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IN VITRO ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF HYDRO ALCOHOLIC LEAVES EXTRACT OF *ROTULA AQUATICA* LOUR

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ABSTRACT

The present study aimed to evaluate the *In vitro* antioxidant and anti-inflammatory activity of hydro alcoholic extract of leaves of *Rotula aquatica Lour*.The antioxidant activity of the extract was assessed by ferric reducing power assay, superoxide radical scavenging assay and hydrogen peroxide radical scavenging assay using ascorbic acid as the standard. Anti-inflammatory activity of the extract was evaluated using HRBC membrane stabilization method and protein denaturation method where diclofenac sodium was used as the standard. The results indicated that the hydro alcoholic extract showed significant antioxidant and anti-inflammatory activity in a dose-dependent manner. The extract demonstrated potent scavenging of free radicals such as hydrogen peroxide and superoxide and possessed high reducing power against the standard ascorbic acid. Potent inhibition of HRBC membrane lysis and protein denaturation proved its anti-inflammatory activity when compared to standard diclofenac sodium. The finding powerfully supports that *Rotula aquatica Lour* leaves extract could be used as a natural antioxidant and anti-inflammatory agent attributed to its rich secondary metabollites.

Key words: *Rotula aquatica Lour*, Hydro alcoholic extract, Antioxidant activity, Anti-inflammatory activity.

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INTRODUCTION

Reactive oxygen species are significant compounds produced in limited quantities in the body and are involved in the control of important functions such as gene expression, signal transduction, receptor activation and maintenance of homeostasis (S Kumar and AK Pandey, 2015) during the cell respiration process (Goossens V *et al.*, 1999). There may also be the production of nitric oxide at the time of hypoxia (Poyton RO *et al.*, 2009). These pro-oxidants in the form of ROS and RNS are efficiently kept in check by the antioxidant levels that act as a defense mechanism in a normal healthy human being (Devasagayam TP *et al.*, 2004) as they can play dual roles, both as beneficial and toxic agents in living systems (Vako M *et al.*, 2007). Normally, the redox state of a biological system is sustained at a negative redox potential (Kunwar A and Priyadasini KI, 2011) which when becomes susceptible to detrimental environmental, physicochemical or pathological agents including atmospheric pollutants, cigarette smoking, ultraviolet rays, toxic chemicals and advanced glycation end products (AGEs), cause an alteration in this finely maintained balance in favor of pro-oxidants that results in oxidative stress (Devasagayam TP *et al.*, 2004). Oxidative stress then targets the lipids, proteins, DNA/RNA ultimately causing changes in these molecules and may induce somatic mutation and neoplastic transformation if

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the ROS are not removed immediately (Chang CH *et al.*, 2010). Immoderate levels of ROS/RNS that are produced during the process of oxidative metabolism induct the process of inflammation which is normally a defense mechanism generated against pathogens and results in the synthesis of proinflammatory mediators which leads to chronic diseases (Hussain T *et al.*, 2016).

There is a decrease in the health status and an increase in the probability of chronic diseases such as cancer, atherosclerosis, Alzheimer's disease, metabolic disorders and so on due to the oxidative stress and oxidative damage that play an important role in the pathophysiology of many inflammatory diseases (He Y *et al.*, 2015) as both oxidative stress and inflammation can produce injury to cells (Ng CY *et al.*, 2012). Ethnomedicinal plants due to its ability to synthesize an array of bioactive constituents such as carotenoids, flavonoids, phenolic acids, phytoestrogens, isoprenoids and alkaloids remains superior to prescription medicicne and shows potent antioxidant and anti-inflammatory effects. Thus, they can act as therapeutic candidates for various diseases (Tiwari BK *et al.*, 2013).

Rotula aquatica Lour belonging to the family *Boraginaceae* is displayed by about 100 genera and 2000 species. This is found throughout Peninsular and Western Ghats of India; in the sandy and rocky beds of rivers. The plant is abundant with compounds such as Baunerol, steroid, alkaloid, rhabdiol and allantoin, flavonoids, phenolic compounds and other nutrients like amino acid and proteins (Vysakh A *et al.*, 2016) which may be responsible for its various actions like anthelmintic activity (Laksmi VK *et al.*, 2012), anti-diarrheal (Singh S *et al., 2012*), anti-diabetic activity (Shyam T *et al., 2013*), antimitotic activity (Patil S *et al.*, 2004), anti-urolithiac activity (Gihotra UK and Christina AJM, 2011), antibacterial and antioxidant activity (Aswathanarayan JB and Vittal RR, 2013) and anti-inflammatory action (Kamurthy H *et al.*, 2014).

The present study was aimed to evaluate the *In vitro* antioxidant and anti-inflammatoryactivity of hydro alcoholic extract of leaves of Rotula aquatica Lour by standard procedures.

MATERIALS AND METHODS

Collection and identification of plant material

The leaves of *Rotula aquatica Lour* were collected from the rocky beds of rivers and streams of Kozhichal, Payyanur, Kannur district, Kerala in the month of November, 2016. The plant was identified and authenticated and the herbarium specimen APSC/COL/08/2016 was deposited in Pharmacology department of college. After authentication the leaves were collected, cleaned and dried in shade for 3 weeks at room temperature. The dried leaves were pulverized in a mechanical grinder to obtain coarse powder.

Extraction of the Powdered Plant Material

The powdered leaves of the plant were sieved through sieve no.40 and the powder was subjected to defatting with petroleum ether for 6 hours. The powder (100g) was subjected for maceration with 500 ml of mixture of ethanol: water (7:3) for 48 hours. The extract was then shaken, filtered through muslin cloth and marc was discarded. The filtrate obtained by maceration was evaporated and stored in refrigerator until further use as hydro alcoholic extract of *Rotula aquatica Lour* (HAERA).

Primary phytochemical screening

For preliminary phytochemical screening, freshly prepared hydro alcoholic extract of leaves were tested for the presence and absence of phytoconstituents such as carbohydrates, alkaloids, glycosides, tannins, flavonoids, phenolic compounds, steroids by using standard methods (Evans *et al.*, 2003).

In vitro Antioxidant Activity

Ferric reducing power assay

- Different concentrations of HAERA (12.5 – 200 µg/ml) were mixed with 2.5 ml of 0.2 M phosphate buffer (pH-6.6) and 2.5 ml of 1% potassium ferric cyanide.
- This mixture was kept at 50^{o}c in water bath for 20 minutes.
- After incubation, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 minutes.
- The upper layer of solution (5 ml) was mixed with 5 ml of distilled water and 1 ml of freshly prepared 0.1% of ferric chloride solution and the absorbance was read at 700nm.
- Control was prepared in a similar manner excluding samples.
- Ascorbic acid was used as the standard (Ferreira ICFR *et al.*, 2007).

The percentage reducing power was calculated by using the formula

$$\text{Reducing power} = \frac{\text{optical density of control} - \text{optical density of test}}{\text{optical density of control}} \times 100$$

Superoxide free radical scavenging assay

- Different concentrations of sample (125-200 µg/ml) from a stock solution of 10 mg/ml, 0.05 ml of Riboflavin solution (0.12 mM), 0.2 ml of EDTA solution [0.1M], and 0.1 ml NBT (Nitro-blue tetrazolium) solution [1.5 Mm] were mixed in a test tube.
- The reaction mixture was diluted up to 2.64 ml with phosphate buffer [0.067 M].
- A control without the test compound but with an equivalent amount of distilled water was taken.
- The absorbance of solution was measured at 560 nm after illumination for 5 minutes in fluorescent light and

also measured after illumination for 30 minutes at 560 nm on UV-Visible spectrophotometer (Valentao P *et al.*, 2001).

$$\% \text{ Inhibition} = \frac{optical\ density\ of\ control - optical\ density\ of\ test}{optical\ density\ of\ control} \times 100$$

Hydrogen peroxide radical scavenging assay

- A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4).
- Different concentrations of extracts (125 -2000 µg/ml) from a stock concentration of 10 mg/ml was added to H_2O_2 solution (0.6 ml).
- A control without the test compound but an equivalent amount of distilled water was taken.
- Optical density was read at 230 nm after 10 minutes (Sasikumar V and Kalaisezhiyen, 2014).

$$\% \text{ Inhibition} = \frac{optical\ density\ of\ control - optical\ density\ of\ test}{optical\ density\ of\ control} \times 100$$

In vitro Anti-Inflammatory Activity

The human red blood cell (HRBC) membrane stabilisation method

- The blood was collected from healthy human volunteer who had not taken any NSAID's for two weeks prior to the experiment.
- The collected blood was mixed with equal volume of Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, 0.42% sodium chloride) and centrifuged at 3000 rpm.
- The packed cells were washed with isosaline and a 10% suspension was made.
- Various concentrations of HAERA were prepared by using distilled water and to each concentration 1 ml of phosphate buffer, 2 ml hypo saline and 0.5% of HRBC suspension were added. It was incubated at 37^{o}c for 30 minutes and centrifuged at 3000 rpm for 20 minutes.
- The haemoglobin content of supernatant solution was estimated spectrometrically at 560 nm.
- Diclofenac sodium was used as the standard and control was prepared by omitting the extracts (Varghese CP *et al.*, 2012; Nagaharika Y *et al.*, 2013).

The percentage of HRBC membrane stabilization or protection was calculated by using the following formula,

$$\% \text{ protection} = \frac{optical\ density\ of\ drug\ treated\ sample}{optical\ density\ of\ control} \times 100$$

$$\%\text{inhibition} = 1 - \frac{optical\ density\ of\ drug\ treated\ sample}{optical\ density\ of\ control} \times 100$$

Protein denaturation method

- Dilutions of HAERA and standard drug (diclofenac sodium) were prepared in the concentrations of 12.5-200 µg/ml.
- To 0.2 ml egg albumin, 2.8 ml phosphate buffered saline (PBS) of pH 6.4 and 2 ml of varying concentration of extracts or standard were mixed.
- Similar volume of distilled water was taken as control.
- The mixtures were incubated at 37^{o}c in the incubator for 15 minutes and then heated at 70^{o}c for 5 minutes.
- After cooling, absorbance was measured at 660 nm by using vehicle as blank (Chandra S *et al.*, 2012).

The percentage inhibition of protein denaturation was calculated by using the formula;

$$\% \text{ Inhibition} = 1 - \frac{absorbance\ of\ test}{absorbance\ of\ control} \times 100$$

RESULTS

Preliminary phytochemical screening

A dry dark green extract with a percentage yield of 14% w/w was obtained. In the phytochemical analysis, HAERA revealed the presence of carbohydrates, proteins, amino acids, steroids, flavonoids, phenolic compounds, tannins, alkaloids, glycosides, anthraquinones and triterpenoids.

Evaluation of Antioxidant Activity

In vitro antioxidant activity of HAERA by Ferric reducing power assay

In vitro antioxidant activity of the HAERA was evaluated using ferric reducing power assay method. The extract exhibited antioxidant activity in a dose dependent manner. HAERA showed a maximum absorbance of 1.456±0.004 and standard, ascorbic acid exhibited the maximum absorbance of 1.628±0.002 at a concentration of 200µg/ml. The results were compared to the standard ascorbic acid and the values are shown in Table 1 and graphically depicted in Figure 1.

In vitro antioxidant activity of HAERA by Superoxide radical scavenging assay method

Antioxidant potential of the HAERA was evaluated using superoxide radical scavenging assay method. The extract exhibited antioxidant activity in a dose dependent manner. HAERA showed a maximum inhibition of 88.52%, while the standard ascorbic acid exhibited the maximum inhibition of 90.77% at concentration of 200µg/ml. The results are summarized in Table 2 and depicted in Figure 2.

In vitro antioxidant activity of HAERA by hydrogen peroxide radical scavenging assay method

In vitro antioxidant activity of the HAERA was evaluated using Hydrogen peroxide radical scavenging method. The extract exhibited antioxidant activity in a dose dependent manner. At a concentration of 200µg/ml,

HAERA showed a maximum inhibition of79.69% of 85.71%. The results are tabulated in Table 3 and depicted in Figure 3.

Evaluation of Anti-Inflammatory Activity

In vitro anti-inflammatory activity of HAERA by HRBC membrane stabilization method

In vitro anti-inflammatory activity of hydro alcoholic extract of leaves of *Rotula aquatica Lour* was evaluated using HRBC membrane stabilization method. HAERA exhibited anti-inflammatory activity in a dose dependent manner. The extract showed maximum inhibition of 74.71%, while standard diclofenac sodium exhibited inhibition of 77.73% at concentration of whereas the standard, ascorbic acid showed an inhibition 200µg/ml. The results are shown in Table 4 and depicted in Figure 4.

In vitro anti-inflammatory activity of HAERA by protein denaturation method

In vitro anti-inflammatory activity of hydro alcoholic extract of leaves of *Rotula aquatica Lour* was evaluated using protein denaturation method. HAERA exhibited anti-inflammatory activity in a dose dependent manner. The extract showed maximum inhibition of 76.69% whereas the standard diclofenac sodium exhibited inhibition of 80.06% at 200 µg/ml concentration. The results are tabulated in Table 5 and depicted in Figure 5.

Fig 1. Reducing power activity of *Rotula aquatica Lour*

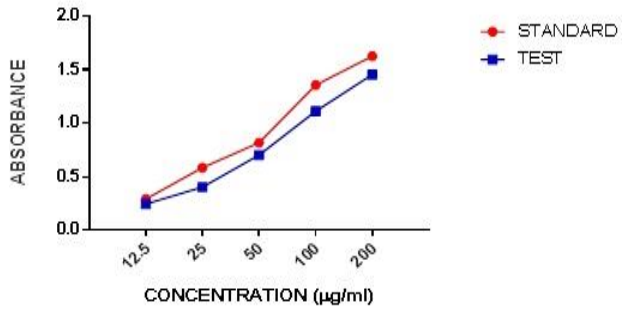

Fig 2. Superoxide Radical Scavenging activity of *Rotula aquatica Lour*

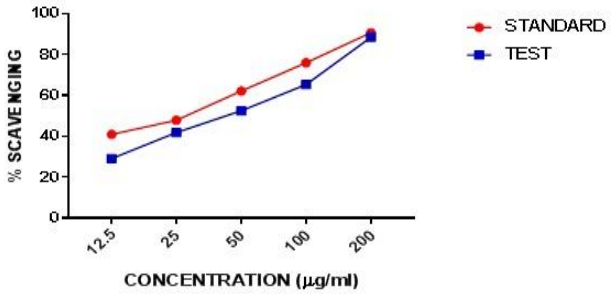

Fig 3. Hydrogen Peroxide Radical Scavenging activity of *Rotulaaquatica* Lour

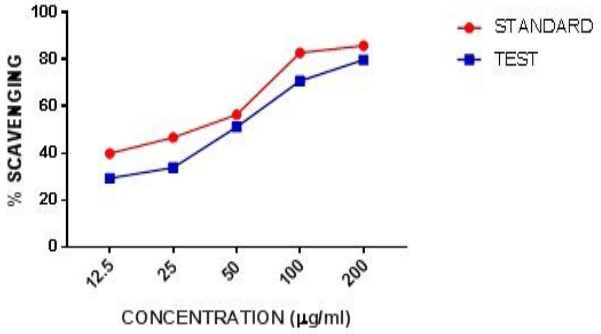

Fig 4. *In vitro* anti-inflammatory activity of *Rotulaaquatica* Lour by HRBC membrane stabilization method

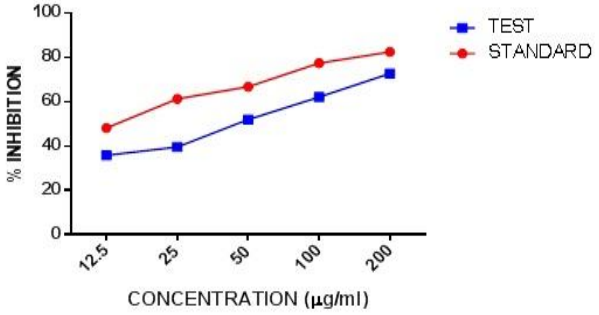

Fig 5. *In vitro* anti-inflammatory activity of *Rotula aquatica* Lour by protein denaturation method

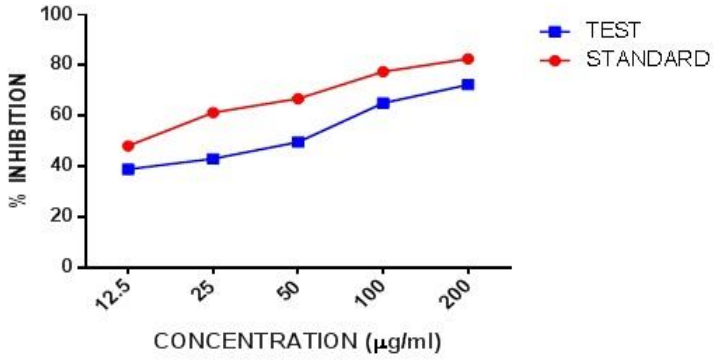

Table 1. Reducing power activity of *Rotula aquatica* Lour

S. No.	Concentration (µg/ml)	Absorbance	
		Ascorbic acid (standard)	HAERA (Test)
1.	Control	1.650±0.060	1.650±0.060
2.	12.5	0.293±0.002	0.246±0.005
3.	25	0.586±0.004	0.404±0.003
4.	50	0.819±0.011	0.794±0.005
5.	100	1.358±0.003	1.112±0.006
6.	200	1.628±0.002	1.456±0.004
EC_{50}		**13.58µg/ml**	**28.79µg/ml**

Values are expressed as mean ± S.D; n = 3.

Table 2: Superoxide radical scavenging activity of *Rotula aquatica Lour*

S. No.	Concentration (µg/ml)	Ascorbic acid (standard)		HAERA (Test)	
		Absorbance	%Scavenging	Absorbance	%Scavenging
1.	Control	0.889±0.008	-	0.889±0.008	-
2.	12.5	0.525±0.004	40.94	0.630±0.004	29.13
3.	25	0.463±0.004	47.91	0.517±0.006	41.84
4.	50	0.336±0.005	62.20	0.422±0.004	52.53
5.	100	0.213±0.003	76.04	0.308±0.003	65.35
6.	200	0.082±0.004	90.77	0.102±0.004	88.52
IC_{50}		**26.09µg/ml**		**47.59µg/ml**	

Values are expressed as mean ± S.D; n = 3

Table 3. Hydrogen peroxide radical scavenging activity of *Rotula aquatica Lour*

S. No.	Concentration (µg/ml)	Ascorbic acid (standard)		HAERA (Test)	
		Absorbance	%Inhibition	Absorbance	%Inhibition
1.	Control	0.133±0.007	-	0.133±0.007	-
2.	12.5	0.080±0.003	39.84	0.094±0.008	29.32
3.	25	0.071±0.003	46.61	0.088±0.004	33.83
4.	50	0.058±0.004	56.39	0.065±0.005	51.12
5.	100	0.023±0.005	82.70	0.039±0.004	70.67
6.	200	0.019±0.003	85.71	0.027±0.002	79.69
IC_{50}		**26.81µg/ml**		**48.90µg/ml**	

Values are expressed as mean ± S.D; n = 3

Table 4. *In vitro* anti-inflammatory activity of *Rotula aquatica Lour* by HRBC membrane stabilization method

Sl. No.	Concentration (µg/ml)	Diclofenac Sodium (STANDARD)		HAERA (TEST)	
		Absorbance	%Inhibition	Absorbance	%Inhibition
1.	Control	0.235±0.003	-	0.235±0.003	-
2.	12.5	0.122±0.003	48.09	0.151±0.002	35.75
3.	25	0.091±0.004	61.28	0.142±0.006	39.58
4.	50	0.077±0.003	66.81	0.113±0.004	51.92
5.	100	0.053±0.005	77.45	0.089±0.004	62.13
6.	200	0.041±0.003	82.55	0.064±0.002	72.77

Values are expressed as mean ± S.D, n = 3

Table 5. *In vitro* anti-inflammatory activity of *Rotula aquatica Lour* by protein denaturation method

S. No.	Concentration (µg/ml)	Diclofenac Sodium (Standard)		HAERA (Test)	
		Absorbance	%Inhibition	Absorbance	%Inhibition
1.	Control	0.260±0.003	-	0.260±0.003	-
2.	12.5	0.146±0.003	48.09	0.159±0.003	38.85
3.	25	0.125±0.004	61.28	0.148±0.003	43.08
4.	50	0.097±0.003	66.81	0.131±0.004	49.62
5.	100	0.077±0.007	77.45	0.091±0.007	65.02
6.	200	0.063±0.006	82.55	0.075±0.008	71.16

Values are expressed as mean ± S.D; n = 3.

DISCUSSION

Oxidative stress is a condition where there is formation of pro-oxidants in the form of ROS and RNS. (Devasagayam TP *et al.,* 2004). It causes tissue damage by different mechanisms including lipid peroxidation, DNA damage and protein modification ultimately resulting in various diseases affecting brain, joints, eyes, heart, blood vessels, kidneys, lungs etc. and conditions such as cancer, aging, diabetes, inflammation and infection (Pham-Huy LA*et al.,* 2008; Ozbek E, 2012). Hence it is very important to inhibit the process of oxidation. Antioxidant constituents present in plants act as scavengers of free radicals and helps in converting the free radicals to less reactive species and thereby play an important role in the defense system of the body (Yadav A *et al.,* 2016). The hydro alcoholic extract of *Rotulaaquatica* was subjected to screen the antioxidant activity using *In vitro* models like ferric reducing power assay, superoxide radical scavenging assay and hydrogen peroxide radical scavenging assay.

The reducing power method measures the ability of antioxidants to reduce the ferric ion. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the measurement of Perl's Prussian blue at 700 nm monitored the Fe^{2+} concentration (Ferreira ICFR *et al.,* 2007).

Superoxide anion is a weak oxidant produced during various biological reactions is highly toxic. Superoxide anion radical is known as an initial radical and plays an important role in the formation of other reactive oxygen-species, such as hydrogen peroxide or singlet oxygen. In the Riboflavin/NADH-NBT system, the superoxide anion derived from the dissolved oxygen from Riboflavin/NADH coupling reaction reduces NBT and results in formation of blue colouredformazan product. A decrease in absorbance indicated the antioxidant activity of the extracts which may be due to the inactivation or consumption of superoxide anion radicals produced in the reaction mixture. The decrease of absorbance indicates the consumption of superoxide anion in the reaction mixture (Mandade R *et al.,* 2011).

Hydrogen peroxide has got the ability to penetrate biological membranes making it very important. Hydrogen peroxide which is normally not very reactive, may give rise to hydroxyl radicals in the cell turning out to be toxic sometimes (Gulcin I *et al.,* 2010). Scavenging of H_2O_2 by extract may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. A decrease in absorbance indicated the scavenging activity or consumption of hydrogen peroxide anion radicals present in the reaction mixture (Sasikumar V *et al.,* 2014).

Inflammation is defined as a reaction seen in living tissues that is shown in response to injury and it comprises systemic and local responses (Saleem TK *et al.,* 2011). Present drugs for the management of pain and inflammation are either narcotics (e.g. opioids), non-narcotics (e.g. salicylate), and corticosteroids (e.g. hydrocortisone) have side-effects (Varghese CP *et al.,* 2013). Inspite of our dependence on local medicine and the tremendous advances in synthetic drugs, a large number of the world populations (80% of people) cannot afford the products of the western pharmaceutical industry and have to rely upon the use of traditional medicines, which are mainly derived from plant material as they have little adverse effects (Nagaharika Y *et al.,* 2013). Hydroalcoholic extract of *Rotula aquatica Lour*. were subjected to analyse the anti-inflammatory activity by HRBC membrane stabilization and protein denaturation method.

As the human red blood cell (HRBC) membrane are similar to the lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis was taken as a measure in estimating the anti-inflammatory property of the extract (Nagaharika Y *et al.,* 2013). The stabilisation of erythrocyte membrane implies that the extract may stabilize the lysosomal membrane as well. The stabilisation of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil. As the RBC membrane is stabilized by the extract, the haemoglobin content of the supernatant

was reduced and hence the absorbance (Varghese CP *et al.,* 2013).

One of the most significant causes of inflammatory and arthritic diseases is the denaturation of tissue proteins. Auto antigens are produced in certain arthritic diseases due to the denaturation of proteins *in vivo* (Opie EL, 1962; Umapathy E *et al.,* 2010). Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development. This method measures the ability of the compound to inhibit thermally induced protein denaturation, a well-known cause of inflammation (Chandra S *et al.,* 2012). Results showed that the extract possess potent inhibitory activity against the adverse effects of inflammation such as tissue damage and protein denaturation.

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