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Evaluation of *In-Vitro* and *In-Vivo* Anti-Inflammatory Activities of Apigenin and Vitexin

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Abstract

The current investigation was carried out to analyse the anti-inflammatory potential of Apigenin (DI) and Vitexin (DII). The anti-inflammatory activities were analysed by in-vitro and in-vivo procedures, the results were found to be very surprising and it shows potential activity. In-vitro anti-inflammatory activity was evaluated using, membrane stabilization assay, albumen denaturation assay and proteinase inhibitory activity at different concentrations. In-vivo anti-inflammatory activity was evaluated using carrageenan induced rat paw model. For the anti-inflammatory activity, Aspirin is used as a standard drug for study purpose. The highest inhibition of albumen denaturation was recorded as 49.2% from DI extract and 54.2% from DII extract. It showed proteinase inhibition as 54.2% and 57.8 % from DI and DII extracts at 500µg/ml respectively. Aspirin showed the highest inhibition as 55.6% at 200µg/ml for proteinase inhibition. The results obtained by carrageenan induced rat paw model indicates DI and DII extracts having anti-inflammatory activity. Therefore, our current in-vitro and in-vivo studies on extracts demonstrate the depression of inflammation. Hence, the Apigenin and Vitexin can be used as a potent anti-inflammatory agent. The extracts showed superior anti-inflammatory potential as the dose varies. Therefore, our studies support the use of active constituents like Apigenin and Vitexin in treating inflammation.

Key Words: Anti-inflammatory activity, Apigenin and Vitexin.

INTRODUCTION

Medicinal plants will continue to produce large source of new drug compounds. Plants are the basic component for the development of a new medicine or they may be used as phyto (plant) medicine for the treatment of disease [1]. Approximately that the plant materials are present in, or have provided the models for 50% Western drugs [2]. The major useful of using plant-derived medicine is safer than the synthetic drugs and also which acts as an alternative, which offer profound therapeutic benefits and less economical treatments [3]. Many plants have proved to successfully aid in different ailments leading to mass screening for their therapeutic components. Today, the research for natural compounds which are originate from plant source are rich in antimicrobial, antioxidant and anti-inflammatory properties is ever-increasing due to their medicinal significance in controlling many related disorder from gene level to tissues level like cancer, diabetes, arthritis, hypertension etc. Anti-inflammatory activities and active principles from plants of North West Himalaya Garhwal region were determined [4,5,6,7,8]. Natural products originated from plants source offer a new component for biological disorders that may have a great impact on human health [9]. Some are inflammatory diseases such as hepatitis rheumatoid arthiritis, asthma and colitis are among the causes of death and disorder in the world [10,11,12]. Inflammation is a normal protective response to tissue injury that is caused by physical trauma, microbiological agents or noxious chemicals. Inflammation is the result of concerted participation of a large number of chemotactic, vasoactive and proliferative factors at diverse stages and there are many targets for anti-inflammatory action [13]. Inflammatory response is a series of well-organized mechanism containing of specific vascular, cellular and humoral trials that is categorized by the movement of fluids, plasma and inflammatory leukocytes (eosinophils, neutrophils, basophils and macrophages) to the site of inflammation [14,15]. A variety of chemical mediators or signalling molecules such as, serotonin, leukotrienes, prostaglandins, histamine and oxygen derived free radicals (O_2, OH, HOO) are produced by inflammatory and phagocytic cells mainly in the sequences which participates in beginning of inflammation [16,17].

In our previous study, two bioactive flavonoids namely Apigenin and Vitexin have been successfully isolated from *Justicia gendarussa* [18].

In the present investigation we have reported the in-vitro and in-vivo anti-inflammatory activities of Apigenin and Vitexin which are isolated from *Justicia gendarussa*.

MATERIALS AND METHODS

The human red blood cell (HRBC) membrane stabilization method

Fresh human blood was collected and mixed with equivalent volume of disinfected Alsever solution (0.42 % sodium chloride in water, 2 % dextrose, 0.8 % sodium citrate and 0.05% citric acid). The collected fresh blood was centrifuged for 10 min at 5000 rpm and the gained packed cells were washed three times with isosaline (0.85%, pH 7.2). The measured fresh blood was reconstituted as 10% v/v suspension with isosaline.

Heat induced hemolysis

The reaction mixture 2ml consists of 1ml of the test drug solution and 1 ml of RBCs suspension 10%. For the control test tube saline buffer is added as a drug. Aspirin is used as standard drug. 2ml reaction mixture in centrifuge tubes were incubated in a water bath for 30min at 56°C. At the completion of the incubation, the tubes were made to attain

room temperature and the mixture was centrifuged for 5 min at 3000rpm. The absorbance of the supernatant liquid (2ml reaction mixture) was measured at 560nm.

The percentage of inhibition of HRBC membrane can be calculated as follows:

Percentage inhibition = (Absorbance of control – Absorbance of sample) X 100/ Absorbance of control

Inhibition of Albumen Denaturation

Method of Mizushima et al was followed with minor modifications. The reaction mixture consists of DI, DII extracts at different concentrations and 1% aqueous solution of bovine albumin fraction. The pH of reaction mixture was then adjusted with diluted hydrochloric acid. The reaction mixture was incubated at 37°C for 20 min and then heated at 57°C for 20 min. After cooling the reaction mixture, the turbidity was measured using spectrophotometer at 660 nm. Percent inhibition of protein denaturation was calculated as follows:

Percentage inhibition = (Absorbance of control – Absorbance of sample) X 100/ Absorbance of control

Proteinase inhibitory activity

The proteinase inhibitory activity was executed with the improved method of Oyedepo et al. The reaction mixture of 2ml was containing 1ml 20mM Tris HCl buffer (pH 7.4), 0.06mg trypsin and 1ml of DI, DII extracts of different concentrations. The reaction mixture was incubated for 5 min at 37°C and 1ml of 0.8% (w/v) casein was added. The mixture was incubated for an another 20 min. For termination of the reaction 2 ml of perchloric acid (70%) was added. Fumy suspension was centrifuged and the reaction mixture supernatant liquid absorbance was taken at 210 nm against buffer as blank. The percentage of inhibition of proteinase activity was calculated.

Percentage inhibition = (Absorbance of control – Absorbance of sample) X 100/ Absorbance of control

In-vivo study by Carrageenan induced rat paw model

Male Wister rats (180–200 g) were used (Experiment is conducted in Livon Biolabs Pvt Ltd, Tumkur, Karnataka, India) to evaluate the extracts DI, DII for in-vivo pharmacological study. The animals were fed *ad libitum* with AMRUT Laboratory Animal Feed manufactured by Pranav Agro Industries Limited, Sangli, Maharashtra and reverse osmosis purified water was provided for drinking *ad libitum. Animals* were kept in rooms maintained at 22±3ºC with a 12 h light and dark cycle (as per CPCSEA guidelines).

After acclimatization period the 20 animals are equally distributed into 4 groups. On day one, 0.1 ml of 1% sterile carrageenan in saline was injected into the sub plantar region of the right hind paw of all group animals after 60 minutes of DI and DII extracts administration. Before administration of the vehicle, DI, DII and Aspirin (standard drug), the size of the left and right hind paw of all the animals were measured using vernier calliper.

After paw measurement G2 and G3 group animals were treated with DI 10mg/kg and DII 10mg/kg. G1 group animals were treated as control and it will be treated with vehicle alone, G4 group animals were treated with known drug Aspirin 10 mg/kg.

The inflammation was measured by determining the volume displaced by the paw, using vernier caliper at time 0, 1, 2, 3 and 4 h after carrageenan injection. After completion of the study, the carcass was disposed of through Medicare Environmental Management Pvt Ltd. The percent inhibition of edema was calculated in comparison to the control male wistar rats. The institutional animal ethical committee has approved the protocol of the study (Protocol No: LBPL-IAEC 137-12/15).

RESULTS AND DISCUSSION

The inhibition of hypotonicity induced HRBC membrane lysis that is stabilisation of HRBC membrane was taken as a measure of the anti-inflammatory activity. The percentage of membrane stabilisation for methanolic extracts DI, DII and Aspirin were done at 100, 200, 300, 400 and 500 µg/ml. The extracts DI and DII are effective in inhibiting as shown in Table 1. With the increasing concentration of extract, the membrane hemolysis is decreased and membrane stabilisation / protection is increased. Hence anti-inflammatory activity of the DI, DII extracts was concentration dependent. Stabilization of RBCs membrane was studied for further establishes the mechanism of anti-inflammatory action of different extract of DI and DII. Heat induced hemolysis of HRBC was well inhibited by the extracts. The results obtained from the membrane stabilization provide additional mechanism of their anti-inflammatory effect. The release of lysosomal content of neutrophils may possibly inhibited at the site of inflammation. The extracts inhibited the heat induced hemolysis of RBCs to varying degree. The maximum inhibition was recorded as 63.4% and 74.1% from DI and DII extracts. The results were compared with standard Aspirin which showed the maximum inhibition as 86.0% at 500 µg/ml.

Table 1: Showing the inhibition of heat induced hemolysis of HRBC at different concentrations

Conc. (µg/ml)	% Hemolysis of DI	% Hemolysis of DII	% Hemolysis of Aspirin	% Stabilization of DI	% Stabilization of DII	% Stabilization of Aspirin
100	64.7	67.2	53.3	35.3	32.8	46.7
200	59.3	57.6	49.7	40.7	42.4	50.3
300	51.8	49.7	26.2	48.2	50.3	73.8
400	40.7	35.8	15.8	59.3	64.2	84.3
500	36.6	25.9	14.0	63.4	74.1	86.0

Table 2: Showing the inhibition of Albumen Denaturation at different concentrations

Test Sample	Conc. (µg/ml)	Absorbance at 660nm	% Inhibition	Test Sample	Conc. (µg/ml)	Absorbance at 660nm	% Inhibition
DI	100	0.355	13.4	DII	50	0.348	15.2
	200	0.313	23.7		100	0.307	25.1
	300	0.29	29.3		250	0.276	32.8
	400	0.256	37.6		500	0.251	38.8
	500	0.208	49.2		1000	0.188	54.2

Table 3: Showing the Proteinase inhibitory activity at different concentrations

Test Sample	Conc. (µg/ml)	Absorbance at 660nm	% Inhibition	Test Sample	Conc. (µg/ml)	Absorbance at 660nm	% Inhibition
DI	100	0.338	15.6	DII	50	0.331	17.3
	200	0.310	22.5		100	0.313	21.7
	300	0.264	34.1		250	0.274	31.5
	400	0.229	42.8		500	0.242	39.4
	500	0.183	54.2		1000	0.169	57.8

Inhibition of Albumen Denaturation

Denaturation of proteins is a well-known cause of inflammation. As part of the study for the mechanism of the anti-inflammation activity, extract inhibition on protein denaturation was conducted. The percentage of Inhibition of Albumen denaturation is done at different extract concentrations. The obtained results were compared with standard Aspirin, which showed as 58.3% inhibition at 200 µg/ml. The results are reported in Table 2. Since proteins are present in the cell gets denatured and cause inflammation, thus here albumen protein is used as a model which is used as a denaturating agent and the inhibition of denaturation done by adding plant extracts it was studied based on concentration.

Proteinase inhibitory activity

Proteinases have been implicated in arthritic reactions. Neutrophils are the group of white blood cells known to be a rich source of proteinase enzyme which carries in their lysosomal granules or vesicles contain many serine proteinases. It was earlier reported that leukocytes proteinase plays a major role in the development of tissue damage during inflammatory reactions and efficient level of protection was provided by proteinase inhibitors. DI and DII methanol extract exhibited efficient anti-proteinase activity at various concentrations as shown in Table 3. It showed maximum inhibition as 54.2% and 57.8 % at 500µg/ml for DI and DII methanol extract respectively. Aspirin showed the maximum inhibition as 55.6% at 200µg/ml.

In-vivo study by Carrageenan induced rat paw model

Summary of average animal body weights (grams) are presented in Table-4 and average percentage of inhibition is presented in Table-5. There were no biologically significant changes observed in body weight (grams) in all group animals. The results obtained by the DI and DII extracts indicates the anti-inflammatory activity.

Table 4: Summary of average body weight of animals

Group	Treatment and Dose (mg/kg)	Average body weight (grams) Day-1
G1	Control and 10 mL/kg	188.40 ± 5.91
G2	Apigenin and 10 mg/kg	191.19 ± 8.10
G3	Vitexin and 10 mg/kg	191.10±5.71
G4	Aspirin and 10 mg/kg	194.63±9.20

Table 5: Average percentage of inhibition

Group	Percentage of Inhibition			
	After 1st hour treatment	After 2nd hour treatment	After 3rd hour treatment	After 4th hour treatment
G1	--	--	--	--
G2	3.74	4.14	11.63	14.87
G3	2.17	2.27	8.58	12.56
G4	6.14	6.89	16.33	21.80

CONCLUSION

Therefore, our present in-vitro and in-vivo studies on extracts shows that there is a reduce in the inflammation. Due to the presence of active components such as flavonoids like Apigenin and Vitexin are responsible for anti-inflammatory activity. Hence, the Apigenin and Vitexin can be used as a potent anti-inflammatory agent.

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