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Research Article

Antioxidant, an anti-inflammatory and anti-arthritic activity of *Centella asiatica* extracts

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ABSTRACT

Centella asiatica is a valuable medicinal herbaceous aromatic creeper which has been valued for centuries in ayurvedic medicine. Phytochemical analysis of Centella asiatica plant extracts revealed the presence of various biochemical compounds such as alkaloids, flavonoids, glycosides, phenolic compounds, triterpenoids and saponins etc. Since phenolic compounds, triterpenoids and flavonoids have remarkable anti-inflammatory, anti-arthritic and antioxidant activites, so our present work aims at evaluating the in vitro anti inflammatory activity by HRBC membrane stabilization and in vitro antioxidant activity of Centella asiatica by three in vitro models such as reducing power, nitric oxide scavenging, DPPH assays. The inhibition of hypotonicity induced HRBC membrane lysis was taken as a measure of the anti inflammatory activity. Protein denaturation, membrane stabilisation and protein inhibitory action were taken as a measure of the in vitro anti-arthritic activity. The total phenolic content was found out to be 33.56 ± 1.56 mg/gm GAE. The DPPH free radical scavenging activity, reducing power and Nitric oxide scavenging activities was concentration dependant with IC50 value being 96.15 ± 1.23 µg/ml, 261.43 ± 0.92 µg/ml and 106.32 ± 2.19 µg/ml respectively. The maximum membrane stabilization of C. asiatica extracts was found to be 94.97 % at a dose of 2000 µg/ml. The maximum percentage inhibiton of protein denaturation, membrane stabilisation and proteinase

inhibitory action for C. asiatica extracts ware found to be 89.76 %, 94.97 % and 91.63% respectively at a dose of 2000 µg/ml. The results show that the extracts of Centella asiatica exhibited Antioxidant activity, Anti-arthritic activity and anti-inflammatory activities.

Keywords: *Centella asiatica*, Antioxidant activity, Anti-inflammatory, Human Red Blood Cell (HRBC), Membrane stabilization, Anti-arthritic activity.

INTRODUCTION

Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as byproducts. Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans[1, 2]. Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity[3]. The intake of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing[4].

Inflammation is the reaction of living tissues to injury, infection or irritation. Lysosomal enzymes released during inflammation produce a variety of disorders which leads to the tissue injury by damaging the macromolecules and lipid peroxidation of membranes which are assumed to be responsible for certain pathological conditions as heart attacks, septic shocks and rheumatoid arthritis etc. The extra cellular activity of these enzymes is said to be related to acute or chronic inflammation. 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[5]. 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 human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an in vitro measure of anti inflammatory activity of the drugs or plant extracts.

Rheumatoid arthritis is a major ailment among rheumatic disorders. It is a chronic condition with multiple causation and affects the people in their most active period of life. The production of auto antigens in certain arthritic diseases may be due to in vivo denaturation of proteins and The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding[6]. So, by controlling the production of auto antigen and inhibiting denaturation of protein and membrane lysis in rheumatic disease leads to anti-arthritic activity. Hence, inhibition of protein denaturation and membrane lysis were taken as a measure of the in vitro anti-arthritic activity. A larger number of herbal extracts are in vogue for the treatment of various types of arthritis.

Centella asiatica is a perennial creeper, faintly aromatic and a valuable medicinal herb of which is distributed throughout tropical and subtropical regions of World such as India, China, Nepal, Madagascar, Srilanka, Indonesia and Eastern South America. Traditionally, *Centella asiatica* has been

valued for centuries in ayurvedic medicine for the treatment of leprosy, ulcer, asthma, bronchitis, elephantiasis, eczemas, anxiety, urethritis[7], cataract, eye troubles, diarrhoea among children, skin diseases, wound healing and for revitalizing the nerves and brain cells, hence primarily known as a "Brain food" or “Memory enchancer” in India. Phytochemical analysis of *Centella asiatica* plant extracts revealed the presence of various biochemical compounds such as alkaloids, flavonoids, glycosides, triterpenoids, saponins, amino acids[8], inorganic acids, vitamins, sterols and lipid compounds[9].

MATERIALS AND METHODS

Collection of Plant Material: The fresh whole plant of *Centella asiatica* was collected from in and around the premises of Andhra University, City of Visakhapatnam, Andhra Pradesh, India. All the other chemicals and reagents were of pure analytical grade and obtained from local supplier.

Extraction and Preparation of Extract: The leaves were garbled and dried under shade and powdered. The 10 g of dried powdered leaves of the plant materials were extracted separately with methanol using soxhlet apparatus for 48 hrs. The solvent was distilled at lower temperature under reduced pressure and concentrated on water bath to get the crude extract which is stored in desiccators for future use. The % of yield is 13.37 % respectively.

INVITRO ANTI-OXIDANT ACTIVITY

Estimation of Total phenolic compounds: Total soluble phenols in the extracts were determined with Folin Ciocalteau reagent using Gallic acid as a standard phenolic compound[10]. 0.1 ml of extract solution taken in a test tube and 1ml of FC reagent was added and 3- 5 min later, 2.5 ml of 20% sodium carbonate was added and the mixture was allowed to stand for 30min with intermittent shaking. The absorbance of the blue colour that developed was read at 765 nm. Gallic acid was used as a standard. The concentration of total phenolic compounds in the extract was determined by using the formula:

$$T = C.V / M$$

T= Total phenolic content mg/gm of plant extract in GAE; V= volume of the extract in mL
C= Concentration of Gallic acid from calibration curve; M= wt of the methanol plant extract.

DPPH free radical scavenging assay: DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the non radical form DPPH[11]. This transformation results in a colour change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple colour is monitored at 517 nm. The reaction mixture (3.0 ml) consists of 1.0 ml of DPPH in methanol (0.3 mM), 1.0 ml of the extract (different concentrations of 50, 100, 250, 500, 1000, 2000 µg/ml) and 1.0 ml of methanol. It is incubated for 10 min in dark and then the absorbance is measured at 517 nm. Ascorbic acid has taken as positive control.

% DPPH radical scavenging activity = [(Absorbance of Control - Absorbance of test Sample) / (Absorbance of Control)] x 100

Reducing Power assay: The reducing power of the extract was determined using Gallic acid as a standard[12]. Different concentrations of stock i.e. 50 - 2000 µg/ml are taken in 1 ml in different tubes and the volumes was made up to 2 ml using distilled water and were mixed in to the mixture of 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. Following incubation, 2.5 ml of 10% trichloro acetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated the increased reducing power.

Reducing power ability (%) = [(Absorbance of Control - Absorbance of test Sample) / (Absorbance of Control)] x 100

Nitric oxide radical scavenging (NO) assay: Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction[13]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 M) in phosphate–buffered saline (1xPBS P^H 7.4) was mixed with 3ml of different plant extracts (different concentrations i.e. 50 - 2000 µg/ml) and incubated at 25°C for 150 min. The samples from the above were reacted with Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm. Ascorbic acid has taken as positive control.

The percentage scavenging of nitric oxide of plant extracts was calculated using the following formula:

NO Scavenging (%) = [(Absorbance of Control - Absorbance of test Sample) / (Absorbance of Control)] x 100

Table 1: Percentage inhibition of various Antioxidant activities at different concentrations

Concentration (µg/ml)	Reducing power activity	DPPH free radical scavenging activity	Nitric oxide radical scavenging activity
50	33.24 ± 1.04	46.82 ± 1.56	31.22 ± 1.44
100	40.36 ± 1.63	52.64 ± 0.85	48.16 ± 2.32
250	48.23 ± 0.76	61.77 ± 1.74	56.38 ± 1.84
500	54.85 ± 1.08	67.96 ± 2.05	63.91 ± 0.55
1000	71.54 ± 1.52	78.24 ± 0.59	75.27 ± 1.67
2000	93.82 ± 1.34	94.86 ± 0.45	92.54 ± 1.72
IC_{50} (µg/ml)	**261.43 ± 0.92**	**96.15 ± 1.23**	**106.32 ± 2.19**

INVITRO ANTI-INFLAMMATORY ACTIVITY

Preparation of Human Red Blood Cells (HRBC) Suspension: Fresh whole human blood was collected and mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05% citric acid and 0.42 % sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min and packed cells were washed three times with isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline.

Heat Induced Hemolysis: The principle involved here is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. The assay mixture contains 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hypo saline [0.36 %], 0.5 ml HRBC suspension [10 % v/v] with 0.5 ml of plant extracts and standard drug diclofenac sodium of various concentrations (50, 100, 250, 500, 1000, 2000 µg/ml) and control (distilled water instead of hypo saline to produce 100 % hemolysis) were incubated at 37^{o}C for 30 min and centrifuged respectively. The hemoglobin content in the

suspension was estimated using spectrophotometer at 560 nm.

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

% Hemolysis = (Optical density of Test sample / Optical density of Control) X 100

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

% Protection = 100 – [(Optical density of Test sample / Optical density of Control) X 100]

INVITRO ANTI-ARTHRITIC ACTIVITY

Inhibition of Protein Denaturation:

1. Test solution (0.5ml) consist of 0.45ml of Bovine serum albumin (5%W/V aqueous solution) and 0 .05ml of test solution.

2. Test control solution (0.5ml) consist of 0.45ml of bovine serum albumin (5%W/V aqueous solution) and 0 .05ml of distilled water.
3. Product control (0.5ml) consists of 0.45ml of distilled water and 0.05 ml of test solution.
4. Standard solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5%w/v aqueous solution) and 0.05ml 0f Diclofenac sodium.

Various concentrations (50, 100, 250, 500, 1000, 2000 µg/ml) of plant extracts (test solution) and diclofenac sodium (standard) of were taken respectively. All the above solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37°c for 20 minutes and the temperature was increased to keep the samples at 57°c for 3 minutes. After cooling, add 2.5 ml of phosphate buffer to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416nm. The control represents 100% protein denaturation. The results were compared with Diclofenac sodium. The percentage inhibition of protein denaturation can be calculated as,

Percentage Inhibition = [100 - (optical density of test solution – optical Density of product control) ÷ (optical density of test control) × 100.

Effect on membrane stabilisation / Inhibition of membranelysis : The principle involved here is stabilization of human red blood cell (HRBC) membrane by hypo tonicity induced membrane lysis. The assay mixture contains 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hypo saline [0.36 %], 0.5 ml HRBC suspension [10 % v/v] with 0.5 ml of plant extracts and standard drug diclofenac sodium of various concentrations (50, 100, 250, 500, 1000, 2000 µg/ml) and control (distilled water instead of hypo saline to produce 100 % hemolysis) were incubated at 37^{o}C for 30 min and centrifuged respectively. The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The percentage inhibition of membrane stabilisation can be calculated as:

Percentage inhibition = 100 - [(optical denity of test solution) ÷ (optical density of control) × 100].

Proteinase inhibitory action: The reaction mixtures (2.0 ml) contained 0.06 mg trypsin, 1.0 ml. 25 mM tris-HCl buffer (pH 7.4) and 1.0 ml aqueous solution of *Centella asiatica* extract of different concentrations (50, 100, 250, 500, 1000, 2000 µg/ml). The mixtures were incubated at 37°C for 5 minutes. Then 1.0 ml of 0.8% (w/v) casein was added. The mixtures were incubated for an additional 20 minutes. 2.0 ml of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged. Absorbance of the supernatant was read at 280 nm against buffer as blank.The percentage of proteinase inhibitory action was calculated as follows:

Percentage inhibition = 100 - [(optical density of sample) ÷ (optical density of control) × 100].

RESULTS AND DISCUSSION

Invitro Anti-Oxidant Activity

Total Phenolic content: Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups and may contribute directly to antioxidative action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1g daily from a diet rich in fruits and vegetables. The standard graph of Gallic acid was shown in **Figure 1.** Our study revealed that 1 mg of methanolic extract of *C. asiatica* contains 33.56 ± 1.56 mg/gm GAE (Gallic acid equivalent).

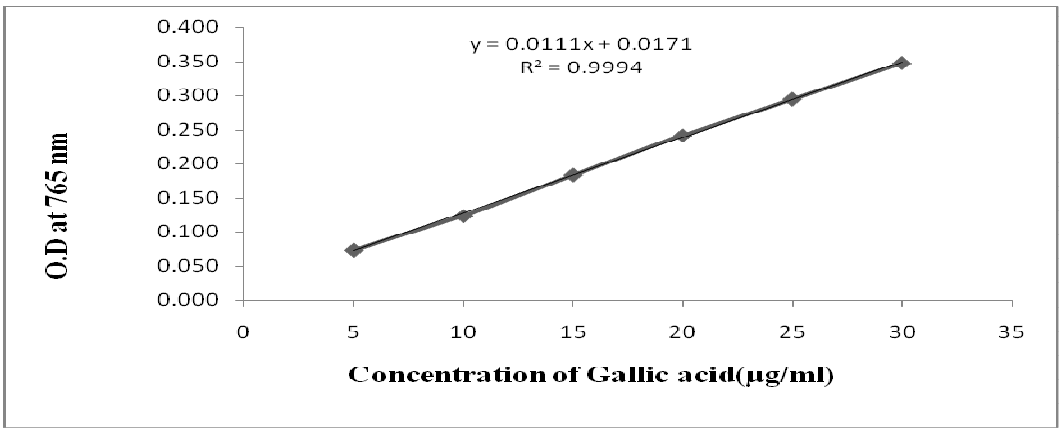

Figure 1: Total phenolic content of Gallic acid extract at different concentrations

PPH free radical scavenging activity: DPPH radicals react with suitable reducing agent, the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up. From the present result it may be postulated that *C. asiatica* extract reduces the radicals to the corresponding hydrazine when it reacts with the hydrogen donor in the antioxidant principles. The activity increased as the concentration increased for each individual of *C. asiatica*. Highest DPPH radical scavenging activity detected in the methanolic extract of *C. asiatica* was 94.86 ± 0.45 % at 2000 µg/ml as shown in **Figure. 2** and IC50 value was found to be 96.15 ± 1.23µg/ml.

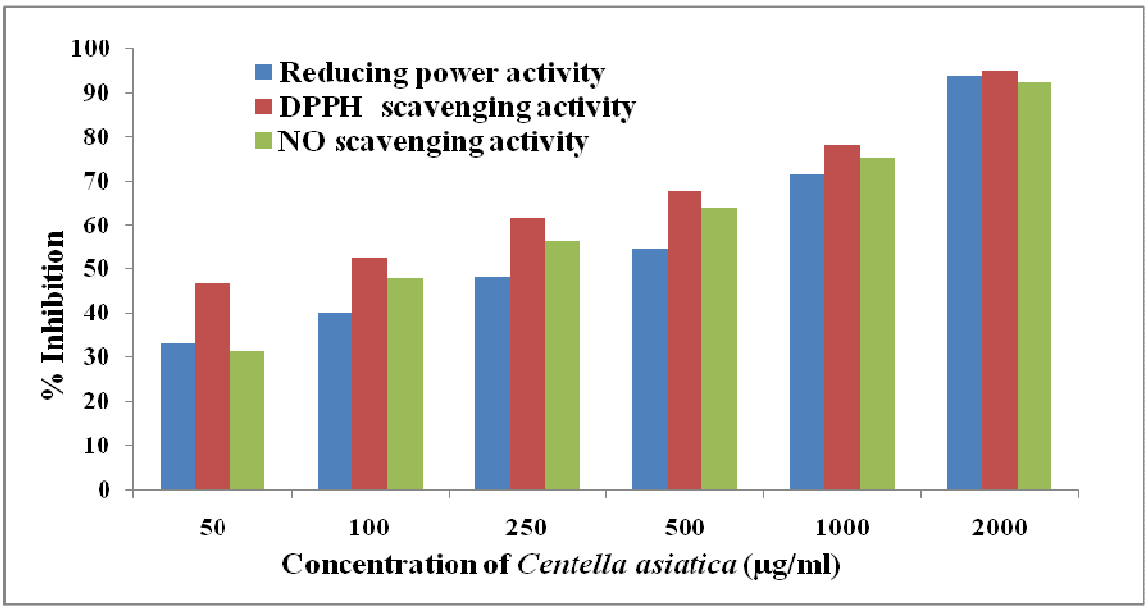

Figure 2: Percentage inhibition of various Antioxidant activities At different concentrations**.**

Reducing power: The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The reducing power increased with increasing amount of the extract. The whole plant extract of *C. asiatica* showed the highest reducing ability of 93.82 ± 1.34 % at 2000 µg/ml as shown in **Figure. 2.** The IC50 value was found to be 261.43 ± 0.92 µg/ml.

Nitric oxide radical scavenging activity: Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals. The activity increased as the concentration increased for each individual of *C. asiatica*. Highest Nitric oxide radical scavenging activity detected in the methanolic extract of *C. asiatica* of was 92.54 ± 1.72 % at 2000 µg/ml as shown in **Figure. 2** and IC50 value was found to be 106.32 ± 2.19 µg/ml.

INVITRO ANTI-INFLAMMATORY ACTIVITY

The inhibition of hypotonicity induced HRBC membrane lysis i.e, stabilisation of HRBC membrane was taken as a measure of the anti inflammatory activity. The percentage of membrane stabilisation for methanolic extracts and Diclofenac sodium were done at 50, 100, 250, 500, 1000, 2000 µg/ml. Methanolic extracts of *C.asiatica* are effective in inhibiting the heat induced hemolysis of HRBC at different concentrations (50-2000µg/ml) as shown in **Table 2.** It showed the maximum inhibition 94.97% at 2000µg/ml. With the increasing concentration the membrane hemolysis is decreased and membrane stabilisation / protection is increased as shown in **Figure. 3.** Hence anti inflammatory activity of the extracts was concentration dependent.

Table 2: Effect of *Centella asiatica* and Standard on HRBC membrane hemolysis and membrane stabilization

Conc. (µg/ml)	% Hemolysis of *C. asiatica*	% Stabilisation of *C. asiatica*	% Hemolysis of Diclofenac sodium	% Stabilisation of Diclofenac sodium
50	32.25	67.74	47.18	52.81
100	20.77	79.22	23.47	76.54
250	16.05	84.05	18.68	81.32
500	12.43	87.56	14.34	85.67
1000	8.45	91.54	7.43	92.58
2000	5.02	94.97	1.24	98.76

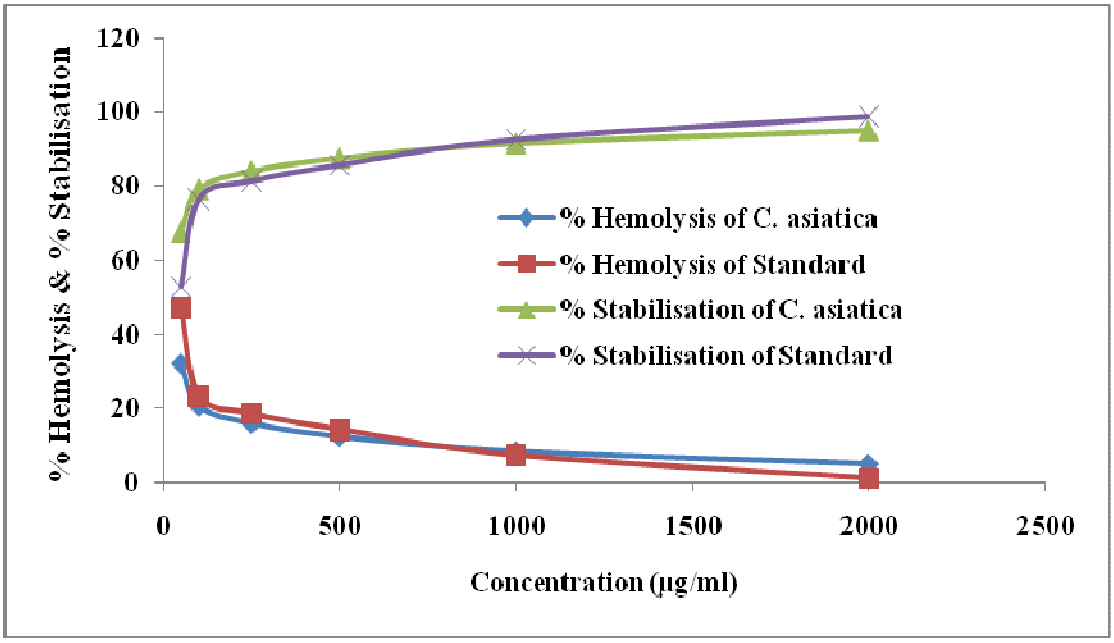

Figure 3: Effect of *Centella asiatica* and Standard on HRBC Membrane hemolysis and membrane stabilization

INVITRO ANTI-ARTHRITIC ACTIVITY

The production of auto antigen in certain arthritic disease may be due to denaturation of protein, membrane lysis and proteinase action. The maximum percentage inhibition of protein denaturation membrane stabilisation and proteinase inhibitory action were observed as 89.76% 94.97 %, 91.63% at 2000 µg/ml respectively as shown in **Table. 3.** From the results of **Figure. 4,** our study reveals that methanol extracts are capable of controlling the production of auto antigen and inhibits denaturation of protein, membrane lysis and proteinase action in rheumatic disease.

Table 3: Effect of *Centella asiatica* and standard on inhibition of protein denaturation membrane stabilization and proteinase inhibitory action.

Conc. (µg/ml)	%Protein denaturation inhibition of *C. asiatica*	% Protein denaturation inhibition of Standard	% Membrane Stabilisation of *C.asiatica*	% Membrane Stabilisation of Standard	%Proteinase inhibitory action of *C. asiatica*	%Proteinase inhibitory action of Standard
50	62.74	64.81	67.74	52.81	39.72	49.67
100	68.29	70.54	79.22	76.54	47.55	54.78
250	76.25	81.32	84.05	81.32	52.92	63.56
500	84.02	85.67	87.56	85.67	67.45	74.83
1000	82.43	92.78	91.54	92.58	79.34	83.67
2000	89.76	96.52	94.97	98.76	91.63	95.89

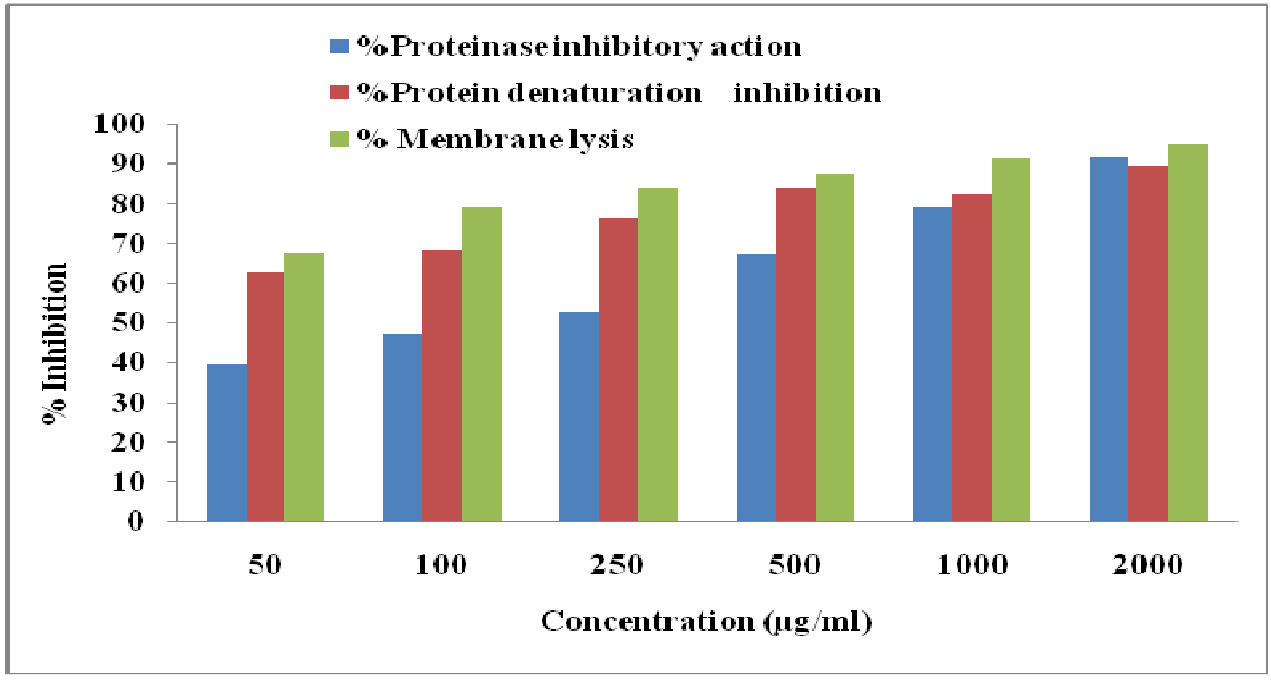

Figure 4: Effect of *Centella asiatica* and standard on inhibition of protein denaturation membrane stabilization and proteinase inhibitory action.

CONCLUSION

Our present studies indicate that *Centella asiatica* methanolic extract exhibits strong Nitric oxide radical scavenging, Reducing power and DPPH activities. The findings of the study suggest that *C. asiatica* could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases. Stabilization of the HRBCs membrane by hypo tonicity induced membrane lysis was studied to establish the mechanism of anti-inflammatory action of *C. asiatica.* Therefore, our in vitro studies on *C. asiatica* extracts demonstrate the depression of inflammation. Hence, *Centella asiatica* can be used as a potent anti-inflammatory agent. Inhibition of protein denaturation, membrane stabilisation and proteinase inhibitory action was studied to establish the mechanism of anti-arthritic effect of *C. asiatica.* Therefore, our *in-vitro* studies on *C. asiatica* extracts demonstrate the significant anti-arthritic activity. Hence, *C.asiatica* can be used as a potent natural anti-arthritic agent. The results show that the extracts of *Centella asiatica* exhibited Antioxidant activity, Anti-arthritic activity and anti-inflammatory activities might be due to the presence of active principles such as polyphenolic content, triterpenoids(asiaticoside, madecassoside etc) and flavonoids.

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