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International Journal of Current Research
Vol. 9, Issue, 11, pp.60234-60238, November, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

TRITERPENOID LUPENONE CHECKS ROS MEDIATED INFLAMMATION IN MURINE PAW EDEMA BY BLOCKING SEQUESTRATION OF NEUTROPHILS

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ARTICLE INFO

Article History:
Received 18th August, 2017
Received in revised form
14th September, 2017
Accepted 06th October, 2017
Published online 30th November, 2017

Key words:
Lupenone,
ROS,
Inflammation.

ABSTRACT

Inflammation underlies a wide variety of physiological and pathological processes and pathological aspects of many types of inflammation are well appreciated. The classic molecular instigators of inflammation is ROS and which trigger the recruitment of leukocytes and causes prognosis of the disease. Current investigation postulated the anti-inflammatory efficacy of a triterpenoid, Lupeonone which exhibited free radicals scavenging activity resulting in reduced oxidative stress. Since inflammatory microenvironment has increased ROS and ability of the lupeonone to counteract this was evaluated in carrageenan induced paw edemas in mice. The lupeonone effectively reduced the inflamed tissue. Down the line, molecular cause of inflammation such as sequestration of neutrophils in edematic paw was drastically reduced and restoring of normal pathology. Overall the current study explore role of herbal based drug Lupenone with is antiflammatory activity with molecular evidence which could be positive hope for the treatment inflammatory disorders.

Citation: Rakesh Hanumaiah, V.Krishna, *Dr. B. T. Prabhakar, 2017. "Triterpenoid lupeonone checks ROS mediated inflammation in murine paw edema by blocking sequestration of neutrophils", *International Journal of Current Research*, 9, (11), 60234-60238.

INTRODUCTION

Inflammation is a response of vascularised tissue to sub-lethal injury (Walsh DA and Pearson CI, 2001). Inflammation can be classified according to duration as either acute or chronic. In acute inflammation, changes in small blood vessels result in fluid and granulocytic cells such as neutrophils accumulate at the site of injury. This reaction may trigger a systemic response such as fever, leucocytosis, protein catabolism, and altered hepatic synthesis of plasma proteins such as C-reactive protein. Chronic inflammation is characterized by tissue infiltration by neutrophils, macrophages and lymphocytes. Inflammation may be considered a homeostatic response designed to destroy or inactivate invading pathogens, remove waste and debris, and permit restoration of normal function, either through resolution or repair. Many of the diseases are directly associated inflammatory conditions such Rheumatoid Arthritis (RA) and cancer. As per the etiopathology is considered oxidative stress is due to ROS, and among them several elements like hydroxyl radical (˙OH), superoxide anion (O2-), hydrogen dioxide (H_2O_2), nitric exerts outrageous effect and there by acting on tissue damage (Xu *et al*., 2012). As an outcome sequestered polymorphonuclear immune cells develops the activating environment for the over release of proinflammatory cytokines. Understanding such molecular causes of the inflammation in pathogenesis and directly targeting the disease is more important in drug development process. Plants have been used for treating various diseases of human beings and animals since time immemorial. More than 50% of all modern drugs in clinical use are of natural product origins, many of which have the ability to control cancer cells. There is a growing interest in natural triterpenoids, also known as phytosterols, due to their wide spectrum of biological activities (Ovesna *et al.,* 2003). During the last decade, there has been an unprecedented escalation of interest in triterpenes. Lupenone is one of the triterpenoid which is a naturally occurring pentacyclic lupane type triterpenoids and is widely distributed throughout the plant kingdom (Ovesna *et al*., 2003). Few reports are available for the pharmacological properties of Lupenone and which exhibits a variety of biological and medicinal properties such as anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic and anti cancer activities (Ovesna *et al*., 2003; Roy *et al*., 2001; Libby and Schönbeck, 2001). Lupenone has been studied for its inhibitory effects on inflammation under *in vitro* conditions and in animal models of inflammation. Recently (Martelanc *et al.,* 2007), showed that the Lupenone on topical application to the mice ears decreased the inflammation induced by 12-O-tetradecanoyl-phorbol acetate (TPA) in an ear mouse model. The anti-inflammatory potential of Lupenone was assessed from the observation of reduced prostaglandin E2 (PGE2). (Geetha *et al.,* 1999),

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reported for the first time about the anti-inflammatory effect of Lupenone in the mouse model of arthritis. A similar report by (Latha *et al.,* 2001), showed that increased activities of many enzymes associated with decreased collagen in arthritic animals which were significantly altered by Lupenone feeding. The anti-inflammatory effect of Lupenone was observed to be equal to dexamethasone, a well known anti-inflammatory agent (Latha *et al.,* 2001), But no study infers the molecular mechanism underlying the pharmacological effect of lupeonone. In the current study we have given experimental evidence of role of herbal based drug Lupenone with its antiflammatory activity particularly targeting ROS mediated neutrophil sequestration which could be used as future medicine.

MATERIALS

Lupenone, DPPH, nitroblue tetrazolium (NBT), NADH (Nicotinamide adenine dinucleotide hydrogen(reduced), phenazine methosulphate, H_2O_2, Sodium , DMSO, Complete freund's adjuvant, Ovalbumin were purchased from Sigma Aldrich USA. Carrageenan and all other chemicals used were molecular Biology grade from Himedia, Mumbai. The images were taken EVOS, Phase contrast microscope , Thermofisher. USA.

METHODS

Antioxidant assays

Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The DPPH scavenging capability of Lupenone was analyzed as followed earlier (Goudarshivanavar *et al*., 2014), In brief different concentration of Lupenone (1-200 µg/mL) with 2.5 mL of 0.2 mM DPPH methanol solution was incubated for 30 minutes in the dark by treating ascorbic acid as standard. Optical density of the samples were measured in dark at 517 nm with a biospectrophotmoeter (Eppendorf, Germany).

Superoxide radical scavenging activity

The assay method was followed as reported earlier (Zabiulla *et al*., 2017). In brief a Tris-HCl buffer (19 mM, pH 8.0) with NBT (108µM), NADH (166µM) solution and Lupenone (1-200 µg /ml) were mixed with phenazine methosulphate (2.7µM) in potassium phosphate buffer (pH 7.4) and incubated for 5 minutes at 25^{o}C and optical density was measured at 560 nm.

Hydrogen peroxide radical scavenging activity

The assay was followed as described antecedently (Ruch *et al*., 1989). The reaction mixture was prepared from of 0.6 ml 2 mM H_2O_2 solution in 0.05 M phosphate buffer (pH 7.4) with 0.1ml of Lupenone concentration ranging from (1-200µg/ml) and final volume was made up to 1 ml and reaction mixture was incubated for 10 minutes and absorbance was measured at 230nm.

Hydroxy radical scavenging assay

Hydroxy Radical Scavenging assay was carried out as reported previously (Shazia Tantary *et al*., 2017). The reaction mixture was prepared with 25mM deoxyribose, 10 mM ferric chloride, 100 mM ascorbic acid, 2.8 mM H_2O_2 in KH_2PO_4 (pH7.4) and Lupenone(1-200 µg/ml) and incubation for an hour. Further 1ml of 1% thiobarbituric and 3% trichloroacetic acid was added and heated for 20 minutes at 100$^{o\ C,}$ and observance was recorded spectrophotometrically at 532nm.

Human erythrocyte membrane stabilization assay

The HRBC membrane stabilization has been used as a method to study the antiinflammatory activity of Lupeonone as reported earlier (Shazia Tantary *et al*., 2017). Briefly blood was collected from healthy human volunteer who had not taken any NSAIDS for two weeks prior to the experiment and mixed with equal volume of sterilized alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.55% sodium chloride). The blood was centrifuged at 3000 rpm and packed cells were washed with isosaline (0.9% w/v NaCl) and a 10% suspension of blood was made with isosaline. Various concentrations of the compounds Lupenone were prepared (50, 100, 150, 200 and 250 µg/ml) using DMSO and to each concentration 1 ml phosphate buffer, 2 ml hyposaline, and 0.5 ml HRBC suspension were added and the mixture incubated at 37° C for 30 min and centrifuged at 3000 rpm for 20 min and the hemoglobin content in the supernatant solution was estimated spectrophotometrically at 560 nm. The experiment used Diclofenac (50, 100, 150, 200 and 250 µg/ml) was used as the reference standard. The hemolysis percentage was calculated by assuming the hemolysis produced by the control group as 100%. The percentage of HRBC membrane stabilization or protection was calculated using the formula, percent protection=100 − ((OD of compound treated sample/OD of control) ×100).

Animals and ethics

The animal models used for the study includes healthy Swiss Albino male mice weighing 25±2.0g and Swiss albino male wistar rat weighing 150±5.0g. All the animals were grouped separately and housed in polyacrylic cages and maintained under standard conditions (25±2°C) with 12±1h dark/light cycle with water ad libitum and standard food pellets procured from Krish Scientist's Shopee, Bengaluru, India. All procedures for animal experiments were carried out in accordance with the CPCSEA guidelines and approved by the Institutional Animal Ethics Committee, National College of Pharmacy, Shimoga, India, in accordance with the CPCSEA guidelines for laboratory animal facility (NCP/IAEC/ CL/101/05/2012-13).

Determination of the Lethal Dose 50 (LD_{50}) of the lupeonone

For the determination of LD_{50} value of Lupeonone, 'staircase' method (Shetty Akhila and Alwar, 2007) was employed. Healthy Swiss albino male mice weighing 25±5g were used and were divided into 12 groups of six animals in group each for all the extract. The test compound were dissolved in DMSO (E-Merk, India) and administered i.p., in increasing levels of 1000, 2000, 3000, 4000, and 5000 mg/Kg body weight of the animals. The animals were then observed continuously for 3h for general behavioral, neurological, and autonomic profiles and then every 30 minutes for the next 3h finally the animals die after 24h (Prabhakar *et al*., 2006). The maximum non lethal and the minimum lethal doses were thus

determined. One tenth and one twentieth of this LD_{50} dose was selected as the therapeutic dose for the evaluation of *in vivo* anti angiogenic activity of the compounds.

Carrageenan-induced Paw Edema in Mice

Male Swiss albino mice were selected for the study of anti-inflammatory effect of luepnone. Against carrageenan induced paw edema (Morris, 2003). Animals were divided into four groups, each group consist of six animals (n=6)

Group I: Normal animals
Group II: Control (Carrageenan)
Group III: Positive Control (Indomethacin)
Group IV: Animal treated with compound Lupeonone.

Prior to experiment animals from group II were injected with saline (5 ml/kg), group III animals were injected with positive control (10 mg/kg) group IV animals were injected with test Lupenone (10 and 20 mg/kg) and animals were left in respective cage for 2 h. After 2 h animals from control, positive control, and test group animals were injected with 100µl of 2% carrageenan at the right paw. Right paw of all the experimental animals were observed with different time interval (2h, 3h and 5h) and volume of the paw were measured using digital plethysmometer.

Histopathology

Animals were sacrificed and hind paw was excised anf frozen in formaldehyde and further processed for paraffin block. A 3mm thick section was prepared and stained with stained with haematoxylin and eosin (H&E) and mounted coverslip with DPX sealing. Images were documented using EVOS Bio-imaging system) (Thirusangu *et al*., 2017)

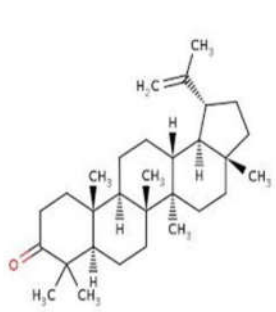
Statistical analysis

Values were expressed as mean ± standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by 2-tailed 13 Student's t-test. MS excel 8.1 version software was used for data analysis and Statistical significant values were expressed as *p < 0.05 and **p < 0.01.

RESULTS

Lupeone encounters reactive oxygen species by scavenging capability

Reminding the ROS plays the pivotal role in the numerous disease including inflamative diseases. The effective scavenging activity of the of Lupenone against ROS shows the proved its potential antioxidant property. Experiments with DPPH assay, free radicals scavenging activity was resulted in IC_{50} value of 9.34±1.52. Similarly the Lupenone was investigated against reactive oxygen species superoxide (O_2^{-}), hydrogen peroxide (H_20_2),hydroxyl radical ($^{\cdot}OH$), and results inferred that respective IC_{50} values 12.89± 0.84, 13.11± 0.76, 15.34± 1.44µg/ml respectively (FIG-2). Postulating the result of Lupenone against free radicals, its been concluded that it has potent antioxidant nature and which could be further tested for ROS mediated inflammation model for its efficacy.



Properties	Description
Empirical Formula	$C_{30}H_{48}O$
IUPAC Name	(1R,3aR,5aR,5bR,7aR,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-2,3,4,5,6,7,7a,10,11,11b,12,13,13a,13b-tetradecahydro-1H-cyclopenta[a]chrysen-9-one
Molecular weight	424.713 g/mol
Solubility	DMSO

Fig.1: Structure of Lupenone and its properties

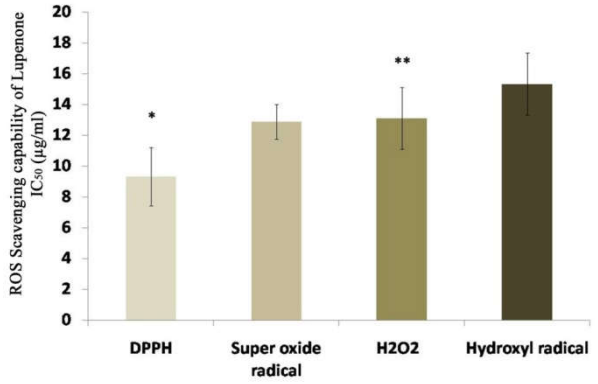


Fig. 2. Lupeone scavenges free radicals: The IC_{50} Value of the antioxidant Lupenone was determined by various *in-vitro* free radical & ROS scavenging assays. IC_{50}values of Lupenone against DPPH, superoxide, hydrogen peroxide radical and hydroxyl radical was represented in graph.

Table 1. Erythrocyte membrane protection by Lupenone

Concentration(mg/ml)	Diclofenac	Lupenone
1mg/ml	53.38 ± 0.32	36.87 ± 0.12
5mg/ml	59.56 ± 0.54	44.67 ± 0.08
10mg/ml	73.23 ± 0.37	50.11 ± 0.83
15mg/ml	82.32 ± 0.90	71.55 ± 0.69
20mg/ml	93.12 ± 0.44	84.33 ± 0.20

Statistical analysis: Data presented as means ± S.D of three consecutive readings and evaluated by ANOVA.

Lupenone protects membrane stabilization

The protection of human blood erythrocyte membrane of Lupenone against lysis evoked by hypotonic solution with comparing the standard diclofenac. At the 20 mg/ml concentration the Lupenone showed 84.33 ± 0.20 % RBC membrane stabilization by protecting against haemolysis which is very much significant protection near to the standard drug diclofenac showed 93.12 ± 0.44. The resulted are tabulated in Table 1.

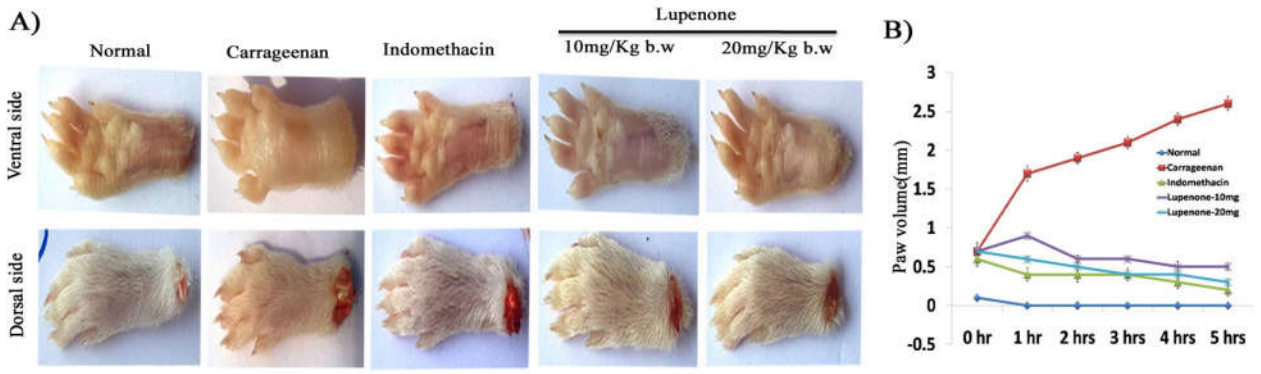


Fig. 3. Anti-inflammatory effect of Lupenone on paw edema in swiss albino mice : Mice were pretreated with Lupenone (10 mg and 20mg/kg b.w.) before carraggenan induction, Normal group received saline. The effect of Lupenone was evaluated by measuring (A) paw redness soft tissue swelling, (B) Paw volume evaluated by vernier caliper

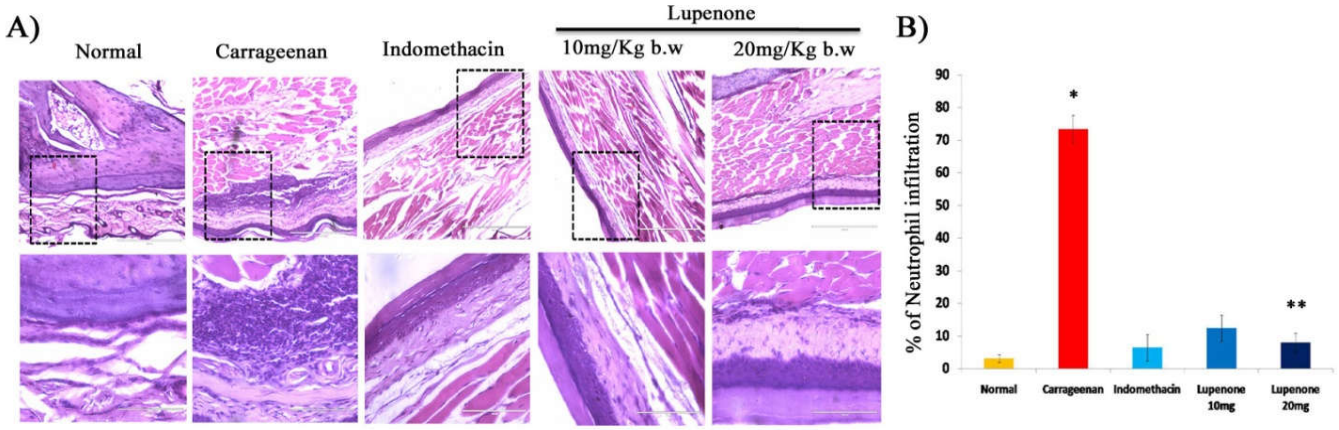


Fig. 4. Lupeone inhibits neutrophil sequestration: A) Histophatological studies of lupeonone treated paw edema tissue showing Neutrophil infiltration. B) Percentage of Neutrophil infiltration

Lupeone downs inflamed paw edema

The animals which received 5000 mg/Kg body weight of Lupenone were immediately died on the other hand animals which received 4000 mg/Kg body weight of lupeonone died after 24h. Abnormal aggressive behavior was observed in the animals which received 3000 mg/Kg body weight and died after 24h. Besides the animals with 2000 mg/Kg body weight of Lupenone died after 24h and the animals which received with same concentration showed abnormal behavior with less food and water consumption. Moreover animals with 200 mg/Kg body weight of Lupenone found to be normal and hence 1/10th of this dose i.e. 10 and 20 mg body weight were selected as therapeutic dose for further analysis. Lupenone on acute phase inflammation was studied in carragennan induced paw edema. Mice were pretreated with Lupenone at of dose 10 & 20mg/kbw and observed for five hours after carrageenan induction. The paw swelling volume was analyzed and measured with vernier caliper. Lupenone treated group animals showed a noticeable inhibition activity of inflammation edema comparing untreated group (Fig. 3) almost on par with Indomethacin treated results.

Lupeonone checks neutrophil sequestration

The molecular mechanism underlying the inflammatory edema is due to accumulation of neutophils. Since Lupenone efficiently blocks the edema, histological analysis was done to check the sequestration of neutrophils, as expected dose dependent inhibition of neutrophils sequestration was evident which is equally comparable with Indomethacin treated results (Fig.4).

DISCUSSION

Natural compounds currently used in medicine exhibit a very wide chemical diversity and together with their analogues and several other natural products, they demonstrate the importance of compounds from natural sources in modern drug discovery efforts. Sample sources and molecular mechanisms are highly important in the development of novel, clinically useful antinflmatory agents (Vivek *et al*., 2010). In the current study efforts has been made to investigate the novel plant product Lupenone and its antinflammtory role with mechanistic approach. Inflammation, has two stages, acute and chronic. Acute inflammation, the initial stage of inflammation, represents innate immunity; it is mediated through the activation of the immune system, lasts for a short period and generally is regarded as therapeutic inflammation. If the inflammation persists for a long period of time, however, the second stage, chronic inflammation, sets in (Xu *et al*., 2007). Chronic inflammation has been linked with most chronic illnesses which include many diseases. Oxidative stress through the overproduction of ROS initiates or increases the pathology in number of diseases, grievous in inflammation. Modern research seeks a novel herbal based phytotherapeutics to target most of the diseases and get the better of side effects from synthetic drugs. Taking these aspects into consideration Lupenone was teaseted to ROS scavaneging activity which scavenged free radicals as verified by antioxidant assays

(Fig.1). The $O_2^{\cdot-}$ plays predominantly role in inflammation, where $O_2^{\cdot-}$ is responsible for the formation of aggressive compounds like peroxynitrite ($ONOO^-$) and hydroxyl radical ($HO^{\cdot}$) (Lin *et al*., 2007, Xu *et al*., 2012). Lupenone showed potent neutralization with DPPH and significant scavenge with O2 $^{\cdot-}$. Similarly Lupenone treated on others reactive species H_2O_2, $^{\cdot}OH$ and nitric oxide, exerts efficiency inactivation of reactivity (Fig.1). This potent nature as antioxidant promised for further studies on acute and chronic inflammation. Scientific studies have shown triterpenoids to be potential anti-inflammatory and anticancer agents and no clear evidence of Lupeone based anti-inflammatory activity except few reports (Aggarwal *et al*., 2009). In the present studies we have studied anti-inflammation activity by the membrane stabilizing capacity of erythrocytes (Table 1) and Lupenone checked acute inflammation model paw edema (Fig.3) wisely. Accumulation of neutrophil at the site of inflammation is molecular event and increases the pathogenesis condition is positive marker and reduction of the same is important hallmark of therapeutic agent. Treating with Lupenone efficiently reduced the nuclear sequestration process in dose dependent manner as evidenced by histopathology. Overall the outcome of research, Lupenone would be a effectual phytochemical triterpenoid for the targeting oxidative stress mediated inflammation with positive drug ouput in near future.

Conflict of interest

Authors exhibits no conflict of interest.

Acknowledgment

Dr.B.T. Prabhakar thankful for the research support given by UGC-MRP (F.No.41-507/2012), Rakesh Hanumaiah acknowledges the Rajiv Gandhi National fellowship(RGNF)(No. F1-17.1/2016-17/RGNF-2015-17-SC-KAR-19249 dated: 12/01/2016) for the financial aid and support.

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