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IN VITRO ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC EXTRACT OF *BRASSICA RAPA SUBSP.RAPA*

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ABSTRACT

Aim: To investigate the anti-inflammatory activity of ethanolic extract of *Brassica rapa* leaves. **Materials and Methods:** The anti-inflammatory activity of Ethanolic Extract of *Brassica rapa* leaves (EEBR) was evaluated by using *in vitro* based assays: Human Red Blood Cell (HRBC) membrane stabilization assay, Heat Induced Hemolysis and Protein Denaturation Inhibition. **Results:** The results showed that the percentage stabilization of EEBR on HRBC membrane under hypotonicity was within the range of 19.32 to 66.21% and EEBR has exhibited a significant % inhibition on heat induced hemolysis (21.3 to 68.7%) when compared to that of standard. The percentage inhibition on protein denaturation of EEBR was within the range of 34.85 to 72.80% and EEBR at a dose of 1500µg/ml has exhibited significantly higher inhibition level. **Conclusion:** The results revealed that EEBR possess anti- inflammatory properties and could have a potential therapeutic effect on disease process causing destabilization of biological membrane.

KEYWORDS: *Brassica rapa*, Protein denaturation, Hemolysis, Membrane stabilization.

INTRODUCTION

Inflammation is body's defense mechanism and an important process that helps organism to respond to harmful stimuli.[1] Inflammation is very helpful in excluding invading pathogens or harmful substances. However, this must be regulated, since uncontrolled inflammation may lead to pathophysiological conditions like cancers.[2] Generally, inflammation is associated with pain, it also involves increase in denaturation of proteins, increase in vascular permeability and alterations in membrane.[3] The emigration of leukocytes from the blood circulation to the site of injury and release of cytokines plays an important role in inflammatory response, these chemicals cause vasodilation and increase blood flow to the site of injury.[4]

Inflammation can be classified as either acute or chronic. Initial stage of inflammation is acute phase which is rapid and persists for a short while[5] Early phase of acute inflammation is characterized by release of pre-inflammatory mediators such as Histamine, Serotonin and Bradykinins. Later phase of acute inflammation begins after one hour in which the early phase mediators activates neutrophils infiltration and release prostaglandins by cyclooxygenase (COX),[6] free radicals, nitric oxide (NO) and pro-inflammatory mediators such as Interleukin-1 (IL-1) and Tumor Necrosis Factor α (TNF- α).[7]

Persistence of this late phase leads to chronic inflammation which is characterized by induction of lipopolysaccharides (LPS), Serotonin and Histamine. LPS induce cellular responses that activate innate or natural immunity which stimulates macrophages.[8] Serotonin regulates inflammation and cell proliferation which are modulated by macrophages.[9] Histamine is an inflammatory mediator which promotes inflammatory and regulatory responses associated with pathological process.[10] Thus, chronic inflammation leads to shift in the type of cells at the site of inflammation leading to healing of tissue from inflammatory process.[11]

Non-Steroidal Anti-inflammatory Drugs (NSAIDs) are most commonly prescribed anti-inflammatory drugs for relieving symptoms associated with inflammation by inhibiting COX. However, NSAIDs have several adverse effects such as gastric irritation leading to gastric ulcers, they affect coagulation of blood and also have adverse effects on kidneys by inhibiting COX-1 enzyme.[12] Newer drugs like specific COX-2 inhibitors claimed to

be devoid of these adverse effects but they failed in reaching the expected outcome as NSAIDs. Thus, the search for natural sources with anti-inflammatory activity, with minimal or no side effects have been increasing in last few years.[13]

Green leaves and roots of *Brassica rapa* commonly called as turnip has medicinal and nutritional values. The data collected from the phytochemical screening studies performed on brassica rapa leaf extract so far reveals the presence of Glucosinolates, isothiocyanates, flavonoids, volatiles, and other compounds.[14,15] Glucosinolates, isothiocyanates, flavonoids, volatiles and phenylpropanoids are the major active constituents of turnip greens. Flavonoids, mainly kaempferol, quercetin and isorhamnetin derivatives were found to be in turnip greens. Turnip is also used as a traditional medicine for the treatment of headaches, chest complaints, rheumatisms, oedemas, gonorrhoea, syphilis, and rabies besides being an important vegetable and source of oil. Glucosinolates and isothiocyanates (mainly 2-phenylethyl, 4-pentenyl, and 3-butenyl derivatives) are the main constituents of turnip with diverse bioactivities, especially for the protective effect against cancers.

The isothiocyanates, are reported to have nematicidal,[16,17] fungicidal,[18,19] and bactericidal[20] properties. *Brassica rapa* was employed as a medicine for constipation, hepatic diseases, gall bladder inflammation, gall stones, and gastritis in Perso-Arabic tradition. The roots of turnip possess antibacterial properties and were used in the treatment of common cold.[21] The powder obtained from the seeds of *Brassica rapa* well-known for its anticancer properties especially used in breast cancer and the extracts obtained from roots of *Brassia rapa* has beneficial effects in skin cancer. Turnip has also been a common diet and herbal remedy in Tibet, where high altitude leads to oxygen deficiency and exhaustion.

MATERIALS AND METHODS

Collection of plant material: Fresh and healthy leaves of *Brassica rapa* belonging to the family Brassicaceae, were collected from the local areas of talakona forest, chittor district, Andhra Pradesh. The plant material was authentified by Dr.K. Madhava chetty, Asst. Prof, Department of Botany, S.V. University, Tirupathi, Andhrapradesh, India with voucher no 0429. It was preserved for future reference in our Pharmacognosy department. Just after collection, the plant material was washed thoroughly with running tap water. They are kept away from direct sunlight to avoid destruction of active compounds, shade dried at room temperature and ground mechanically into a coarse powder and stored in air tight container for future use.

Preparation of plant extract

Brassica rapa leaves were allowed to dry in shade. Dried leaves were powdered by electric grinder. The experiment is started by building a rig using stands and clamps to support the extraction apparatus. Following this the solvent (ethanol- 500ml) was added to the round bottom flask, which was attached to a soxhlet extractor and condenser on an isomantle. The plant material was loaded onto the thimble, and was placed inside the soxhlet extractor. The side arm was lagged with glass wool. The solvent was heated using the isomantle and it started to evaporate, moving through the condensor. The condensate then dripped into the reservoir containing the thimble. Once the level of solvent reached the siphon tube it was poured back into the flask and the cycle begins again. The process was runned for 16 hours. The alcohol was evaporated using a rotary evaporator, leaving a small yield of extract (3.78%) in glass bottom flask**.** and the extract was stored at 4-5^0 C until used. Double- distilled water was used to redissolve the extract prior to experimentation to evaluate anti inflammatory activity.[22]

Preliminary Phytochemical Screening

Standard screening test of the Ethanolic extract of *Brassica rapa* leaves (EEBR) was carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites using standard procedures.[23]

In vitro methods

Human Red Blood Cell (HRBC) - membrane stabilization assay

The anti-inflammatory activity of the extracts was determined using human red blood cell (HRBC) - membrane stabilization assay developed by Shinde et al.[24] and modified by Sikder et al.[25] Venous human blood was collected from a normal male adult who had not consumed anti- inflammatory medicaments during two weeks before taking the sample. The blood was mixed with equal volume of Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride in water). The resulting mixture was centrifuged at 3000rpm for 10min; the supernatant was removed and the packed cells washed 3 times with isosaline solution (0.9%, pH 7.2). The assay mixture was prepared by mixing 1mL phosphate buffer (pH 7.4), 2mL hyposaline solution (0.36%) and 0.5mL HRBC suspension (10% v/v) with 1 mL of EERB of various concentrations (500, 1000 and 1500 µg/mL) or standard drug diclofenac sodium (125, 250 and 500µg/mL) respectively. A reaction mixture with distilled water instead of plant sample was used as control and phosphate buffer as blank. The mixtures were incubated at 37°C for 30 minutes and then centrifuged at 3000rpm. The hemoglobin content in the supernatant solution was estimated spectrophotometrically at 560nm. The percentage of hemolysis produced in the presence of distilled water was considered as 100%.

Preparation of Erythrocyte Suspension

Erythrocyte suspension was prepared according to the method described in Shin de et al.[26] with some modifications. Whole human blood was collected from a

healthy human subject. The blood was centrifuged at 3000 rpm for 5 min in heparinized centrifuge tubes, and washed three times with equal volume of normal saline (0.9% NaCl). After the centrifugation, the blood volume was measured and reconstituted as a 10% (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4). Composition of the buffer solution (g/l) used was NaH_2PO_4 (0.2), Na_2HPO_4 (1.15) and NaCl (9.0).

Heat-Induced Hemolysis

This test was carried out as described by Okoli et al.[26] with some modifications as described in Gunathilake et al.[27] Briefly, 0.05 mL of blood cell suspension and 0.05ml EERB of various concentrations (500, 1000 and 1500 µg/mL) or standard drug (aspirin 90 ug/ml), were mixed with 2.95 ml phosphate buffer (pH 7.4). The mixture was incubated at 54 ℃ for 20 min in a shaking water bath. After the incubation, the mixture was centrifuged (2500 rpm for 3 min), and the absorbance of the supernatant was measured at 540 nm using a UV/VIS spectrometer. Phosphate buffer solution was used as a control for the experiment.

The level of hemolysis was calculated using the following equation.

$$\%inhibition\ of\ denaturation = 100x(1 - \frac{A2}{A1})$$

where A1 = absorption of the control, and A2 = absorption of test sample mixture.

Effect on Protein Denaturation

Protein denaturation assay was done according to the method described by Gambhire et al.[26] with some modifications as described in Gunathilake et al.[27] The reaction mixture (5 mL) consisted of 0.2 mL of 1% bovine albumin, 4.78 mL of phosphate buffered saline (PBS, pH 6.4), and 0.02 mL EERB of various concentrations (500, 1000 and 1500 µg/mL) or standard drug (aspirin 200 ug/ml, 400 ug/ml, 800 ug/ml, and was incubated in a water bath (37 ^{0}C) for 15 min, and then the reaction mixture was heated at 70^{0}C for 5 min. After cooling, the turbidity was measured at 660 nm using a UV/VIS spectrometer. Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula.

$$\%inhibition\ of\ denaturation = 100x(1 - \frac{A2}{A1})$$

where A1 = absorption of the control, and A2 = absorption of test sample mixture.

RESULTS

Preliminary Phytochemical Screening

The preliminary phytochemical screening of the ethanolic extract of *Brassica rapa* revealed the presence of alkaloids, steroids, flavonoids, flavonones, glycosides, saponins, proteins and tannins.

Effect on Human Red Blood Cell (HRBC) - membrane stabilization assay

We have evaluated the effect of ethanolic extract of EEBR on stabilization of HRBC membrane. It was found that the different concentrations of EEBR have an ability to stabilize the RBC membrane in hypotonic solution and can inhibit haemolysis. EEBR at a concentration of 1000µg/ml and 1500µg/ml have shown a significant percentage of inhibition with 30.14% and 66.21% respectively. The percentage stabilization of EEBR at a concentration of 1500 µg/mL was comparable to that of standard diclofenac 500 µg/mL.

Table 1: Effect of EEBR on HRBC Membrane stabilization Assay.

S.no	Extract/Standard	Concentration(µg/mL)	% stabilization
1	EEBR	500	19.32±0.68*
2	EEBR	1000	30.14±0.53**
3	EEBR	1500	66.21±0.74*
4	Std (Diclofenac)	125	23.40±0.79*
5	Std (Diclofenac)	250	45.08±0.98*
6	Std (Diclofenac)	500	71.76±0.45

Values are expressed as Mean ± S.E.M; n=6. Statistical significance: (**p<0.01 and p*<0.05) One way ANOVA followed by Dunnett's test.

Effect of EEBR on heat induced haemolysis

Inhibition of heat induced haemolysis by EEBR at different concentrations were depicted in table-2. Compared to the control EEBR showed a significant reduction in heat induced haemolysis. EEBR at a concentration of 1500 µg/mL showed 70% of inhibition when compared with that of standard.

Table 2: Effect of EEBR on Heat induced Haemolysis.

S.no	Extract/Standard	Concentration(µg/ml)	% Inhibition (Haemolysis)
1	EEBR	500	21.3±0.72*
2	EEBR	1000	34.4±0.34**
3	EEBR	1500	68.7±1.8*
4	Std(Aspirin)	90	65.9±2.7

Values are expressed as Mean ± S.E.M; n=6. Statistical significance: (**p<0.01 and p*<0.05) One way ANOVA followed by Dunnett's test.

Effect of EEBR on Protein Denaturation

The inhibitory effect of different concentrations of EEBR on protein denaturation was shown in table-3. EEBR at a concentration of 500, 1000 and 1500 µg/ml and standard (aspirin) at a concentration of 200, 400 and 800 µg/ml showed significant inhibition of denaturation of albumin in concentration dependent manner. In this study EEBR showed maximum inhibition of 72.80% at 1500 µg/ml. aspirin a standard anti-inflammatory drug showed the maximum inhibition of 79.22% at a concentration of 800 µg/ml.

Table 3: Effect of EEBR on Protein Denaturation.

S.no	Extract/Standard	Concentration(µg/ml)	% inhibition
1	EEBR	500	34.85±0.81*
2	EEBR	1000	58.66±0.65*
3	EEBR	1500	72.80±0.23*
4	Std(Aspirin)	200	48.62±0.17**
5	Std(Aspirin)	400	66.2±0.48*
6	Std(Aspirin)	800	79.22±0.76

Values are expressed as Mean ± S.E.M; n=6. Statistical significance: (**p<0.01 and p*<0.05) One way ANOVA followed by Dunnett's test.

DISCUSSION

The present study reports the potential anti-inflammatory activity of *Brassica rapa leaves* against heat and hypotonicity induced haemolyis and protein denaturation.

The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract can stabilize lysosomal membranes.[28] Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release.[29]

On the basis of in vitro evaluated results EEBR showed significant anti-inflammatory activity as compared to control.

Certain flavonoids possess potent inhibitory activity against a variety of enzymes such as proteinkinase C, protein tyrosine kinases, phospholipase A2, phosphodiesterases. The anti- inflammatory activity of the extract may be due to the presence of flavanoids, tannins etc. either singly or in combination.[30] In vitro result suggests that the leaf extract of *Brassica rapa* possess potential anti-inflammatory activity.

The haemolysis of red blood cell can be induced by heat as well as hypotonicity. In hypotonicity induced haemolysis, cells undergo shrinkage, characterized by the release of intracellular electrolytes and fluid components that takes place as a result of osmotic loss.[31] EEBR has shown to inhibit the haemolysis triggered by both heat and hypotonicity in a dose dependent manner probably owing to the ability of EEBR to stabilize the membranes.

Denaturation of proteins is a well-documented cause of inflammation. Protein denaturation is a process in which proteins loose their tertiary structure and secondary structure by application of external stress or compound such as strong acid or base a concentration inorganic salt, an organic solvent or heat most biological protein lose their biological function when denatured. Phenylbutazone, salicylic acid, flufenamic acid (anti-inflammatory drugs), etc., have shown dose-dependent ability to thermally-induced protein denaturation.[32] Thus in our study, EEBR significantly inhibited the protein denaturation when compared with standard drug aspirin.

CONCLUSION

On the basis of above experimental results it is evident that the ethanolic extract of *Brassisca rapa* leaves possess anti-inflammatory activity. The Extract inhibits hypotonicity and heat induced hemolysis and protein denaturation. The Extract of *Brassica rapa* leaves shows dose dependent significant activity when compared with the standard drug (like aspirin and diclofenac). The anti-inflammatory activity of *Brassica rapa* leaves may be attributed to the presence of bioactive compounds such as flavonoids, polyphenols etc. the present study indicates that *Brassica rapa* leaf extracts possess an ability to stabilize biological membranes and can restrict disease progression.

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