro-inflammatory activating enzyme inhibitory herbal lead molecule, in safe and effective manner. The study has shown that the adverse changes (paw edema and levels of lysosomal enzyme) in arthritic rates were substantially reserved by oral administration of drug combination. It provides the evidence of its anti-inflammatory property.

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Source of Support: Nil. **Conflict of Interest:** None declared.