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In vitro, in vivo and *in silico* antiarthritic studies of polyprenol from *Kirganelia reticulata Baill.*

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Abstract

Kirganelia reticulata is a medicinal shrub which has been valued for centuries in ayurvedic medicine. *In vitro, in vivo* and *in silico* antiarthritic activity of a phytoconstituent, polyprenol isolated from the leaves of *Kirganelia reticulata* was screened. Various *in vitro* models such as inhibition of protein denaturation, effect of membrane stabilization and proteinase inhibitory actions were studied. Polyprenol with two different concentrations (100µg/ml and 250µg/ml) was used and results were compared with acetyl salicylic acid. The *in vivo* antiarthritic activity of polyprenol was evaluated against formaldehyde induced arthritis in albino rats. 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 behavioral observations. The effect of polyprenol was compared with standard drug aspirin. HIF-2α promotes degradative pathways that foster osteoarthritis. The articular cartilage resides in hypoxic, avascular conditions within the synovial joint. Chondrocytes, cells of the articular cartilage are affected by various forms of stress. The biological role of this mediator is clearly understood thus offering new target for inhibiting incurable osteoarthritis. The inhibitory effect of polyprenol was studied using Autodock and efficiency was compared with standard drug in terms of interation and binding. The isolated compound polyprenol showed dose dependent activity which was found to be significant to that of the standard drugs and supports the traditional use of plant for rheumatism.

Keywords: autodock, formaldehyde, osteoarthritis, polyprenol

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Introduction

The free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation (Chatterjee 1997). Most clinically important medicine belongs to steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammation related diseases, eventhough these have potent activity and long term administration is required for treatments of chronic diseases. Furthermore, these drugs have various and severe adverse effects. Therefore, naturally originated agents with very little side effects are desirable to substitute chemical therapeutics. For a long time, plants have been an important source of natural products for human health. *Kirganelia reticulata* Baill. (Synonym- *Phyllanthus reticulatus* Poir.) is a large, often scandent, shrub of the family Euphorbiaceae. The plant grows throughout tropical areas of India, Bangladesh, China, and the Malay Islands (Kirtikar and Basu 1980). 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 bark showed significant antiviral (Renuka et al. 1998) and antiplasmodial activity (Omulokoli et al. 1997). The antibacterial potential of the aerial parts of this plant has been evaluated (Direkbusarakom et al. 1998). The bark is used to treat rheumatism, dysentery and venereal diseases (Yoganarasimhan 1996). The plant is used for a variety of ailments, including smallpox, syphilis, asthma, diarrhoea, bleeding from gums (Nandkarni 1982; The Wealth of India 2005). It is also claimed to have antidiabetic activity in tribal areas, which has been validated by Kumar et al. (2008). The antibacterial potential of the leaf extracts of this plant has been evaluated recently (Shruthi et al. 2010).

Plant Polyprenols are biologically active and have low toxicity. Polyprenol has been proved to be able to lower tumour cell resistance to chemotherapy *in vivo*. Polyprenol opens up

possibilities for targeted regulation of drug resistance. Ropren is the first commercial polyprenol drug approved as a pharmaceutical in the world. It is a natural product approved as a hepatoprotector pharmaceutical product in Russia. Based on its unique characteristics including efficacy, mechanism of action and undiscovered side effects, Ropren should become a leading premium drug in the multibillion global hepatoprotector market. Polyprenol named ficaprenol-11 is isolated from methanolic extract of *Leucaena leucocephala* (Chung and Yau 2010). The polyprenol has showed remarkable antibacterial activity, in assay conditions, on different microorganisms studied, and it has also presented to be having best antihelminthic activity (Shruthi et al. 2012).

Arthritis is an autoimmune disease in which there is joint inflammation, synovial proliferation and destruction of articular cartilage (Tripathi 2003). It is a common disease having peak incidence in 3rd to 4th decades of life with 3-5 times higher preponderance in female (Mohan 2000). Its prevalence depends upon age (Pandey 2010). Arthritis is highly inflammatory poly arthritis, often leading to joint destruction, deformity and loss of function which has a worldwide distribution with an estimated prevalence of 1 to 2% (Satish and Vivek 2011). However, side effects remain one of the problems for long-term use of medicines; thus, there is a need for antiarthritic drugs with less severe side effects. In addition, recent interest in alternative treatments for arthritis (Gaby 1999; Jacobs et al. 2001) has promoted their use in the US, but scientific evidence of antiarthritic efficacy is lacking. 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). Recent study showed that piascledine, mixture of nonsaponifiable components of avocado and soybean oils, exerts promising effect to relief inflammatory arthritis symptoms (Boileau et al. 2009); several groups also have studied small anti-inflammatory molecules derived from natural sources (Gaby 1999; Jacobs et al. 2001) with the aim of developing new treatments, but scientific evidence is still insufficient.

Animal models of arthritis are used to study pathogenesis of disease and to test potential new therapies for clinical use. The three most commonly used models for the testing of potential arthritic therapeutic agents are adjuvant-induced arthritis in rats, collagen-induced arthritis in rats and mice (Bendele 2001; Hegen et al. 2008). 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 cell populations, including B cells, monocytes/macrophages, T cells, endothelial cells, and fibroblasts, participate in the ongoing inflammatory process (Scott et al. 2010), suggesting the presence of multiple cellular targets for immunotherapy of arthritis. These autoimmune changes are receiving increased attention in drug discovery and development as the progress has been made in understanding immune and inflammatory processes (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, including type X collagen and the type II collagen–degrading protease MMP-13. 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 developed a disease progression model to provide an understanding of the relationship between target modulation and efficacy in the animal model.

The objective of this study was to investigate the antiarthritic effect and mechanisms of action of polyprenol 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 vitro*, *in vivo* 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 locally from Bhadra Wild Life Sanctuary, Karnataka (Southern India) in Dec 2011. The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number YLR429). 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 chloroform extract was used for isolation of polyprenol. A portion of the polyprenol was used for the *in vitro* and *in vivo* antiarthritic assays.

Isolation of polyprenol

The chloroform soluble fraction was fractionated by column chromatography (CC) over silica gel (60-120 mesh) using *n*-hexane and ethyl acetate mixtures of increasing polarities to give 50 fractions, collecting each 25 ml. Preparative thin layer chromatography (stationary phase- silica gel F_{254}, mobile phase - 30 % ethylacetate in hexane, thickness of plates-0.5 mm) of fractions 18-20 afforded compound polyprenol. The PP was characterized by subjecting to IR, NMR, MASS spectral analysis and TLC (ethylacetate:hexane; 3:7) with iodine vapor as the detector.

In vitro antiarthritic activities

Inhibition of protein denaturation

The assay was conducted as per the method followed by Vallabh et al. (2009). The reaction mixture consisted of 0.45 ml bovine serum albumin and 0.05 ml of polyprenol (100 and 250 µg/ml) in DMSO. The samples were incubated at 37°C for 20 min and then heated at 57°C for 3 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm, for control tests 0.05 ml distilled water was used instead of compound while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation (Snedecor and Cochran 1967) was calculated as follows:

$$Percent\ inhibition = 100 - \frac{(\text{OD of test} - \text{OD of product control})}{\text{OD of control}} * 100$$

The control represents 100% protein denaturation. The results were compared with acetyl salicylic acid (250 µg/ml) treated samples.

Effect on membrane stabilization

The assay was conducted as per the method followed by Vallabh et al. (2009). The reaction mixtures consisted of 2 ml hypotonic saline (0.25% NaCl), 1 ml 0.15 M phosphate buffer (pH 7.4) and 1 ml of polyprenol (100 and 250 µg/ml) in DMSO. 0.5 ml of 10% rat RBC in normal saline was added. For control tests, 1 ml of isotonic saline was used instead of test solution while product control tests lacked red blood cells. The mixtures were incubated at 56^{o}C for 30 mins. The tubes were cooled under running tap water for 20 mins, centrifuged and the absorbance of the supernatants read at 560 nm. Percent membrane stabilizing activity (Mizushima 1966) was calculated as follows:

$$Percent\ stabilization = 100 - \frac{(\text{OD of test - OD of product control})}{\text{OD of control}} * 100$$

The control represents 100% lysis. The results were compared with acetyl salicylic acid (250 µg/ml) treated samples.

Proteinase inhibitory action

The assay was conducted as per the method followed by Vallabh et al. (2009). The reaction mixtures contained 0.06 mg trypsin, 1.0 ml 25 mM tris-HCl buffer (pH 7.4) and 1 ml of polyprenol (100 and 250 µg/ml) in DMSO. The mixtures were incubated at 37^{o}C for 5 mins and then 1.0 ml of 0.8% (w/v) casein was added. The mixtures were incubated for additional 20 mins and 2.0 ml of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged and absorbance of the supernatant was read at 280 nm against buffer as blank. The percentage of proteinase inhibition (Brown and Mackey 1968) was calculated as follows:

$$Percent\ inhibition = 100 - \frac{(\text{OD of test - OD of product control})}{\text{OD of control}} * 100$$

The control represents 100% inhibition. The results were compared with acetyl salicylic acid (250 µg/ml) treated samples.

Evaluation of in vivo antiarthritic activity Animals

Studies were carried out using Wistar strain albino male rats (150-200 g), which were procured from ALN Rao Ayurvedic Medical College Koppa, Karnataka. They were housed under standard laboratory conditions (25 ± 20^{0}C) with dark light circle (14/10hr). Animals were allowed free access to standard pellet diet (Sai Durga Feeds, Bangalore) and water *ad libitum*. Food was withdrawn 2 hrs 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 toxicity study

This study was carried out to determine the therapeutic dose of extract (Khare et al. 2000). The animals were divided into 6 groups containing 8 animals per each group. Experiment was conducted for polyprenol at different concentrations by stair case method (Ghosh 1984). 200 mg/kg b.w was taken as the therapeutic oral dose for the compound.

Formaldehyde induced arthritis

Male Wistar rats weighing between 150-200 g were randomly selected. They were grouped into 6 animals each into 4 groups. On the 0th day, the basal paw volume of left hind paw of each animal will measured using Plethysmometer. On day 1 and day 3, they were injected into the sub-plantar region of the left hind paw with 0.1 ml of 2 % v/v formaldehyde in normal saline. Dosing with standard drug, aspirin and extracts were started on same day and continued for 20 days. Group I served as - Arthritis control, Group II - Aspirin treated, Group III & IV - 100µg/ml and 250µg/ml respectively. Paw volume of injected paw was measured daily. The health status parameter included body weight and behavioral 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 a certain distance. The percentage inhibition of edema in the test drug treated group was calculated by using the formula (Eduardo and Tania 2004):

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

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

Molecular docking studies

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 polyprenol 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 were generated with AutoGrid. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters (Vidya et al. 2011).

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 (Nitha et al. 2007). Values of $P < 0.05$ were considered as statistically significant, using software ezANOVA ver. 0.98.

Results and Discussion

The chloroform extract was subjected to column chromatography to furnish orangish red coloured waxy mass of polyprenol (Fig 1).

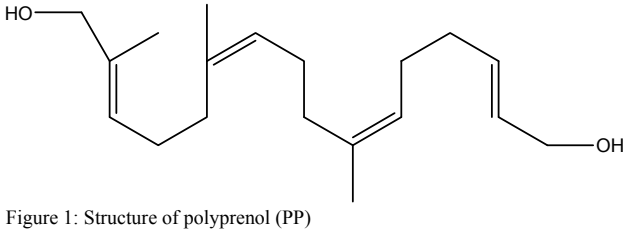

Figure 1: Structure of polyprenol (PP)

From the experimental evaluations such as molecular weight, ^{1}H-NMR, ^{13}C-NMR, IR spectral data the compound was identified to

be polyprenol. Polyprenol at two different concentrations provided significant protection against denaturation of proteins, membrane stabilisation 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). Neutrophils are known to be a rich source of proteinases which carry in their lysosomal granules many neutral serine proteinases. It was previously reported that leucocyte proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors (Vallabh et al. 2009). The maximum percentage inhibition of protein denaturation, membrane stabilisation and proteinase inhibitory action were observed at 250 µg/ml as shown in table 1. From the results, our study reveals that polyprenol is capable of controlling the production of auto antigen and inhibits denaturation of protein, membrane lysis and proteinase action in rheumatic disease. Obtained data states that polyprenol can be used as potent anti-arthritic agent.

Table 1: Effect of polyprenol on *in vitro* antiarthritic test models.

Treatment	Protein Denaturation (%)	Membrane Stabilitization (%)	Proteinase inhibition (%)
Polyprenol	61.98 ± 0.20	62.75 ± 0.15	60.21 ± 0.23
Polyprenol	52.72 ± 0.21**	49.29 ± 0.23*	48.51 ± 0.23*
Acetyl Salicylic acid	32.78 ± 0.15	27.24 ± 0.11	28.32 ± 0.17

The values are the mean of triplicates ± S.E. * P<0.05 are considered significant compared to standard. Polyprenol (100 µg/ml); Polyprenol (250 µg/ml); Acetyl Salicylic acid (250 µg/ml)

Figure 2: Parameters taken into consideration for observing inhibition of paw volume, body weight and movement of rats in formaldehyde induced arthritic model.

In formaldehyde induced arthritis model, rats 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 polyprenol 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 2. It has close similarities to human rheumatoid disease (Singh and Majumdar 1996). 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 chronic inflammation involves the release of number of mediators like cytokines, GM-CSF, interferons

Table 2: Effect of polyprenol 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	0.42 ±0.01	38.3	0.38 ±0.02	48.7	0.36 ±0.01	56.7	0.32 ±0.01	64.5	0.24 ±0.01	74.0
Polyprenol	0.56 ±0.02	17.7	0.50 ±0.02	22.4	0.44 ±0.02	38.9	0.42 ±0.01	43.4	0.39 ±0.01	57.7*
Polyprenol	0.50 ±0.02	26.5	0.48 ±0.02	38.2	0.45 ±0.02	47.7*	0.42 ±0.01	58.2**	0.40±0.01	66.6*

Values are expressed in mean ±SEM, n = 6, * P<0.05 are considered significant compared to standard. Polyprenol (100 µg/ml); Polyprenol (250 µg/ml)

Table 3: Effect of Polyprenol 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	
	BW (gms)	M (secs)	BW (gms)	M (secs)	BW (gms)	M (secs)	BW (gms)	M (secs)	BW (gms)	M (secs)
Control	180 ±0.02	20	160 ±0.01	30	150 ±0.02	35	100 ±0.02	50	92 ±0.02	55
Standard	170 ±0.01	20	160 ±0.02	25	140 ±0.01	30	130 ±0.01	30	175 ±0.01	25
Polyprenol	160 ±0.02	20	150 ±0.02	30	140 ±0.02	30	120 ±0.01	40	100 ±0.01	40
Polyprenol*	200 ±0.02	20	180 ±0.02	25	160 ±0.02	25	165 ±0.01	30	195 ±0.01*	25

Values are expressed in mean ±SEM, n = 6, * P<0.05 are considered significant compared to standard. Polyprenol (100 µg/ml); Polyprenol* (250 µg/ml); BW-Body weight; M-Movement

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

Molecule	Binding energy	Docking energy	Internal energy	H-bonds	Bonding
PP	125.85	126.74	122.43	2	PP::DRG1:OAT:HIF:A:ARG260:HH12 PP::DRG1:OAT: HIF:A:ARG260:HH22
AP (std.)	11.85	11.82	10.92	3	ASP::DRG1:OAC: HIF:A:ARG260:HH21 ASP::DRG1:OAB: HIF:A:ARG260:HH22 ASP::DRG1:OAB: HIF:B:ARG366:HH12

and PGDF. These mediators are responsible for pain and destruction of bone, cartilage that can leads to severe disability (Eric and Lawrence 1996). As the incidence and severity of arthritis increased, the changes in the body weights of the rats also occurred during the course of the experimental period. 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. In, polyprenol injected group of animals, body weight declined after 3 days of study and significant loss of weight was observed on 5th and 10th day. Aspirin treatment significantly restored loss in body weight. In non-treated group of rats, no significant change in behaviour was observed. During 3rd and 5th day of study significant decrease in the movement of rats were noted both in polyprenol treated as well as aspirin treated groups. However, on 20th day of study restoration of the normal movement was observed when compared with non treated groups. The results of which are shown in table 3.

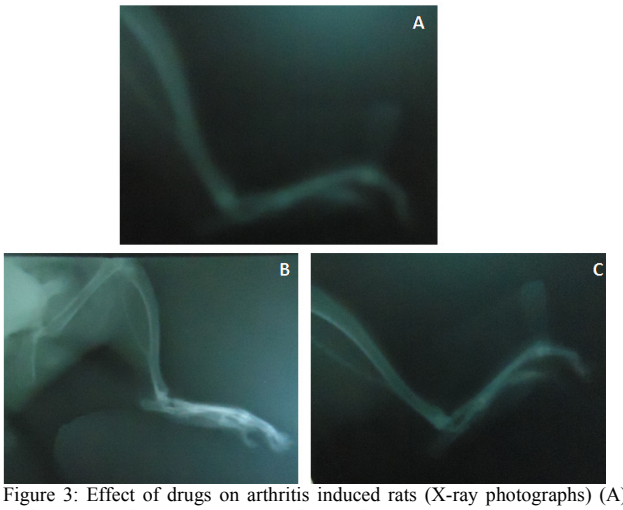

Figure 3: Effect of drugs on arthritis induced rats (X-ray photographs) (A) Arthritic control; (B) Aspirin; (C) Polyprenol

Two different doses of polyprenol exhibited anti arthritic activity which was maintained until the experiment was terminated on day 20. The bone modulation observed from 10th to 20th day was more effective in polyprenol 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. The injection of formaldehyde into rat paw produced localized inflammation and pain which is biphasic in nature i.e as early neurogenic component followed by a later tissue mediated response (Nair et al. 2011; Owoyele et al. 2011). Polyprenol 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. The docking of polyprenol with HIF-2α reveals that, our compound exhibited interactions with one or the other amino acids in the active pocket (Fig 4). The docking results for polyprenol and aspirin are documented in table 4. Practically, polyprenol showed good docking energy and ligand efficiency compared to standard. The polyprenol 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 polyprenol and standard, which is lined by interacting amino acids as predicted from the ligplot (Fig 4c). By *in silico* analysis, it seems that polyprenol is promoting the remarkable anti arthritic activity through the inhibition of HIF-2α protein. Hence, polyprenol has been proved to be one of the potent anti arthritic agents.

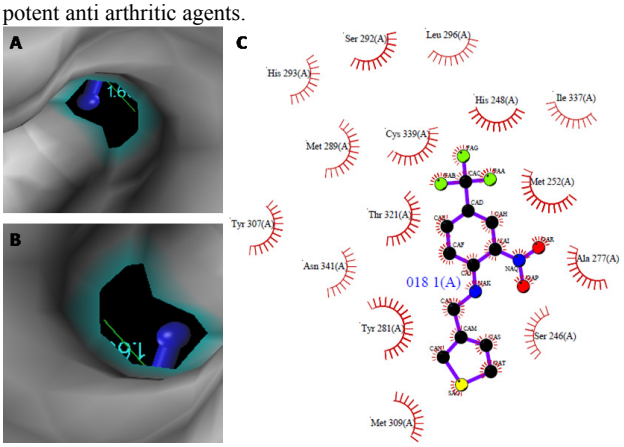

Figure 4: (A) Orientation of polyprenol in the active pocket of HIF-2α; (B) Enfolding of aspirin in active pocket; (C) Interacting amino acids as predicted from the ligplot.

Conclusion

On the basis of the results obtained in this study we conclude, and propose that possibly, the potent anti arthritic effect of polyprenol 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. We would also like to conclude that significant and promising anti arthritic activity of polyprenol is possibly mediated through HIF-2α inhibition. The study confirms the *in vitro, in vivo* and *in silico* antiarthritic activites of polyprenol in dose dependent manner. However, experimental validation of the predicted compound in evaluating its biosafety and clinical potentials is needed.

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