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Anti-inflammatory study of Solamargin isolated from *Solanum xanthocarpum*

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Abstract

Solanum xanthocarpum is also called from different name in various different languages in India. The *Solanum xanthocarpum* is a very prickly diffused bright-green, perennial herb of the family *Solanaceae*. It active constituents Solamargin reported Ayurvedic system of medicine has been found to have anti-asthematic, hypoglycemic, hepatoprotective, anti-inflammatory, antipyretic and nephron-protective activities. Inflammation is a complex response to local injury or other trauma; it is characterized by redness, heat, swelling, and pain. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical and/or microbial agents. Inflammation is the body response towards foreign substances such as pathogens, allergens, chemical irritants as well as injury which involves infiltration of leukocytes and generation of pro-inflammation factors to the injured site. Major compounds co-eluted at 40% acetonitrile as a white-powder and were separated using preparative TLC to afford compoundof steroidal alkaloid.

The identities of the isolated compounds were confirmed using Nuclear Magnetic Resonance Mass-Spectrometry analysis. Compounds were analysed using different NMR techniques such as H-1-NMR, C-13-NMR and correlation spectroscopy (COSY) to accurately determine structural moieties. Mass Lynx 4.1 software was used for analysis of mass spectrometry data and the fragmentation patterns of the isolated compounds were identified using Waters Mass Fragment software as Solamargin. High resolution mass spectrometry (HRMS) data for compound 1 yielded the molecular formula $C_{45}H_{73}NO_{15}$ with a molecular mass of *m/z* (mass to charge ratio) 868.5077 ($[M + H]^+$, 100%) which was identified as the steroidal alkaloid, solamargine. The highest percentage of inhibition for Group 2 was at the 5^{th} hour followed by the 1^{st} hour of monitoring and Group 6 showed the highest percentage of inhibition amongst the Solamargin pretreated groups (Group 3, 4 and 5) at all the monitored time intervals and manifested a similar pattern as Group 2.

Key words : *Solanum xanthocarpum* , Solamargin, Anti-inflammatory, Steroidal alkaloids

Introduction

The *Solanum xanthocarpum* is a very prickly diffused bright-green, perennial herb of the family *Solanaceae*. It is found abundantly throughout India in plains of dry regions, by roadsides, wastelands and rubbish heaps[1]. The *S. xanthocarpum*, a plant from dashmula[2] of Ayurvedic system of medicine has been found to have anti-asthematic, hypoglycemic, hepatoprotective, anti-inflammatory, antipyretic and nephron-protective activities[3]. The leaves and fruits of *S. xanthocarpum* contains steroidal alkaloids such as Solamargin has been shown to possess such properties[4]. This work was aimed to estimate whether or not seeds from raw fruits and leaf of *S. xanthocarpum in vitro* exhibits anti-inflammatory and antioxidant activity. Inflammation is a complex response to local injury or other trauma; it is characterized by redness, heat, swelling, and pain. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical and/or microbial agents. It is the body's response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair[5,6]. The response can sometimes be alarming including allergies; autoimmune diseases, microbial infections, transplants, and burns may initiate a chronic inflammatory response. Various therapeutic approaches are available for reducing long-term inflammatory responses and thus the complications associated with them. The major approach used presently includes use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). The duration of their use is limited by gastrointestinal side effects that include unease and abdominal pain and in a few serious cases bleeding or perforation of the stomach or upper GI tract[7,8]. The NSAIDs has been reported to cause transient imbalance in electrolyte and water levels as well as liver and renal toxicity[8,9].

Inflammation is the body response towards foreign substances such as pathogens,

allergens, chemical irritants as well as injury which involves infiltration of leukocytes and generation of pro-inflammation factors to the injured site. Inflammation helps in mitigating the effects of harmful micro-organism and remove dead cell which may prevent further development of irritation and allows the injured tissue to recover to normal condition. Reactive oxygen species (ROS) is the product of cellular aerobic metabolism giving rise to both harmful and beneficial effects to the body. Inequality between antioxidants and oxidants in the body is detrimental to the integrity of macromolecules and body cells[10]. Free radicals generation is one of the causes of inflammation, while excessive and persistent inflammation leads to undesirable pathologic conditions such as rheumatoid arthritis, neurogenerative diseases, cancer, asthma and inflammatory bowel disease[11].

MATERIALS AND METHOD

Plant material- Ripened leaves and fruits of *S. xanthocarpum* were gifted from the field and road side of Ayodhya, where the identity was confirmed by Prof. R.K. singh, Advance Study Centre, Department of Botany, B.H.U. Varanasi. Specimens are available within the botanical gardens, while plant material is stored within the Department of Pharmacology as section of Chemistry Department and powdered plant material. Leaves and Fruits were cut and air- dried at ambient temperature. Plant material was ground to a fine powder (Yellowline A10, Merck (Pty) Ltd) and stored in air-tight, amber containers until needed.

Preparation of crude extract: Ground plant material (150 g) was sonicated in 1.5 L methanol for 30 min, after which it was agitated for 2 h on a shaker. The solution was incubated for an additional 16 h at 4 °C. The supernatant was collected, and the marc re-extracted five more times. Supernatants were pooled, centrifuged at 500 *g* for 1 min,

vacuum filtered (0.2 µm filters, Waters Corporation) and concentrated using in vacuo rotary evaporation. Dried crystals were resuspended in distilled water (dH_2O) and lyophilized (Freezone® 6 Freeze Dry System, Labconco) to yield a dry, yellow powder (17.98% *w*/w).

Preparation of alkaloid-enriched fractions: Alkaloid-enriched fractions were prepared as described by Munari[12] with modifications to the volumes used. Two organic fractions were obtained by sequential liquid-liquid extraction with diethyl ether and chloroform. The crude extract (26.72 g) was acidified with 2% acetic acid (267.2 mL) on a shaker for 2 h. Diethyl ether (534.4 mL) was added to the acidified mixture, shaken for 20 min and the organic phase siphoned off after separation. This procedure was repeated four times, and all di- ethyl ether fractions combined. After fractionation, the aqueous phase was fractionated with chloroform as described above. Organic fractions were clarified with anhydrous sodium sulphate (10% *w*/*v*). The two organic fractions (diethyl ether [8.98% *w*/w] and chloroform [0. 15% w/w]) and the aqueous alkaloid-enriched fraction were concentrated using in vacuo rotary evaporation and lyophilisation, respectively. All samples were dissolved in dimethyl sulfoxide (DMSO). Aliquots (20 mg/mL) were stored at -80 °C until needed.

Test for the presence of steroidal alkaloids: Samples (20 µg) were spotted onto a C10 silica plate (5×10 cm, Agilent Technologies South Africa) and developed in a mobile phase consisting of chloroform, acetone and methanol (4:4:2). Plates were visualized using ultraviolet light (UV, at 254 and 366 nm), sprayed with Dragendorff's reagent and developed in an oven at 60 °C.

Isolation of active constituents

Silica gel was mixed with chloroform and methanol (3:2) and poured into a cotton wool plugged glass column (2.7×50.5 cm). The aqueous alkaloid-enriched fraction was dissolved in a hydromethanolic solution (10% dH_2O), mixed with silica, left at room temperature to dry and loaded on top of the packed silica gel column. Sub- fractions were collected in glass tubes, monitored using TLC, and compared to sub-fractions 10 and 11 as reference. Similar sub-fractions were pooled together and further purified by solid phase extraction chromatography using a ISOLUTE flash C18 column with a mobile phase of acetonitrile and dH_2O (starting at 100% dH_2O, followed by 5% acetonitrile, then 10% acetonitrile and increasing to 100% acetonitrile in 10% increments). Major compounds co-eluted at 40% acetonitrile as a white-powder and were separated using preparative TLC (solvent system: methanol, ethyl acetate and acetone; 4:4:2) to afford compound of steroidal alkaloid.

The identities of the isolated compounds were confirmed using Nuclear Magnetic Resonance (NMR, 600 MHz VNMRS, Varian) Mass-Spectrometry (Synapt G1 UPLC-QTOF-HDMS system, Waters, USA) analysis. Compounds were analysed using different NMR techniques such as H-1-NMR, C-13-NMR and correlation spectroscopy (COSY) to accurately determine structural moieties. Mass Lynx 4.1 software was used for analysis of mass spectrometry data and the fragmentation patterns of the isolated compounds were identified using Waters Mass Fragment software.

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RESULTS

Structural elucidation of steroidal alkaloids

Intense black and violet spots were observed for the crude extract and alkaloid-enriched fraction on TLC plates under short (254 nm) and long (366 nm)-wave-length UV light, respectively. Orange spots were seen after spraying with Dragendorff's reagent. The aqueous alkaloid-enriched fraction was sub-fractionated into eleven fractions. Major compounds were identified in sub-fractions 10, which were not visible under UV light, but appeared after vanillin-spraying. Compound 1 appeared as white crystals.

The structure of compound 1 was identified by H-1-NMR, C-13-NMR, 2-D data analysis. C-13-NMR revealed that compound 1 possesses an aglycone backbone related to a steroidal spirazolane-type alkaloid. Four quaternary carbons at chemical shifts (δ_c's) 38.2, δ_c 41.8 ppm including one linked to oxygen and nitrogen at δ_c 99.6 as well as one attached to a double bond at δ_c 142.1, nine methine groups at δ_c's 31.8, 31.8, 42.9, 51.9, 57.9, 64.2, 79.5, 80.5, 122.8, ten methylene groups at δ_c's 22.2, 30.9, 31.1, 32.9, 33.1, 33.4, 38.7, 39.7, 41.2, 48.5 ppm and four methyl groups at δ_c's 15.6, 17.0, 19.9, 19.9 ppm were observed. An ether function with a trisaccharide moiety showing an anomeric carbon at δ_c 100.6 linked to the oxygen of C-3 at δ_c 79.5 was also present (Table-1). The 1D NMR chemical shifts of the trisaccharide moiety indicated the structure *O*-[α-L-rhamnopy-ranosyl-(1→2)-*O*-[□-L-rhamnopy -ranosyl-(1→4)]-□-D-glucopyranoside. The proton NMR showed four distinctive aglycone methyls at δ_c 0.83 (3H, s), 0.85 (3H, d, *J* [coupling constant] 6.4), 0.97 (3H, d, *J* 7.2) and 1.05 (3H, s) (Table-1).

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Table-1: H-1NMR and C-13-NMR resonances of compound 1 compared to solamargine

		Solamargine (deuterated chloroform and methanol)		Compound 1 (2015) deuterated methanol)	
	Carbon no (type)	**C13-NMR**	**H-1-NMR (J in Hz)**	**C13-NMR**	**H-1-NMR (J in Hz)**
Aglycone	C-1 (CH_2)	37.9		38.70	
	C-2 (CH_2)	30.5		31.13	
	C-3 (CH)	79		79.50	
	C-4 (CH_2)	40.4		41.19	
	C-5 (C)	141.1		142.07	
	C-6 (CH)	122.2	5.37 (d, J 4.2)	122.78	5.38 (d, J 4.6)
	C-7 (CH_2)	32.5		32.94	
	C-8 (CH)	32.1		31.81	
	C-9 (CH)	50.7		51.90	
	C-10 (C)	37.5		38.20	
	C-11 (CH_2)	21.4		22.15	
	C-12 (CH_2)	38.9		39.67	
	C-13 (C)	41.1		41.77	
	C-14 (CH)	57.1		57.90	
	C-15 (CH_2)	30.1		30.90	
	C-16 (CH)	79.7	4.33 (m)	80.45	4.34 (m)
	C-17 (CH)	63.2		64.21	
	C-18 (CH_3)	16.8	0.84 (s)	17.00	0.83 (s)
	C-19 (CH_3)	19.6	1.05 (s)	19.91	1.05 (s)
	C-20 (CH)	42		42.85	
	C-21 (CH_3)	15.4	0.97 (d, J 7.0)	15.60	0.97 (d, J 7.2)
	C-22 (C)	98.9		99.60	
	C-23 (CH_2)	34.4		33.36	
	C-24 (CH_2)	32.7		33.14	
	C-25 (CH)	31.3		31.81	
	C-26 (CH_2)	47.8	2.6 (m, br)	48.47	2.53 (m)
	C-27 (CH_3)	19.6	0.87 (d, J 6.0)	19.98	0.85 (d, J 6.4)
Glucose (glu)	C-1′ (CH)	99.9	4.48 (d, J 7.8)	100.63	4.50 (d, J 7.6)
	C-2′ (CH)	79.5		80.23	
	C-3’(CH)	77.3		78.19	
	C-4′ (CH)	75.7		76.73	
	C-5′ (CH)	78.6		79.50	
	C-6′ (CH_2)	61.4		62.12	
Rhamnose A (rha A)	C-1″ (CH)	102.4	4.87 (s)	103.16	4.84 (s)

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	C-2″ (CH)	71.8		72.60	
	C-3″ (CH)	71.5		72.35	
	C-4″ (CH)	73.3		74.10	
	C-5″ (CH)	69.1		69.94	
	C-6″ (CH_3)	17.7	1.29 (m)	18.11	1.26 (m)
Rhamnose B (rha B)	C-1‴(CH)	101.5	5.23 (s)	102.44	5.21 (s)
	C-2‴(CH)	71.6		72.52	
	C-3‴(CH)	71.3		72.35	
	C-4‴(CH)	73		73.89	
	C-5‴(CH)	70.1		70.85	
	C-6‴(CH_3)	17.6	1.29 (m)	18.0.	1.26 (m)

Two multiplets observed at δ_c 1.26 were attributable to 6-deoxyhexose methyls whereas a doublet at δ_c 5.38 with *J* value of 4.6 could be attributed to an olefinic proton at position C-6. Four anomeric H-1-NMR resonances were observed at δ_c 4.34 (1H, m), 4.50 (1H, d, *J* 7.6), 4.84 (1H, s) and 5.21 (1H, s) (Table-1). High resolution mass spectrometry (HRMS) data for compound 1 yielded the molecular formula $C_{45}H_{73}NO_{15}$ with a molecular mass of *m/z* (mass to charge ratio) 868.5077 ($[M + H]^+$, 100%) which was identified as the steroidal alkaloid, solamargine (Fig.-1).

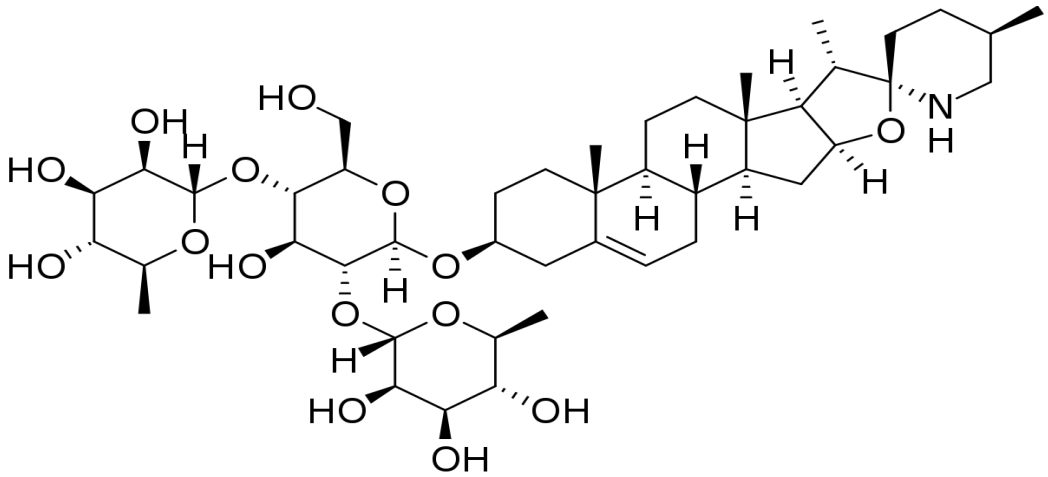

Fig.1: Solamargin

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Biological study: The leaves and fruit of *S. xanthocarpum* showed evidence of anti-inflammatory activity, which was reported by careenage induced albino rat[13]. The leaves and active compound were raised to form callus and Solamargin was used to detect antioxidant activity and anti-inflammatory activity by thiobarbituric acid reactive substances[13]. Solamargin exhibit anti- inflammatory activity as mention in many literatures. The sequential extraction with hexane, benzene, chloroform, ethyl acetate, acetone, ethyl alcohol and water of *S. xanthocarpum* leaves and fruits exhibited antioxidant activity[14]. In this study for the first time, Solamargin were evaluated for their anti-inflammatory activity on albino rats.

Table-2: Effects of different dose of Solamargin treatments on the increase of paw edema thickness (cm) and the percentage of inhibition that monitored at every time interval (hr).

Group	**Dose (mg/kg) b.w.**	**Increases thickness (1st hour)**		**Increases thickness (2nd hour)**		**Increases thickness (3rd hour)**		**Increases thickness (4th hour)**		**Increases thickness (5th hour)**	
		cm	**(%)**	**cm**	**(%)**	**cm**	**(%)**	**cm**	**(%)**	**cm**	**(%)**
Control	0	0.154	0.00	0.229	0.00	0.270	0.00	0.283	0.00	0.283	0.00
2	0.2	0.111	27.92	0.188	17.90	0.233	13.70	0.219	22.62	0.187	33.92
3	0.4	0.146	5.19	0.217	5.24	0.254	5.93	0.274	3.18	0.269	4.95
4	0.6	0.151	1.95	0.214	6.55	0.253	6.30	0.259	8.48	0.248	12.37
5	0.8	0.116	24.68	0.200	12.66	0.250	7.41	0.243	14.13	0.229	9.08
6	1.0	0.116	24.68	0.197	13.97	0.236	12.59	0.221	21.91	0.200	29.33

The albino rats (150-200 gm/kg body weight) were pre-treated with either aspirin, different doses of alkaloid extract of *S. xanthocarpum* alkaloid leaf extract or vehicle via oral force feeding one hour prior to subplantar injection to 0.2 ml of 1% (w/v) carrageenan suspension to their right hind paw. The time when the injection was given was recorded as 0 h, while the paw thickness of each rat was measured using vernier caliper just before the injection was carried out. One hour after induction of the edema, the thickness of the paw of each rat was measured and the

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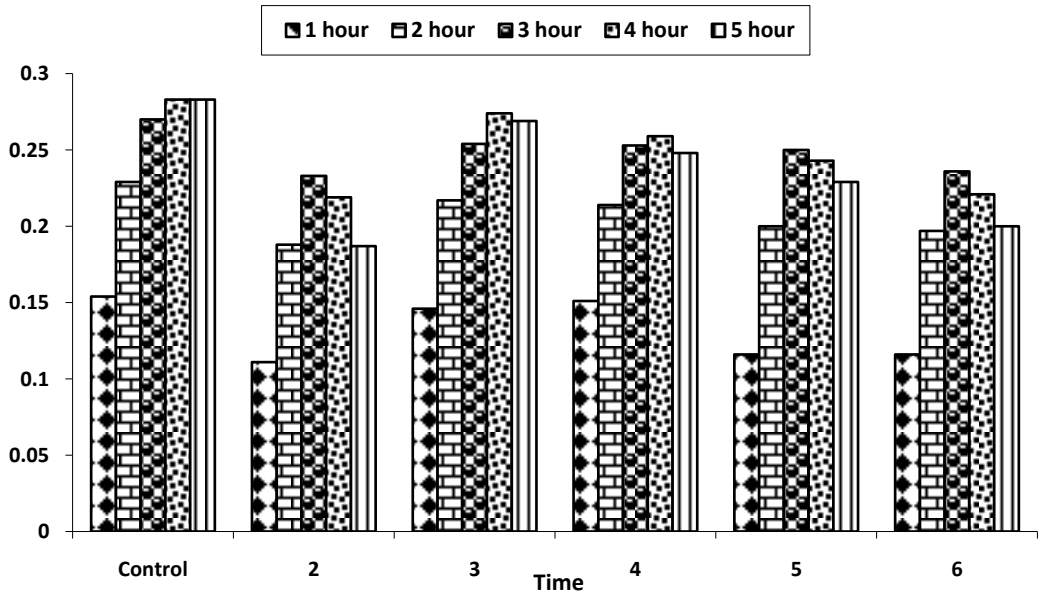
time was recorded as first (1st) hour, then the paw's thickness was subsequently measured at hourly basis up to the 5th hour. The increase of paw thickness for every hour was calculated as difference of paw thickness at particular (t) hour to 0h and presented as mean increase of paw thickness (cm). The anti-inflammatory effect of standard drug and Solamargin alkaloid leaf extract was expressed as percentage of inhibition (%) which was using the calculated formula = [1-(C_t-C_0) treated group/(C_t-C_0) control group]×100% where C_0 is mean paw thickness measured at time 0 h and Ct is the mean paw thickness measured at particular time point[3]. Observations of above experiment are mentioned in Table-2.

Fig.2: Effects of different dose of Solamargin treatments



The gross photographs showed the paw edema thickness of the various pretread groups of rats at the 5th hour after the induction of edema, Group 2, 5 and 6 showed better effects in reducing the edema upon the carrageenan induction when compared to Group 1. Group 1 rats pretreated with 2% Tween 20 showed paw edema thickness increased in time dependent manner

upon 1 h of induction, reached its maximum at 4th hour and remained the same at the last hour of monitoring. Group 2 pretreated with Aspirin showed significant difference of paw edema thickness when compared to Group I at the 1st, 2nd, 4th, and 5th hour of time interval after induction of edema. Groups 3 and 4 did not show any significant differences when compared to Group 1 at all-time intervals. Group 6 showed significant anti-inflammatory effect when compared to Group 1 at time interval of the 1st, 4th and 5th hour and Group 5 showed similar pattern as Group 6 except at the 4th hour. The highest percentage of inhibition for Group 2 was at the 5th hour followed by the 1st hour of monitoring and Group 6 showed the highest percentage of inhibition amongst the Solamargin pretreated groups (Group 3, 4 and 5) at all the monitored time intervals and manifested a similar pattern as Group 2.

Inhibition of albumin denaturation

The denaturation of proteins as one of the causes as inflammation has been well documented. A number of anti-inflammatory drugs have been known to inhibit protein denaturation[15]. To investigate the ability of anti-inflammatory activity of Solamargin albumin denaturation inhibition assay was performed. The inhibition of albumin denaturation by Solamargin at concentration 0.8 mg/kg b.w. was effective (25%) than 1.0 mg/kg b.w. of Solamargin (Table-2).

Discussion

Inflammation is a complex response to local injury or other trauma; it is characterized by redness, heat, swelling, and pain. Lysosomal enzymes released during inflammation produces a variety of disorders which leads to the tissue injury by damaging the macromolecules and lipid peroxidation of membranes. Stabilization of lysosomal membrane is important in limiting the

inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane. Human red blood cells (HRBC) or erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of HRBC by heat induced membrane lysis can be taken as an *in vitro* measure of anti-inflammatory activity of the drugs or plant extracts[16]. Erythrocyte membrane stabilization was studied by incubating erythrocyte with SE and LE. Results shown in Figure-2 LE was able to stabilize erythrocyte membrane better to that of SE. In a parallel experiment, erythrocyte membrane stabilization studies were carried with acetone, ethyl acetate and aqueous extracts prepared from seeds. Results shown that membrane stabilization was found with acetone extracts only whereas both ethyl acetate and aqueous extracts failedto stabilize erythrocyte membrane.

It is known that during inflammatory disorders there is an excessive activation of phagocytes, production of reactive oxygen species, nascent oxygen, hydroxyl and hydrogen peroxide, which could harm surrounding tissue. This in turn would initiate lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors. The reactive oxygen species are also known to activate MMP. The collagenase and MMP is known to cause increased tissue destruction demonstrated in various arthritic reactions. Hence, the agents that can scavenge these reactive oxygen species would be of greater interest in treatment[17].

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