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ANTI-INFLAMMATORY ACTIVITY OF 3, 3'- DIINDOLYLMETHANE-AN *IN VITRO* STUDIES

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ABSTRACT

Inflammation is one of the common causes of disease. Every chronic disease is an inflammatory disease. Targeting the inflammation process is one of the good strategies to prevent disease occurrence and progression. The present study was focused to evaluate the anti-inflammatory property of diindolyl methane (DIM), a naturally occurring phytochemical by using in vitro studies such as albumin denaturation assay, heat induced haemolysis and hypo tonicity effect on human erythrocytes (membrane stabilization). Three different concentrations of DIM (20, 40, 80µg/ ml) was used to study the *In-vitro* anti-inflammatory activities and aspirin (40µg/ ml) was used as reference drug. The experiment was conducted in triplicate. The percentage of inhibition of protein denaturation and hemolysis (55, 35, and 20) is more in 80µg DIM treated group as compared to the effect of standard drug (68, 43, and 21). But no significant difference between 40µg and 80µg DIM treated groups (52, 32, and 18). The results reveal the better anti-inflammatory effect at minimum dose of DIM (40µg/ml) and further *In vitro* studies are needed to understand the mechanism of action.

KEYWORDS

Inflammation, Diindolyl methane, Aspirin, Albumin denaturation and Hemolysis.

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INTRODUCTION

Chronic inflammation causes to generate the free radicals that resistant to the anti-oxidant and damage DNA due to cause of disease. Environmental pollutants are consisting of toxic substances. Such as synthetic fibers, latex, glues, adhesives, plastics, air fresheners, cleaning products and perfume are examples on a daily basis of chemicals that can produce an inflammatory

response (Denko.1992)[1]. Chronic exposure, at even low doses, can drive your immune system weak, resulting in inflammatory diseases. Inflammation is the reaction of living tissues to redness, swelling, injury, infection or irritation. It is a complex process, frequently associated with pain and increase in vascular permeability, increase of protein denaturation and membrane alterations (Umapathy *et al.,* 2010)[2].

Leukocytes, the important of inflammatory reaction, can reduce microbes and dead cells by phagocytosis, followed by their damage in phagolysosomes. Destruction is caused by free radicals generated by activated in the neutrophils, monocytes and lysosomal enzymes (Dray *et al.,* 1995)[3]. These are involved during inflammation produce a variety of disorders which leads to the tissues are damaged by the macromolecules and lipid peroxidation of membranes which are thought to be responsible for certain pathological conditions. The extracellular activities of these enzymes are associated to acute or chronic inflammation. Stabilization of lysosomal membrane is important from the limited to inflammatory response by preventing the release of lysosomal constituents and they are activated neutrophils such as bactericidal enzymes and proteases (Rajendran *et al.,* 2008)[4]. Such mediators are mainly involved in arachidonic acid metabolites, generated through cyclooxygenase and lipoxygenase pathways (Chaplin *et al.,* 2010)[5].

3, 3'-diindolylmethane (DIM) is a compound that is formed (I-3C) during the absorption of foods that the nutrient of indole-3-carbinol. It is found in cruciferous vegetables such as broccoli, cabbage, cauliflower and Brussels sprouts. DIM is also sold in supplemental form and is thought to supply a variety of health-promoting benefits and also exerts anti-carcinogenic effects in the body and is one of the reasons this vegetable family is seen as healthy.

DIM, a major acid-condensation product and has been shown to have multiple anticancer effects in experimental models. Recurrent or chronic inflammation is implicated in the development of human cancer (Han Jin Cho *et al.,* 2007)[6]. Naturally occurring phytochemcials are having anti-inflammatory property which plays an important role in prevention of chronic diseases. Hence the present study was hypothesized to analyse the anti-inflammatory effect of diindolylmethane through per cent inhibition of protein denaturation and hemolysis (membrane stabilization) in vitro experiments.

MATERIAL AND METHODS

Analysis of *in vitro* anti-inflammatory activity

Inhibition of albumin denaturation

The anti-inflammatory activity of DIM was considered by using inhibition of albumin denaturation technique which was studied according to Mizushima *et al*. (1968)[7] and Sakat *et al*. (2010)[8] followed with minor modifications. The reaction mixture was consists of 1ml of DIM different concentration and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N Hcl. The samples were incubated at 37ºC for 20 min and then heated to 51ºC for 20 min, after cooling the samples the turbidity was measured at 660nm using colorimeter. The experiment was performed in triplicate. Aspirin used as standard.

The percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition = (OD of Control –OD of Sample) X 100/ OD of control.

Membrane stabilization

Preparation of Red Blood Cells (RBCs) suspension

The Blood was collected from healthy human volunteer who has not taken any NSAIDs (Non-steroidal anti-inflammatory drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 revolution per 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline **(**Sakat *et al.,* 2010[8], Sadique *et al.,* 1989)[9].

Heat induced haemolysis

The reaction mixture (2ml) consisted of 1 ml test sample of different concentrations (20 - 80 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56ºC for 30min. At the stop of the incubation the tubes were chilled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was read at 560 nm. The experiment was performed in triplicates for all the test samples (Sakat *et al*[8], 2010, Shinde *et al.,* 1999)[10].

The percentage inhibition of haemolysis was calculated as follows:

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

Hypotonicity-induced haemolysis

Three different concentration of DIM (20-80 µg/ml) and control were separately mixed with 1ml of phosphate buffer, 2ml of hyposaline and 0.5ml of HRBC suspension. Aspirin (40µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37ºC for 30 min and centrifuged at 3000 rpm. The supernatant was decanted and the haemoglobin content was estimated by a spectrophotometer at 560nm **(**Azeem *et al.,* 2010)[11]. The percentage hemolysis was estimated by assuming the haemolysis produced in the control as 100%.

Percentage protection = 100- (OD sample/OD control) x 100

RESULTS

Inhibition of albumin denaturation

Protein denaturation is a process and losing their tertiary structure and secondary structure by the compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological molecules lose their function as a result, due to causes of inflammation. As part of the investigation on the mechanism of the anti-inflammation activity, ability of DIM to inhibit protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation. Maximum inhibition of 55% was observed at 80µg/ml. Aspirin, a standard anti-inflammation drug showed the maximum inhibition 68% at the concentration 40 µg/ml (Table No.1).

Heat induced haemolysis

The DIM was effective in inhibiting the heat induced haemolysis with increasing the concentration. Maximum 35% of inhibition of erythrocyte membrane lysis induced by heat was observed. Aspirin 40µg/ml offered a significant 43% inhibition of erythrocyte lysis.

Hypotonicity induced haemolysis

The results showed that DIM at concentration range of 20-80µg/ml protects the erythrocyte membrane against lysis induced by hypotonic solution. Aspirin (40µg/ml) offered a significant protection against the damaging effect of hypotonic solution. At the concentration of 80µg/ml, DIM showed 21% protection, whereas, Aspirin (40µg/ml) showed 20% inhibition of RBC haemolysis when compared with control.

DISCUSSION

Most of the phytochemicals are possessing anti-inflammatory properties. They are used to therapy of various chronic and infectious diseases. Epidemiology and experimental studies have been implicated oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems as the primary cause of cardiovascular, diseases, cancer, aging etc (Halliwell, 1996)[12].

Similarly, Leelaprakash *et al*. (2010)[13] reported that methanol extract of whole plant *Enicostemma axillare* was produce significant anti-inflammatory activity in dose dependent manner in inhibition of protein denaturation. Padmanaban *et al*. (2012)[14] reported that inhibition of albumin denaturation of alcoholic extract showed significant inhibition of albumin denaturation.

The *In vitro* anti-inflammatory activity of DIM reported in the study can be attributed that DIM capable of stabilizing red blood cell membranes against heat and hypotonic induced lyses. DIM exhibited membrane stabilization effect by

inhibiting hypo tonicity induced lysis of erythrocyte membrane. The erythrocyte membrane is related to the lysosomal membrane and its stabilization implies as well as stable. Stabilization of lysosomal membrane is important from the limited to inflammatory response by preventing the release of lysosomal constituents. The membrane stabilization by the process of hypo tonicity-induced hemolysis may arise from shrinkage of the cells due to osmotic loss of intracellular electrolyte and fluid components.

Similarly, Seema Chaitanya Chippada *et al.* (2011)[15] studied the stabilization of the HRBCs membrane by hypo tonicity induced membrane lysis and heat induced haemolysis to establish the mechanism of anti-inflammatory action of *C. Asiatica*.

The results indicate that the plant extract inhibit the HRBCs membrane lysis by stabilizing membrane integrity due to its anti-inflammatory. Results of our findings confirmed that the use of DIM inhibits albumin heat denaturation and hemolysis via maintaining the stability of membrane compared with standard NSAID which clearly indicating its anti-inflammatory potential. A study also reported that DIM significantly decreased the release of nitric oxide (NO), prostaglandin (PG) E2, tumor necrosis factor-α (TNF-α)interleukin (IL)-6, and IL-1β by RAW264.7 cells treated with LPS.

Table No.1: Effect of DIM on heat induced protein denaturation

S.No	Treatment(s)	Concentration (µg/ml)	Absorbance at 660nm	% inhibition of Protein denaturation
1	Control	-	0.129 ± 0.02	-
2	DIM	20	0. 065 ± 0.03	49
3	DIM	40	0.062 ± 0.05	52
4	DIM	80	0.059 ± 0.06	55
5	Aspirin	40	0.041 ± 0.04	68

Each value represents the mean ± SD. N=3

Table No.2: Effect of DIM on heat induced haemolysis of erythrocyte

S.No	Treatment(s)	Concentration (µg/ml)	Absorbance at 560nm	% inhibition of haemolysis
1	Control	-	0.120 ± 0.03	-
2	DIM	20	0.080 ± 0.05	31
3	DIM	40	0.078 ± 0.04	33
4	DIM	80	0.076 ± 0.02	35
5	Aspirin	40	0.064 ± 0.06	43

Each value represents the mean ± SD. N=3

Table No.3: Effect of DIM on hypo tonicity induced haemolysis of erythrocyte

S.No	Treatment(s)	Concentration (µg/ml)	Absorbance at 560 nm	% inhibition of haemolysis
1	Control	-	4.14 ± 0.05	-
2	DIM	20	3.66 ± 0.03	11
3	DIM	40	3.40 ± 0.06	18
4	DIM	80	3.23 ± 0.02	21
5	Aspirin	40	3.33 ± 0.04	20

Each value represents the mean ± SD. N=3

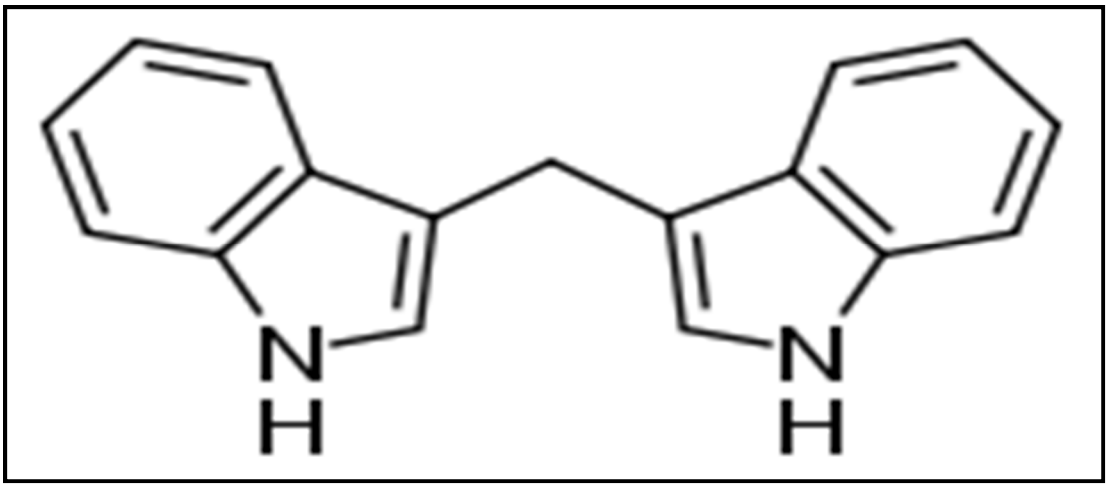

Figure No.1: Structure of 3, 3'-diindolylmethane

CONCLUSION

In the present study, results indicate that the 3, 3'diindolylmethane possess anti-inflammatory properties. DIM inhibited the heat induced albumin denaturation, haemolysis and hypo tonicity of erythrocytes may be due to free radical inhibitors /scavenger/ acting possibly as primary oxidants. The compound of 3, 3'diindolylmethane can be used designing a potent anti-inflammatory drug which can be used for treatment of various diseases such as cancer, neurological disorder, aging and inflammation.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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