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# *In vivo, In vitro* anti-arthritic studies of Ellagic acid from *Kirganelia reticulata* Baill and its molecular docking

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**ABSTRACT**

*Kirganelia reticulata* is a useful shrub having various medicinal properties. *In vivo, in vitro* and *in silico* antiarthritic activity of a phytoconstituent, ellagic acid (EA) isolated from the leaves of *K. reticulata* was screened. EA is a naturally occurring plant polyphenol found at high concentrations that act as potential protectors against variety of human diseases. Formaldehyde induced paw edema, assumed to be one of the most suitable test procedures to screen chronic anti-inflammatory agents as it closely resembles human arthritis, and was employed for this study. The course of treatment was followed for over and 4 weeks post inoculation period using health, clinical and behavioural methods of study. Estimation of change in body weight was considered as health parameters and clinical observations included paw edema volume, change in the movements was studied in behavioural observations. The effect of EA was compared with standard drug aspirin. Various *in vitro* models such as inhibition of protein denaturation, effect of membrane stabilization and proteinase inhibitory actions were studied. EA with two different concentrations (100 µg/ml and 250 µg/ml) was used and results were compared with acetyl salicylic acid. Hypoxia-inducible factor (HIF-2α) promotes degradative pathways that foster osteoarthritis. The inhibitory effect of EA was studied using automated docking and efficiency was compared with standard drug in terms of interaction and binding. The isolated compound EA showed anti-arthritic activity which was found to be significant to that of the standard drugs and supports the traditional use of plant for rheumatism.

## INTRODUCTION

*Kirganelia reticulata* Baill. (Synonym-*Phyllanthus reticulatus* Poir.) is a large, often scandent, shrub of the family Euphorbiaceae. It is having elliptic to oblong or obviate leaves. The plant bears unisexual flowers and fleshy sub-globose, purplish black fruits. It grows into a good hedge plant. The plant also has certain medicinal uses. Besides the use of it in traditional medicine, the plant still represent a large source of natural anti-oxidants that might serve as leads for the development of the novel drugs. The leaves and bark are used as astringent and diuretic. Juice of leaves is used for the treatment of diarrhea in children (Ghani, 2003). The stems are used to treat sore eyes and the powdered leaf is used in sores, burns, suppurations and chafing of skin (Chopra *et al.*, 1956).

The plant is used for a variety of ailments, including smallpox, syphilis, asthma, diarrhoea, bleeding from gums etc (Nadkarni, 1954). The antibacterial potential of the leaf extracts of this plant has been evaluated recently (Shruthi *et al.*, 2010). Different chemical compounds such as alkaloids, tannins, flavonoids, phytosterols and glycosides were detected, which could make the plant useful for treating different ailments and having a potential of providing useful drugs of human use. By virtue of their photosynthetic machinery, leaves serves as a sink for several metabolites and as an important source of several bioactive compounds (Sujan *et al.*, 2009; Murti *et al.*, 2010).

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Several antiinflammatory, digestive, antinecrotic, neuro-protective and hepatoprotective drugs have recently been shown to have an antioxidant or radical scavenging mechanism as part of their activity (Kirtikar and Basu, 1980). The antiarthritic, analgesic, antioxidant, antihelminthic activities of the leaf extracts have been investigated (Shruthi *et al.,* 2012a &, 2012b).

Ellagic acid (EA) is a polyphenol found at high concentrations in a number of fruits like grapes, strawberries, black currants and raspberries (Yizhong *et al.,* 2013). EA is a naturally occurring plant polyphenol that exhibits antioxidative properties both *in vivo* and *in vitro*. Recently dietary polyphenols are receiving increasing attention as potential protectors against a variety of human diseases like cancer and chemotherapy induced toxicity in animal models (Ahmet *et al.,* 2010). EA is a dietary supplement, reported to have anti-inflammatory, antinociceptive properties via cyclooxygenase inhibition (Gainok *et al.,* 2011). It has been found to have anticarcinogenic, antifibrosis and antioxidative properties (Dong *et al.,* 2006). The effects of EA on cell cycle events and apoptosis were studied in cervical carcinoma (CaSki) cells (Narayanan *et al.,* 1999; Te-Mao *et al.,* 2005).

Arthritis is a chronic, systemic inflammatory disease predominantly affecting the joints and peri-articular tissues. Arthritis still remains a formidable disease, being capable of producing severe crippling deformities and functional disabilities. Arthritis is classified as an inflammatory arthritis, the disease comprises of 3 basic inter-related processes like inflammation, synovial proliferation and joint tissue destruction. Arthritis factor containing immune complexes found in the joints activate the pathological process. Tumour necrosis factor alpha (TNF-alpha) is the product of macrophages has been demonstrated to play an important role in the pathogenesis of Arthritis (Lavanya *et al.,* 2010). Conventional treatments for arthritis, including Non-steroidal Anti-inflammatory Drugs (NSAID's), disease modifying anti-rheumatoid drugs (DMARD's) and corticosteroids, aim to reduce the patient's pain and joint inflammation, minimize loss of function and decrease the progression of joint damage. However, such treatments are rarely totally effective and some pharmacological therapies have the potential to cause side effects (Agarwal, 2010).

Even though several studies have been performed, still an efficient medicine is not found out. The mediators bind to specific receptors, causing gene transcription, and form complicated signaling interactions which contribute to the progression of inflammatory arthritis, e.g. leukocyte infiltration, cytokine networks formation, cartilage catabolism elevation and anabolism suppression (Kapoor *et al.,* 2011). The onset of arthritis is rapid, typically developing 10-13 days after immunization with homologous or heterologous type II collagen, peaking at about days 15-20 and then gradually declining. The resulting polyarthritis is characterized by marked cartilage destruction associated with immune complex deposition on articular surfaces, bone resorption, periosteal proliferation and moderate to marked synovitis and periarticular inflammation (Bendele, 2001). Many immune cell populations, participate in the ongoing inflammatory process (Scott *et al.,* 2010), suggesting the presence of multiple cellular targets for immunotherapy of arthritis. The progress has been made in understanding immune and inflammatory processes and hence these autoimmune changes are receiving increased attention in drug discovery and development (Havagiray and Nitin, 2009).

In the articular chondrocytes in the synovial joint, HIF-1α promotes homeostatic pathways, and HIF-2α promotes degradative pathways that foster osteoarthritis. HIF-2α promotes chondrocyte hypertrophy, a terminal differentiation state characterized by a unique gene expression program. This switch to hypertrophy seems to be a relatively early signal to ignite and drive osteoarthritis in stressed cartilage (Matthew *et al.,* 2010). Therefore, we have employed a disease progression model using formaldehyde-induced arthritis in rats to provide an understanding of the relationship between target modulation and efficacy in the animal model.

As part of this research, significant attention has been paid to the natural drug as these drugs elicit few side effects and are inexpensive (Dharamsiri *et al.,* 2003). The objective of this study was to investigate the anti-arthritic effect and mechanisms of action of EA isolated from traditionally proven plant and compare the mode of interactions existing, in the hunt of better therapies against arthritis and provide scientific evidence to folkloric claim of the plant using *in vivo*, *in vitro* and *in silico* pharmacological models.

## MATERIALS AND METHODS

### Plant materials and Preparation of extract

Fresh leaf materials of *K. reticulata* were collected in winter season in and around HSR layout, Bangalore, Karnataka (Southern India). The taxonomic identification of the plant was confirmed and processed for further investigations. Collected leaves were shade-dried and then powdered using a mechanical grinder (Sieve No. 10/44). Then subjected for successive extraction using hexane, chloroform and methanol (LR grade, Merck, India) separately using soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). Obtained methanol extract was used for isolation of EA. A portion of the EA was used for the *in vivo* and *in vitro* anti-arthritic assays.

### Isolation of EA

The pure compound was isolated from the methanol leaf extract of the plant following the method of Anonymous (Anonymous, 1998). The residue was dissolved in methanol and adsorbed on silica gel powder and loaded on a silica gel column (Merck, 100-200 mesh). The column was eluted first with chloroform followed by graded mixtures of chloroform: methanol in the ratio of 8:2. The elution was monitored by TLC sheets (commercially available aluminum foiled sheets, Merck) and fractions 12-15 afforded compound EA. The EA was characterized by subjecting to IR, NMR and MASS spectral analysis.

**Infrared spectroscopy (IR)**

The vibrational state of a molecule can be probed in variety of ways. The most direct way is infrared spectroscopy because vibrational transition typically requires an amount of energy that corresponds to the infrared region of the spectrum between 4000–400 $cm^{-1}$. Radiation in this region can be utilized in structure determination in coordination chemistry by making use of the fact that interatomic bond in ligand absorb it. Infrared spectra of ligand and complexes were recorded in the region of 4000–400 $cm^{-1}$ on a FT-IR 8400s SIMADZU spectrometer in KBr pellets, CDRI Lucknow, India.

**Nuclear magnetic spectroscopy**

The $^{1}H$ and $^{13}C$-NMR spectra were recorded in DMSO-d6 at 100-400 MHz using Bruker NMR spectrometer with tetramethylsilane as an internal standard at sophisticated Instrumentation Facility Indian Institute of Science, Bangalore, India.

**Mass spectroscopy**

The exact molecular weight and fragmentation of the ligand and their coordination compound was examined by mass spectra and were recorded with MAT 312 spectrophotometer.

**In vivo anti-arthritic activity**

***Procurement of animals***

Wistar strain albino male rats (150-200 g) were collected from ALN Rao Ayurvedic Medical College Koppa, Karnataka. The animals were housed in solid bottomed polypropylene cages and acclimatized to animal house conditions ($25 \pm 20^{0}C$) with dark light circle (14/10 h). The rats were fed with commercial pellets and water *ad libitum*. The standard pellet diet was supplied by Sai Durga Feeds, Bangalore. Food was withdrawn 2 h before and during experimental duration. All experimental protocols were prepared and performed based on ethical guidelines of Institutional Animal Ethics Committee (No. IAEC-BT-01/2011-2012).

***Acute oral toxicity study***

Acute toxicity test were performed on rat of either sex weighing 160-180 g body weight. The animals were divided into 6 groups containing 6 animals per each group. The EA was subjected to acute oral toxicity studies as per revised OECD Organization of Economic Co-operation and Development guidelines (OECD No. 423) and acute class method (Ecobichon, 1997). The animals were fasted overnight, provided only water after which EA was administered to the groups orally by gastric intubation. The EA were devoid of any toxicity up to 2500 mg/kg body weight in albino rats for a single oral dose monitored for 14 days. If mortality was observed in 5 or 6 animals among 6 animals, then the dose administered was assigned as a toxic dose. If mortality was observed in 3 animals, then the same dose was repeated again to confirm the toxic dose. The animals were observed for toxic symptoms such as behavioral changes, locomotion, convulsions and mortality for 72 h. The optimum conditions for experiments were decided on the basis of pilot experiments carried out using six animals per group. 250 mg/kg b.w was taken as the therapeutic oral dose for the compound.

***Formaldehyde induced arthritis (Santos et al., 1994)***

Male Wistar rats weighing between 150-200 g were taken for the experiment. They were divided into 4 groups of 6 animals each. On the day of experiment, the basal paw volume of left hind paw of each animal was measured using Plethysmometer. 0.1 ml of 2% v/v formaldehyde in normal saline was injected into the sub-plantar region of the left hind paw to all the rats. Dosing with standard drug, aspirin and EA were started on same day and continued for 20 days. Group I served as the control, Group II received the standard drug aspirin, Groups III and IV received EA at two doses (100 and 250 µg/ml). Formaldehyde (0.1 ml 2% v/v) was again injected into the same paw of all the rats on the third day. Paw volume of injected paw was measured daily. The health status parameter included body weight and behavioural observations included change in the movements. The body weights of all the animals were recorded in grams on weekly basis by using single pan weighing balance (Kale and Kale, 1999). Body movement was measured by observing the time taken by individual animal to move 2 meter distance. The percentage inhibition of edema in the test drug treated group was calculated by using the formula (Snedecor and Cochran, 1967).

$$\% \text{ Inhibition} = 1-(Vt/Vc) \times 100$$

Where Vt = Edema volume in the test drug treated animals.
Vc = Edema volume in the control group animals.

**Evaluation of in vitro anti-arthritic activities**

***Inhibition of protein denaturation***

The test solution consisted of 0.45 ml of Bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of EA (100 and 250 µg/ml) in DMSO. Test control (0.5ml) consisted of 0.45 ml of bovine serum albumin and 0.05 ml of distilled water. Whereas product control (0.5 ml) consisted of 0.45 ml of distilled water and 0.05 ml of EA and standard consisted of 0.45 ml of Bovine serum albumin and 0.05 ml of Acetyl salicylic acid. The samples were incubated at 37 °C for 20 min and the temperature was increased to 57 °C for 3 min. After cooling, 2.5 ml of phosphate buffer saline (pH 6.3) was added to each tube. The absorbance was measured spectrophotometrically at 660 nm. The control represented 100% protein denaturation. The results were compared with acetyl salicylic acid. The percentage inhibition of protein denaturation (Eduardo and Tania, 2004) was calculated as below:

Percent inhibition =

$$\frac{100- (\text{O.D. of test solution} - \text{O.D. of product control}) \times 100}{\text{O.D. of test control}}$$

***Effect on membrane stabilization/ Inhibition of membrane lysis***

The principle involved here is stabilization of human red blood cell (HRBC) membrane by hypo tonicity induced membrane lysis. The test solution consisted of 1 ml phosphate buffer (pH 7.4, 0.15 M), 2 ml hypo saline (0.25% NaCl), 0.5 ml rat RBC

suspension (10 % v/v) with 0.5 ml of EA (100, 250 µg/ml) for assay and for standard, 0.5 ml of acetyl salicylic acid (250 µg/ml) was taken. For test control, 0.5 ml of isotonic saline was used instead of EA to produce 100% hemolysis. While product control lacked red blood cells. They were incubated at 56°C for 30 min, cooled under running tap water for 20 min and centrifuged further. The haemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The control represented 100% lysis. The results were compared with acetyl salicylic acid. Percent membrane stabilizing activity (Mizushima, 1966) was calculated as below:

Percent Stabilization =

$$\frac{100 - (\text{O.D. of test solution} - \text{O.D. of product control}) \times 100}{\text{O.D. of test control}}$$

***Proteinase Inhibitory Action***

The test solution (2.0 ml) contained 0.06 mg trypsin, 1.0 ml 25 mM tris-HCI buffer (pH 7.4) and 1.0 ml of EA (100 and 250 µg/ml) in DMSO and standard consisted of 0.5 ml Acetyl salicylic acid (250 µg/ml). The mixtures were incubated at 37°C for 5 min. Then 1.0 ml of 0.8% (w/v) casein was added. The mixtures were incubated for an additional 20 min and 2.0 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and absorbance of the supernatant was read at 280 nm against buffer as blank (Chatterjee, 1996). The product control lacked trypsin in reaction mixture and test control represented 100% inhibition. The results were compared with acetyl salicylic acid (250 µg/ml) treated samples. The percentage inhibition of protein was calculated as below:

Percent inhibition =

$$\frac{100 - (\text{O.D. of test solution} - \text{O.D. of product control}) \times 100}{\text{O.D. of test control}}$$

***In silico* anti-arthritic activity**

With the *in vivo* and *in vitro* anti-arthritic results in hand it was thought worthwhile to perform *in silico* studies which will further support the results obtained. Automated docking was used to determine the orientation of inhibitors bound in the active site of HIF-2α as target for antiarthritic activity. A Lamarckian genetic algorithm method, implemented in the program AutoDock 3.0, was employed. The ligand molecules EA and aspirin were designed and the structure was analyzed by using ChemDraw Ultra 6.0. 3D coordinates were prepared using PRODRG server (Ghose and Crippen, 1987). The protein structure file (PDB ID: 3H7W) was taken from PDB (www.rcsb.org/pdb) was edited by removing the heteroatoms, adding C terminal oxygen (Binkowski *et al.,* 2003). For docking calculations, Gasteigere– Marsili partial charges (Gasteiger and Marsili, 1980) were assigned to the ligands and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map was centered at particular residues of the protein which was predicted from the ligplot and was generated with AutoGrid. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters (Vidya *et al.,* 2012).

**Statistical analysis**

The values were expressed as mean ± SEM. Statistical analysis of data was performed using ANOVA followed by student t-test to study the differences amongst the means. Values of $P < 0.05$ were considered as statistically significant, using software ezANOVA ver. 0.98.

## RESULTS AND DISCUSSION

The methanol extract was subjected to column chromatography to furnish creamish coloured powder. The $^{1}H$ NMR spectrum explains the symmetry over the axis, proton in aromatic region and hydroxyl proton attached to aromatic group has been observed on same position. The peak at δ7.44 is due to benzene ring and δ10.58-10.75 corresponding to OH attached to benzene ring shows broad singlet. The δ159.59 shows the presence of carboxyl group. The δ110.71, 112.79, 136.86, 140.05, 148.58 shows the presence of benzene ring and CH carbon in benzene ring at δ108.11. The compound has molecular weight 302.19 evidenced by pseudo molecular ions at m/z300.8 for $[M-H]^-$ in the negative mode of LCMS.

$^{1}H$ NMR (400 MHz, DMSO-$d_6$):δ7.44(s, 2H, Benzene), 10.58-10.75(s, 4H, OH).

$^{13}C$ NMR: 159.59(C-Carboxyl), 148.58(C-benzene), 140.05(C-benzene), 136.86(C-benzene), 112.79(C-benzene), 110.71(C-benzene), 108.11(CH-benzene).

IR (KBr) cm-1 = 3435 (–OH), 2976 (Ar–CH2), 1606 (C=O), 1547 (C=C).

LCMS: m/z = 300.8 ($M^+$).

Based on spectral data the compound is characterized as EA (**Fig 1**).

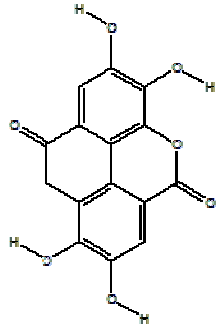

**Fig. 1:** Structure of EA.

The rat models induced with arthritis developed a chronic swelling in multiple joints with the influence of inflammatory cells, erosion of joint cartilage, bone destruction and remodeling. These inflammatory changes ultimately result in the complete destruction of joint integrity and functions in the affected animal (Carl, 1963). The EA at 250 µg/ml inhibited rat paw edema which is comparable with standard drug aspirin after 20 days. The results of which are shown in **Table 1**. The determination of rat paw swelling is apparently simple, sensitive and one of the quick procedures for evaluating the degree of inflammation and the therapeutic effects of drugs. The release of number of mediators

like cytokines, GM-CSF, interferons and PGDF occurs in chronic inflammation. These mediators are responsible for pain and destruction of bone, cartilage that can leads to severe disability (Eric and Lawrence, 1996). The changes in the body weights of the rats were observed during the course of the experimental period due to the incidence and severity of increased arthritis. The loss of the body weight during arthritic condition was also supported by earlier observations (Walz *et al.,* 1971), on alterations in the metabolic activities of diseased rats. The body weight in standard group remained same during 20 days of study. Parameters taken into consideration for observing inhibition of paw volume, body weight and movement of rats in formaldehyde induced arthritic model are shown in **Fig 2**.


**Fig. 2:** Parameters taken into consideration for observing movement of rats (A), inhibition of paw volume (B), body weight (C) and movement difficulty of limb (D) in formaldehyde induced arthritic model.

In, EA injected group of animals, body weight declined after 3 days of study and significant loss of weight was observed on 5$^{th}$ and 10$^{th}$ day. Aspirin treatment significantly restored loss in body weight. In control group of rats, no significant change in behaviour was observed. During 3$^{rd}$ and 5$^{th}$ day of study significant decrease in the movement of rats were noted both in EA treated as well as aspirin treated groups. However, on 20$^{th}$ day of study restoration of the normal movement was observed when compared with control group. The results of which are shown in **Table 2**. The bone modulation observed from 10$^{th}$ to 20$^{th}$ day was more effective in EA at a dose of 250 µg/ml when compared with aspirin. The inhibition of formaldehyde induced joint edema is one the most suitable methods to evaluate antiproliferative activity and screen anti arthritic agents. EA inhibited the proliferative global edematous response to formaldehyde in dose dependent manner, suggesting that it may alter certain aspects of the inflammatory response similar to that of aspirin with possible antiarthritic potential. X-ray photographs of effect of drugs on arthritis induced rats are shown in **Fig 3**. Proper bone remodulation in tibiotarsal joint is observed in standard drug treated animals **Fig 3B.** Whereas **Fig 3C** shows EA treated animal with some amount of regeneration of bone cells; which is very less with arthritic control animal **Fig 3A.**

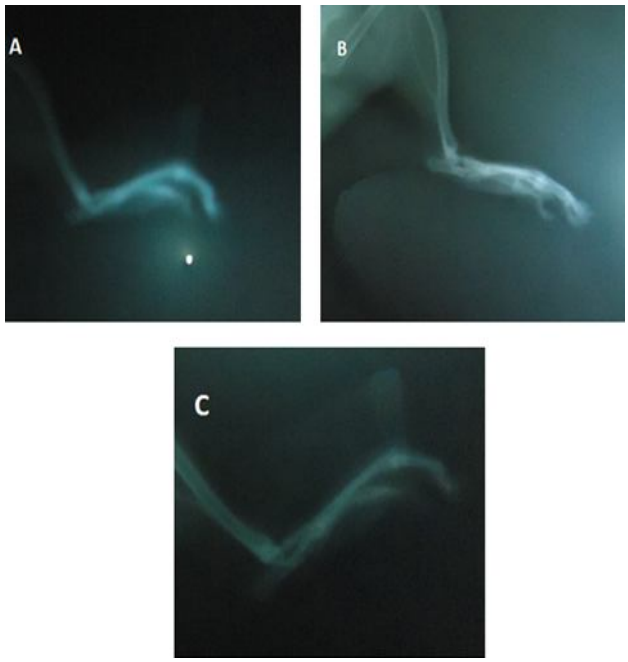


**Fig. 3:** Effect of drugs on arthritis induced rats (X-ray photographs). (A)Arthritic control; (B) Aspirin; (C) EA

EA at two different concentrations provided significant protection against denaturation of proteins, membrane stabilization and proteinase inhibition. Mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding (Madan *et al.,* 2011). The production of auto antigen in certain arthritic disease may be due to denaturation of protein, membrane lysis and proteinase action (Seema and Meena, 2011). Protective effect on heat and hypotonic saline-induced erythrocyte lysis is known to be a very good index of anti-inflammatory activity of any agent. Since the membrane of RBC is structurally similar to the lysosomal membrane, the effect of any substance on stabilization of RBC membrane may be extrapolated to the stabilization of lysosomal membrane (Brown and Mackey, 1968). The maximum percentage inhibition of protein denaturation, membrane stabilisation and proteinase inhibitory action were observed at 250 µg/ml as shown in **Table 3**. From the results, our study reveals that EA is capable of controlling the production of auto antigen and inhibits denaturation of protein, membrane lysis and proteinase action in rheumatic disease.

The similar claims are reported by Jiao et al, (2013) saying that topical formulations of pomegranate rind extracts and EA are promising therapies for contact dermatitis and can be applied as an alternative treatment for cutaneous disorders; after testing the compound on topical anti-inflammatory models. Ea has proved to be an effective anti-inflammatory agent in the carrageenan-induced rat paw edema, with a prolonged onset and duration of action (Corbett *et al.,* 2010).

**Table. 1:** Effect of EA on formaldehyde induced arthritis in rats showing clinical observations.

| Group | Paw edema volume | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0th day | | 3rd day | | 5th day | | 10th day | | 20th day | |
| | Mean ±SEM | % PEI | Mean ±SEM | % PEI | Mean ±SEM | % PEI | Mean ±SEM | % PEI | Mean ±SEM | % PEI |
| Control | 0.68 ±0.02 | ---- | 0.74 ±0.01 | ----- | 0.86 ±0.02 | ----- | 0.90 ±0.02 | ----- | 0.92 ±0.02 | ---- |
| Standard(Aspirin) | 0.42± 0.01** | 38.3 | 0.38± 0.02** | 48.7 | 0.36± 0.01** | 58.2 | 0.32± 0.01** | 64.5 | 0.24± 0.01** | 74.0 |
| EA (100 µg/ml) | 0.58± 0.12 | 14.8 | 0.53± 0.03 | 28.4 | 0.47± 0.05 | 45.4 | 0.42± 0.01** | 53.4 | 0.38± 0.01** | 58.7 |
| EA (250 µg/ml) | 0.43 ± 0.03 | 36.8 | 0.43± 0.03* | 41.9 | 0.42 ± 0.03* | 51.2 | 0.38 ± 0.01** | 57.8 | 0.35 ± 0.02** | 62 |

Values are expressed in mean ±SEM (n = 6), * P<0.05, ** P<0.01 are considered significant, compared to control .Abbreviations: PEI= percentage edema inhibition

**Table. 2:** Effect of EA on formaldehyde induced arthritis in rats showing health and behavioural observations.

| Group | Physical and behavioural changes | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0th day | | 3rd day | | 5th day | | 10th day | | 20th day | |
| | Body Weight (g) | Movement (s) | Body Weight (g) | Movement (s) | Body Weight (g) | Movement (s) | Body Weight (g) | Movement (s) | Body Weight (g) | Movement (s) |
| Control | 180 ±0.02 | 20.87 ± 0.30 | 160 ±0.01 | 30.07 ± 0.50 | 150 ±0.02 | 35.87 ± 0.22 | 100 ±0.02 | 50.67 ± 0.59 | 92 ±0.02 | 55.73 ± 0.62 |
| Standard | 170 ±0.01** | 20.33 ± 0.27** | 165 ±0.02** | 23.60 ± 0.18** | 161 ±0.01** | 27.67 ± 0.33** | 155 ±0.01** | 30.60 ± 0.23** | 168 ±0.01** | 21.73 ±0.84** |
| EA (100 µg/ml) | 170±0.03 | 20.53 ± 0.60 | 157±0.02 | 25.53 ± 0.17 | 150±0.02 | 30.00 ± 0.58 | 143±0.01* | 32.77 ± 0.30* | 163±0.03** | 28.73 ±0.19** |
| EA (250 µg/ml) | 170 ±0.04 | 20.33 ± 0.22 | 165 ±0.02* | 24.73 ± 0.24* | 158 ±0.01* | 29.87 ± 0.18* | 148 ±0.01** | 31.27 ± 0.34** | 165 ±0.01** | 25.27 ±0.30** |

Values are expressed in mean ±SEM (n = 6) * P<0.05, ** P<0.01 are considered significant compared to control

**Table. 3:** Effect of EA on in vitro antiarthritic test models.

| Treatment | Protein Denaturation (%) | Membrane Stabilization (%) | Proteinase inhibition (%) |
|---|---|---|---|
| EA(100 µg/ml) | 88.96 ± 2.38** | 87.61 ± 1.11** | 89.21 ± 0.66** |
| EA(250 µg/ml) | 62.12 ± 0.31** | 56.28 ± 0.13* | 68.51 ± 0.22* |
| Acetyl Salicylic acid (250 µg/ml) | 32.78 ± 0.15 | 27.24 ± 0.11 | 28.32 ± 0.17 |

Values are expressed in mean ±SEM, * P<0.05, ** P<0.01 are considered significant, compared to standard Acetyl Salicylic acid.

**Table. 4:** Molecular docking results with HIF-2α protein.

| Molecule | Binding energy | Docking energy | Intermol energy | H-bonds | Bonding |
|---|---|---|---|---|---|
| EA | -9.38 | -9.39 | -0.01 | 4 | HIF:A:ASN341:OD1:EA::DRG1:HAR<br>HIF:A:SER292:O:EA::DRG1:HAS<br>HIF:A:THR321:HG1:EA::DRG1:OAM<br>HIF:A:SER246:HG:EA::DRG1:OAQ |
| AP (std.) | 11.85 | 11.82 | 10.92 | 3 | ASP::DRG1:OAC: HIF:A:ARG260:HH21<br>ASP::DRG1:OAB: HIF:A:ARG260:HH22<br>ASP::DRG1:OAB: HIF:B:ARG366:HH12 |

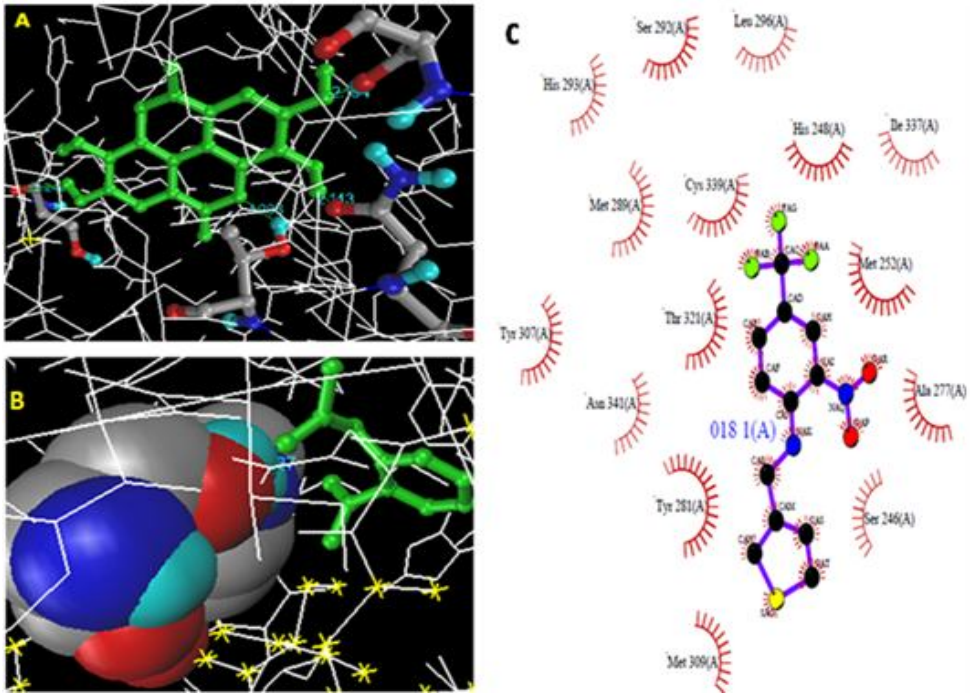


**Fig. 4:** (A) Orientation of EA in the active pocket of HIF-2α; (B) Enfolding of aspirin in active pocket; (C) Interacting amino acids as predicted from the ligplot.

Another research also revealed that EA took an important part in the anti-inflammatory, antiedematous and analgesic effects of an extract of Lafoensia pacari St. Hil. (Rogerio *et al.*, 2006). So, as the above details also correlate with our results obtained we state that EA can be used as potent anti-arthritic agent.

The molecular docking results of EA reveal that, our compound exhibited interactions with amino acids in the active pocket of HIF-2α (**Fig 4**). The *in silico* results obtained by EA with the formation of 4 hydrogen bonds and binding energy -9.38 are documented in **Table 4**. Practically, EA showed good docking energy and ligand efficiency compared to standard. The EA was completely enfolded in the entire active pocket of HIF-2α (**Fig 4a**) as compared to aspirin (**Fig 4b**). The topology of the active site of HIF-2α was similar in both EA and standard, which is lined by interacting amino acids as predicted from the ligplot (**Fig 4c**). The obtained data supports that EA inhibits the activity of HIF-2α protein thereby reducing remarkable anti-arthritic activity. Hence, EA has been proved to be one of the potent anti-arthritic agents.

## CONCLUSION

The study confirms the *in vivo*, *in vitro* and *in silico* anti-arthritic activities of EA. On the basis of the results obtained in this study we conclude, and propose that, the potent anti-arthritic effect of EA may be through maintenance of synovial membrane and vascular permeability, thereby inhibiting cytokines and leukotriene infiltration inhibition as evidenced in paw edema volume and protecting synovial membrane, destruction of cartilage and improving health status; and is possibly mediated through HIF-2α inhibition. However, experimental validation of the predicted compound in evaluating its clinical potentials is needed and the clinical trials are necessary to determine the toxicity of the active constituents, their side effects, circulating levels, pharmacokinetic properties and diffusion in different body sites.

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