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Original Article

PHYTOCHEMICAL COMPOSITION, ANTIOXIDANT AND *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY OF ETHANOL EXTRACT OF *RUTA GRAVEOLENS* L. LEAVES

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ABSTRACT

Objective: The uses of medicinal herbs in prevention and treatment of numerous diseases attracting attention of scientists worldwide. It is necessary to bring vital healthcare to the people and the better substitute for various infectious diseases. *Ruta graveolens* is traditionally used as a medicinal plant as well as a flavoring agent in food. In the present study *R. graveolens* leaves extracts were evaluated for phytochemical and anti-inflammatory activity.

Methods: The phytochemical, antioxidant (DPPH), ferrous reducing antioxidant property, Anti-lipid peroxidation and superoxide scavenging activity of extracts were assayed by spectrophotometerically. Anti-inflammatory activities; Human plural fluid phospholipase A_2 (HPF-PLA_2) inhibition by egg yolk plate method and lipoxigenase (LOX) inhibition were assayed by *IN VITRO* method.

Results: The phenolics were significantly higher than the flavonoids, saponins and alkaloids contents in all the extracts. Among extracts of *R. graveolens*, ethanol and water extracts exhibits more phytochemicals. The ethanol extract had the highest antioxidant activity followed by the aqueous extract. The ethanol extract shows highest DPPH free radical scavenging, ferric reducing, superoxide scavenging and anti-lipid peroxidation activity and IC_{50} was 3.27±0.03, 3.58±0.05, 3.87±0.04 and 4.77±0.04 µg/µl respectively at dose-dependent manner. Further ethanol and water extracts were subjected for LOX inhibition in the concentration dependent manner and IC_{50} values are 4.25±0.05, 5.15±0.05 µg/µl for 5-LOX and 4.15±0.04, 4.66±0.05 µg/µl for 15-LOX respectively.

Conclusion: The result shows *R. graveolens* has strong antioxidant property and anti-inflammatory activity. It suggests that the strong correlation between antioxidant activity and the phytochemical contents of the extracts.

Keywords: HPF, Human Pleural Fluid, Anti-inflammatory, $sPLA_2$, Secretory Phospholipase A_2, HPF-PLA_2, Human Pleural Fluid-PLA_2. LOX Lipoxigenase, Lipid peroxidation, Anti-inflammation.

INTRODUCTION

Plants are the local heritage with global importance. World is endowed with a rich wealth of medicinal plants. Plant extracts have been the source of remedy for various clinical abnormalities from the time immemorial. Over three quarters of the world population relies mainly on plant-based medicines for the primary health care [1]. Roughly more than 30% of the plant species have been used for therapeutic purposes and about 25% of all prescribed medicines using today are substances derived from plants. Many of these indigenous medicinal plants are used as spices and food plants [2]. They are sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes [3]. The medicinal properties of herbal extracts are attributed to the presence of diverse classes of bio-active molecules, many of which have attained the level of drugs [4]. Phytochemicals are employed for the treatment of hypertension, cardiovascular disorders, cancer, neuronal disorders, inflammation, diabetes, malaria, influenza, arthritis, and severe pain [5].

Inflammation is a localized reaction that produces redness, warmth, swelling, and pain result in infection, irritation or injury. It contributes to all disease process including immunity, vascular pathology and leads to several undesirable consequences such as systemic shock, circulatory collapse and local tissue injury in many organs [6, 7]. Inflammation is mainly mediated by secretory phospholipase A_2 ($sPLA_2$). Several snake venom PLA_2 enzymes are responsible for local tissue damage such as edema and hemorrhage. Elevated level of $sPLA_2$ enzymes are detected in many inflammatory disorders [8, 9] and play a key role by releasing arachidonic acid and lysophospholipid, which are rate-limiting precursors for the production of pro-inflammatory lipid mediators. Cyclooxigenase-1/2 (COX-1/2) and lipoxigenase (LOX) catalyze arachidonic acid into pro-inflammatory mediators such as prostoglandins, thrombaxanes, and leukotriens respectively. Lysophospholipid is further converted into platelet activation factor (PAF) by acetyltransferase [10]. Further, Phospholipase A_2 (PLA_2) plays a crucial role in a number of diverse cellular as well as chemical and metabolic injuries. Apart, they involved in phospholipid digestion and metabolism, host defense, signal transduction and provide precursors for eicosanoid generation.

Present anti-inflammatory therapies include the non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit either Cyclo-oxygenase-1/2 (COX-1/2) or Lipoxygenase (LOX) catalysis have severe side effects such as intestinal ulceration, bleeding and cardiovascular complications [11]. In addition to these problems, COX-1/2 or LOX inhibitors cannot regulate the production of leukotrienes or PAF that continue to cause inflammation [12]. It appears rational that effective inhibitors of $sPLA_2$ could deplete the sources of arachidonic acid and therefore its downstream metabolites and PAF would not affect the homeostasis of COX-1/2 and LOX enzymes.

Therefore, the great demand for natural products for PLA_2 inhibition rather than NSAIDs to treat inflammatory disorders. The literature survey showed that several endogenous and exogenous agents have been reported to inhibit $sPLA_2$ enzymes [13]. In addition, several laboratories are synthesizing compounds to inhibit $sPLA_2$ [14]. But none of them are made into the market, which demands researchers to look for new specific $sPLA_2$ inhibitors.

Ruta graveolens belonging to family Rutaceae has long been used traditional medicine for various ailments including hysteria, gastrointestinal disorders, and menstrual problems. The juice of fresh rue has been used to relieve toothaches and earaches. It has been reported to possess antifungal [15], antibacterial [16], anti-inflammatory [17], antitumour [18] and cytotoxic activities [19]. Although, no information available on the effect of R. graveolens on inflammatory enzymes. In the present study, effect of R. graveolens leaves ethanol and aqueous extract on LOX and $sPLA_2$ activity was estimated.

MATERIALS AND METHODS

Diphenyl Picryl Hydrazyl radical ($DPPH^{\cdot}$), Thiobarbituric acid, Quercetin, Gallic acid, were purchased from Sigma-Aldrich Chemical

Laboratories, St. Louis, MO, USA. Aluchrosep silica Gel 60/UV_{254} TLC plates were obtained from S D FINE CHEM. Limited, Mumbai. Human pleural fluid was obtained from Chest Disease Hospital, Mysore, India. Dimethyl sulphoxide (DMSO) from Merck and all other chemicals and reagents used in this study were of analytical grade or better.

Preparation of plant extracts

R. graveolens plants were collected from kodagu district, washed, fresh leaves were shade dried and powdered mechanically. The powder (75g) was subjected to soxhlet extraction with solvents such as hexane, benzene, chloroform, acetone, ethanol, methanol and water in the ratio of 75g/500 ml (w/v). The extracts of organic solvents were concentrated to powder by flash evaporator and water sample was reduced to powder by lyophilization. The yield was calculated and expressed as % w/w.

Phytochemical analysis

The concentration of total phenolics was estimated by the methods of Singleton [20] using Gallic acid as standard. The photochemical analysis of different solvents extracts of *R. graveolens* was done by the method described in Yusuf AZ. *et. al* [21]. TLC for all the extracts was eluted with solvent-n-hexane: ethyl acetate (8:2) and observed under UV light.

Estimation of flavanoids

The flavonoids were estimated calorimetrically using Quercetin to make the calibration curve [22]. 100µg/ml of Quercetin was prepared by dissolving 10 mg in 80% ethanol. The reaction mixture contains 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water was incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm. The aluminum chloride (10%) was substituted with distilled water in blank. Flavonoids in plant extract reacted with aluminum chloride was determined as described above.

Reducing power assay

The reducing power ability of extracts was determined by the method as described in Jayaprakash [23]. The reaction mixture contains, extract (100µg/ml) with equal volume of 0.2M phosphate buffer pH 6.6 and potassium ferricyanide was incubated at 50° C for 20 min. Centrifuged at 3000 rpm for 10 min by adding equal volume of 10%TCA to the mixture. To the upper layer, distilled water and 0.1% ferric chloride in a ratio of 1:1:2(v/v/v) were added. The absorbance was measured at 700 nm and increased absorbance of the reaction mixture compare to blank indicates increased reducing power activity.

Superoxide radical scavenging

The reduction of NBT was assayed according to Dasgupta [24]. Superoxide radicals generated by non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system often reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 ml reaction mixture contains phosphate buffer (20 mM, pH 7.4), NBT (50 µM), NADH (73 µM), PMS (15 µM) and plant extracts (100µg/ml). Incubate for 5 min at room temperature and the absorbance was measured at 562 nm against an appropriate blank to determine the quantity of formazan generated. Quercetin was used as positive control.

Estimation of antioxidant activity

Antioxidant activity of *R. graveolens* was determined using 2, 2-Diphenyl-1-picryl hydrazyl radical ($DPPH^{\cdot}$) as described by Blios [25]. Briefly, 100µg Extracts of *R. graveolens* were mixed with 5 ml of 0.1 mM methanolic solution of DPPH and incubated at 20°C for 20 min in complete dark. The DPPH alone serves as control and methanol were used for the base line correction. The absorbance of the samples was measured at 517 nm and radical scavenging activity was expressed as percentage activity using the following formula.

$$\%\ \text{radical scavenging activity} = (\text{Control absorbance} - \text{sample absorbance}) / (\text{Control absorbance} - \text{sample absorbance}) \times 100$$

Anti-lipid peroxidation activity

Anti-lipid peroxidation activity of R. graveolens leaf extracts were estimated by TBARS method [26]. 0.5 ml of egg homogenate and extracts (100µg) were made up to 1 ml with distilled water. 100µl of 0.07M $FeSO_4$ was added and incubated for 30 min at room temperature. To all test tubes 1.5 ml of acetic acid, 1.5 ml of TBA and 50µl of TCA were added. Vortexed and kept in boiling water bath for 1 hour. By adding 5 ml of butanol test tubes were centrifuged at 3000 rpm for 10 min. Absorbance of supernatant was measured at 530 nm and percentage was calculated using the formula,

$$\%\ \text{Anti lipid peroxidation} = (1 - \text{Extract absorbance}) / (\text{Control absorbance}) \times 100$$

Purification OF $sPLA_2$

$sPLA_2$ from human pleural fluid (HPF) was purified by the modified method described by Vishwanath [27]. HPF was centrifuged at 10,000 rpm for 10 min. Supernatant was separated and equal volume of 0.36N H_2SO_4 was added. The sample was kept on the stirrer overnight, centrifuged at 10,000 rpm for 10 min. Supernatant was dialyzed using 7-8 KD cut off membrane against phosphate buffer (10 mM, pH 4.6). Dialyzed sample was kept on a water bath for 5 min at 60-70°C. The boiled sample was further centrifuged at 10,000 rpm for 10 min and the supernatant will be used as a source of $sPLA_2$

$sPLA_2$ inhibition

Inhibition of $sPLA_2$ by agarose egg yolk plate method was done according to Gutierrez [28]. 1g of agarose was boiled in 100 ml of 0.1M Tris HCl (pH 7.4) contains 5 mM $CaCl_2$. Add 6 drops of egg yolk agarose after it reached to room temperature. Stirred and poured into sterile petriplates and allowed to solidify. Make well using gel puncture and load 25µl HPF-PLA_2 into the wells. Incubate the plates for overnight at 37°C, measure the zone of clearance using normal scale carefully. Zone of HPF-PLA_2 alone serves as control and the well without HPF-PLA_2 serves as a negative control. HPF-PLA_2 pre-incubated with 50µg R. graveolens ethanol and aqueous extracts were used as test. Percentage of PLA_2 inhibition was calculated using the formula.

$$[(\text{Control diameter} - \text{Test diameter})/\text{Control diameter}] \times 100$$

Inhibition of 5-lipoxygenase

The Poly Morpho Nuclear Leukocytes (PMNLs) 5-lipoxigenase (5-LOX) assay was performed according to the method published by Aharony and Stein [29]. 1 ml of standard reaction mixture contains 100 mM phosphate buffer pH 7.4, 50µg of DDT, 200µg of ATP, 300µg of $CaCl_2$, 150µg of arachidonic acid and PMNLs lipoxigenase (5µg). The reaction was carried out at room temperature. Absorbance was read for every 30 seconds up to 2 min at 234 nm using Shimadzu spectrophotometer. The enzyme activity was expressed as µgole of 5-HETE formed/min/mg protein. The 5-lipoxigenase was subjected to the inhibition by *R. graveolens* extract (100-500 µg). The Quercetin, a known inhibitor of LOX, was used as a positive control.

Inhibition of 15-lipoxygenase

The enzyme assay was performed according to the method of Axelrod [30]. The standard reaction mixture for the 15-LOX assay (1.0 ml final volume) contained 100µg linoleic acid and soybean 15-lipoxygenase enzyme (5µg) in 200 mM borate buffer pH 9.0. The reaction was carried out at room temperature. The absorbance was measured continuously for 3 min at 234 nm. The enzyme activity was expressed as µg ole of 13-HPODE formed/min/mg protein. For inhibition study, different concentrations (100-500 µg) of ethanol and aqueous extracts of R. graveolens were pre-incubated with 15-LOX for 5 min. The reaction was initiated by adding linoleic acid to the respective assay mixtures. Quercetin, a known inhibitor of LOX was used as a positive control.

Neutralization of indirect hemolytic activity

The indirect hemolytic activity of *R. graveolens* was measured as described by Boman and Kaletta [31]. The substrate was prepared by suspending 1 ml of packed fresh human RBC and 1 ml fresh hen egg yolk in 8 ml of PBS. Aqueous extract of *R. graveolens* was pre-incubated with 30µg of PLA_2 for 30 min at 37 °C. The substrate (1

ml) was added to the pre-incubated sample and allowed to react for 45 min at 37ºC. The reaction was stopped by adding 9 ml of ice cold PBS and the suspension was mixed and centrifuged at 1,500 g for 20 min. The released hemoglobin was read at 530 nm. The substrate with $sPLA_2$ enzyme served as positive control.

Statistical analysis

The experimental results were presented as mean±SD of three determinations. The IC_{50} concentration was calculated using Graph pad version 5.0 USA.

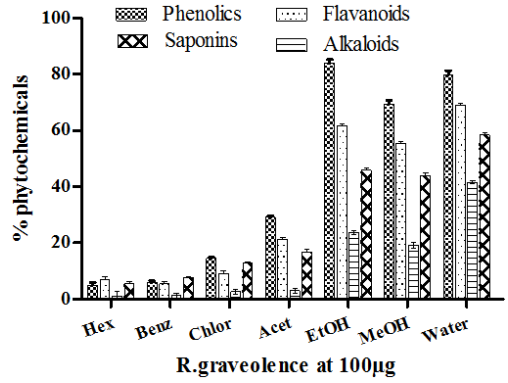

Fig. 1: Estimation of phenolics, flavonoids, saponins and alkaloids (%w/w) of *R. graveolens* extracts at 100µg. Data represents mean±SD (n=3)

RESULTS

The *R. graveolens* possesses anti-ulcer activity and promotes incision wound healing in mice [32] but the mechanism is unclear. The $sPLA_2$ enzyme is subjected to inhibition as a function of anti-inflammatory activity. Initially, different solvent extracts of *R. graveolens* were prepared and the phyto chemicals were estimated quantitatively and qualitatively. Total phenolics, flavonoids, saponins and alkaloids were estimated for all the extract. Ethanol extract of *R. graveolens* leaf has excellent phytochemical followed by water extract. Whereas hexane extract has very low phytochemicals (Fig.1). Among phytochemicals, phenols have higher concentration followed by flavonoids, saponins and alkaloids.

Phytochemical separation of the extracts was carried out by Thin Layer Chromatography (TLC). Hexane: ethyl acetate solvent ratio (8:2) was found to be a good for the separation of the active constituents and the plant extract on the chromatogram was carried out. The TLC of different solvent extracts of *R. graveolens* shows different spots under UV light (fig. 2). Rf values of extracts spots separated in TLC were calculated and ethanol extract shows maximum spots between Rf values 0.228 to 0.906 (table. 1).

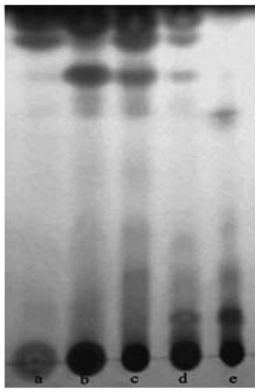

Fig. 2: TLC of *R. graveolens* leaf extracts: a) Chloroform, b) Acetone, c) Ethanol, d) Methanol and e) Water extract

Table 1: The Thin layer chromatography retention factor of *R. graveolens* leaf extracts

S. No.	*R. graveolens* Extracts	Number of spots	Retention factor (Rf)
1	chloroform	1	0.906
2	acetone	4	0.967, 0.906,0.811, 0.717
3	Ethanol	7	0.906, 0.811, 0.717, 0.517, 0.367, 0.228
4	Methanol	3	0.967, 0.906, 0.111
5	Water	4	0.689, 0.367,0.228, 0.111

Antioxidant and anti-inflammatory activity of extracts of *R. graveolens* were estimated. The DPPH scavenging, reducing power, lipid peroxidation and superoxide scavenging activity of ethanol extract and aqueous extracts of *R. graveolens* showed 92.34/85.81%, 89.7/75%, 80.8/61.8% and 78.9/69.21% respectively at 100 µg concentrations (fig. 3).

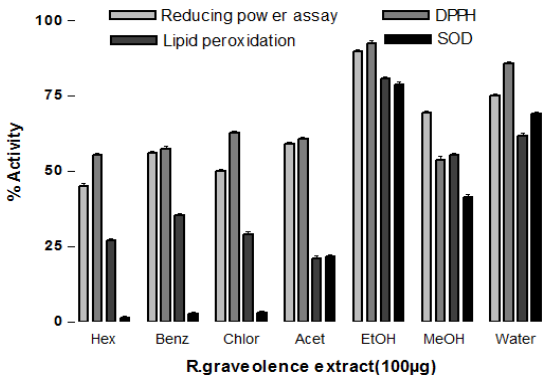

Fig. 3: Estimation of DPPH scavenging, anti-lipid peroxidation, reducing power and superoxide scavenging activity of *R. graveolens* extracts at 100µg. Data represents mean±SD (n=3)

To confirm the anti-inflammatory activity of *R. graveolens* ethanol and aqueous extract, the secretory PLA_2 enzyme from Human Pleural Fluid (HPF) was subjected for inhibition. The ethanol extract inhibited $sPLA_2$ enzyme at 50µg concentration in egg yolk plate method followed by water extract. Percentage of HPF-PLA_2 inhibition was 66.6% and 46.6% for ethanol and water extract respectively (fig. 4).

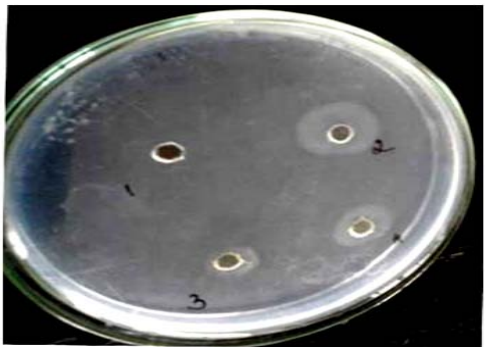

Fig. 4: Inhibition of $sPLA_2$ by egg yolk plate method. The well contains 1) control 2) 25µg of HPF-PLA_2 3) 25µg of HPF-PLA_2 and 50 µl ethanol extract 4) 25µg of HPF-PLA_2 and 50 µl aqueous extract

The pro-inflammatory enzymes 5-LOX and 15-LOX activity was measured in terms of amounts of formation of 5-HETE and 13-HPODE respectively. The ethanol extract of *R. graveolens* inhibited both the LOX enzymes in concentration dependent manner followed by aqueous extract. IC_{50} values of *R. graveolens* extracts for PMNLs 5-LOX is 4.25, 5.15±0.05 µg/µl (fig. 5) and 15-LOX is 4.15, 4.66±0.05 µg/µl (Fig.6) for ethanol and water extracts respectively.

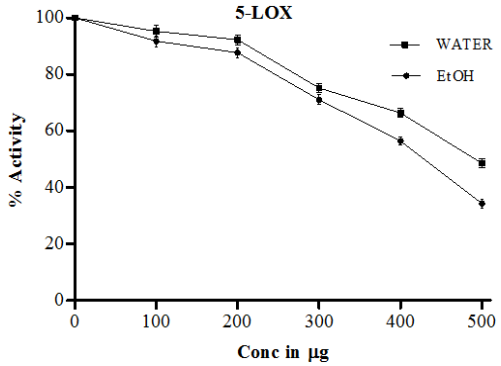

Fig. 5: Inhibition of 5-LOX by ethanol and aqueous extracts of *R. graveolens* at 500 µg. 1 ml of reaction mixture contains 100 mM phosphate buffer (pH 7.4), DDT (50µg), ATP (200µg), $CaCl_2$ (300µg), arachidonic acid (150µg) and 5-LOX (5µg). Absorbance measured at 234 nm by µgole of 5-HETE formed/min/mg protein

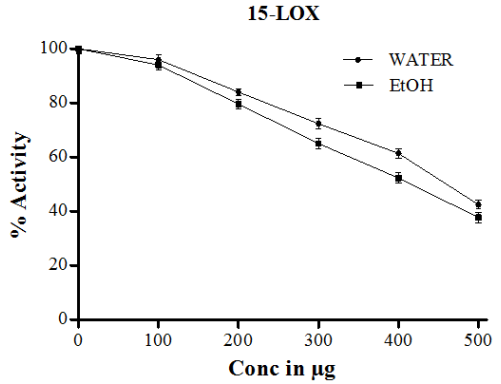

Fig. 6: Inhibition of 15-LOX by ethanol and aqueous extracts of *R. graveolens* at 500 µg. The reaction mixture contained linoleic acid (100µg) and 15-LOX (5µg) in 200 mM borate buffer (pH 9.0). Absorbance measured at 234 nm by µgole of 5-HETE and 13-HPODE formed/min/mg protein

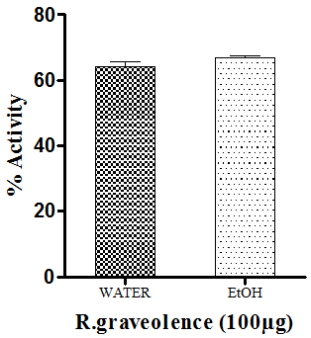

Fig. 7: Indirect hemolytic activity of *R. graveolens* leaf extract. The substrate contains 1 ml RBC, 1 ml egg yolk and 100 µg *R. graveolens* leaf extracts were pre incubated with 30µg HPF-PLA_2. The released hemoglobin was read at 530 nm

The *in situ* hemolytic activity is an indirect way of measuring PLA_2 activity using egg yolk phospholipids dispersed as micelles together with washed erythrocytes. Ethanol extract inhibited the indirect hemolytic activity 66.8% and water extract 64.22% at 100µg concentrations (fig. 7).

DISCUSSION

Since ancient times, plants have been a veritable source of drugs. However, human tends to ignore the value of herbal medicine. Successive isolation of bio active compounds from plant material is mainly dependent on the type of solvent used in the extraction procedure. Phytochemical analysis of the chloroform, Acetone, Ethanol and water extracts of *R. graveolens* showed the presence of some phytochemical parameters like phenolics, flavanoids, saponins and alkaloids. Therefore, such screening experiments make a primary platform for further phytochemical and pharmacological studies that may open the possibility of finding new clinically effective anti-inflammatory compounds.

The thin layer chromatographic study of the extract shows maximum spots in ethanol extract. This result supports that phytochemical content is high in ethanol extract of *R. graveolens.* Further, all solvent extracts were subjected to check the *In vitro* anti-oxidant activity. *R. gravoelens* extracts found to scavenge hydroxyl radical and inhibited lipid peroxidation at lower concentration. These results strongly suggest the correlation between antioxidant activity and phytochemical contents of the extracts.

The most NSAIDs target the either COX-1/2 or LOX enzymes or both the enzymes. Therefore the ethanol extracts of *R. graveolens* was checked for the two of the inflammatory enzymes Poly Morpho Nuclear Lymphocytes LOX (PMNLs 5-LOX) and soybean LOX (15-LOX).

R. graveolens ethanolic and aqueous extracts might possess potent active principles which inhibits 5-LOX and 15-LOX enzyme. IC_{50} values of ethanol and water extracts of *R. graveolens* were found to be 4.25, 5.15 µg/µl for 5-LOX and 4.15, 4.66±0.05 µg/µl respectively.

The phytochemical and anti-oxidants derived from plants are potential interest in therapeutic intervention for chronic inflammatory disorders. They act either by inhibiting pro-inflammatory enzymes (PLA_2, COX and LOX) or by inhibition of cytokines (IL-1β, TNF-α) release and inhibition of mast cell degranulation that are known contributors to chronic inflammatory disorders. The $sPLA_2$ enzyme catalyze rate limiting step in the production of pro-inflammatory eicosanoids and free radicals. The PLA_2 catalyzed reaction is considered to be a significant pathway for reactive oxygen species (ROS), it turn activates PLA_2 as well as lipid peroxidation and thereby augment the chronic inflammatory diseases to several folds [32]. Hence The PLA_2 inhibition is legitimate in the neutralization of inflammation. The anti-oxidants such as flavonoids, retinoids and vitamin E and their potential benefit in intervention of inflammatory reactions arise if it inhibits the key enzyme PLA_2 along with neutralizing the free radical generation reaction. Among the solvents ethanol exhibited the higher degree of $sPLA_2$ inhibitory action. In the present study, we have established the anti-inflammatory activity of the ethanol extract of *R. graveolens* leaf by an egg yolk agar well diffusion method. The ethanol extracts of *R. graveolens* leaf shows $sPLA_2$ inhibition along with LOX inhibition and anti-oxidant activity at low concentration.

CONCLUSION

In the present day, anti-oxidative properties of plants extracts have become a great interest due to their possible uses as natural additives to replace synthetic ones and NSAIDs. The above data summarized that ethanol extracts of *R. graveolens* exhibited very good *in-vitro* HPF-PLA_2 inhibition at very low concentration and the pro-inflammatory enzymes like 5-LOX and 15-LOX in concentration dependent manner. Although, the extract used in the assay is crude there is always a possibility of the cumulative effect, which may be the other reason for $sPLA_2$ inhibition. However, several important questions remain open and further investigations are necessary to understand the mechanism underlying the effects of the extract and their active compound/s responsible for anti-inflammatory activity.

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CONFLICT OF INTERESTS

Declared None

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