Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2015; 7(2); 65-73

ISSN: 0975-5160

Research Article

Evaluation of Pharmacological Activities of *Rivea hypocrateriformis* in Experimental Animal Models

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Available Online: 2nd March, 2015

ABSTRACT
The present study aimed to investigate the potential pharmacological activities of the ethanolic extract of aerial parts of *Rivea hypocrateriformis* (EERH) and also their phenolic and flavonoid contents measured. The use of this plant in traditional medicine prompted us to study the antioxidant activity by using *in vitro* assay of total antioxidant assay, 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging assay and reducing power assay. In DPPH assay, the IC_{50} values of EERH, ascorbic acid and Butylated hydroxyanisole (BHA) were 43.23, 46.70 and 46.61µg/mL respectively. Reducing power of the extracts increased by increasing their concentration. The analgesic activity employed by hot plate and tail flick methods and *in vitro* and *in vivo* methods of anti-inflammatory activity by human red blood cell membrane stabilization (HRBC) and carrageenan induced paw edema were screened. Percentage protection of inhibited by 250 µg/mL concentration was more and corresponds to standard diclofenac sodium. In EERH in doses of 200 and 400 mg/kg of body weight showed 28.57% and 71.42% inhibition of paw edema, respectively at the end of the 4 hours compared to that of standard diclofenac sodium (85.71%). In hot plate and tail flick methods, the ethanolic extract at high dose 400 mg/kg of body weight increased the pain threshold significantly ($P < 0.001$) after 4 hours of administration and the effect was retained for 6 hours. The EERH shows the dose dependent effect in all the experimental simulations. The results of this study provide pharmacological application of the *Rivea hypocrateriformis* in pain and inflammatory disorders.

Keywords: *Rivea hypocrateriformis*, antioxidant, analgesic, anti-inflammatory, phenolics and flavonoids

INTRODUCTION

Natural phenolic and flavonoid constituents in plant have been receiving increased interest from consumers and researchers for their beneficial health effects on coronary heart diseases and cancer mainly due their antioxidant activity. Oxidative-free radicals are byproducts of the normal reactions within our body. These reactions include the generation of calories, the degradation of lipids, and catecholamine response under stress and the offensive processes (Ikeda and Long 1990). If the balance between oxidative free radical production and eradication was maintained, the harmful effects of free radicals would be minimized in the body. However, if the unwanted free radicals are not eradicated efficiently, oxidative stress would occur. Oxidative stress caused by reactive oxygen or free radicals, has been shown to be associated with the progression of many diseases that include cancer, heart diseases, and depression, among others (Kovacic and Jacinatho 2001; McCord 2000; Parola and Robino 2001). This increases the chance of occurrence of age-related disorders, cancer, atherosclerosis, neurodegenerative diseases and offensive (Cai et al.2004; Tang et al.2004). Consumption of antioxidants, through diet and supplements, was expected to remove ROS from the living system and provide health benefits (Tang et al.2004). Inflammatory diseases, such as rheumatoid arthritis (RA), chronic asthma, multiple sclerosis, inflammatory bowel disease and psoriasis are common worldwide (Ferrero-Miliani et al.2007). To alleviate pain and stiffness in patients with inflammatory disorders, medications such as cyclo-oxygenase inhibitors and cytokinase were often applied (Willich et al.2010). Most of these drugs are expensive and exhibit side effects (Mukharjee et al. 2011).Therefore there is needed to develop new drugs with lower the cost with minimum side effects. For instance, Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used medications to treat pain and inflammatory conditions. Inhibitors of cytokine production can exhibit disease modifying activities, thus representing an alternative therapeutic strategy for the control of inflammatory diseases. Pain is one of the classic signs of the inflammatory process. Inflammatory sting was induced by different chemical mediators released during an inflammatory process, which cause nociceptive sensitization (Julius and Basbaum 2001). The inflammatory process involves a complex cascade of biochemical and cellular events that occur in response to cellular injury (Carvalho and Lemonica 1998).This

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process triggers formation of inflammatory nueromediators that activate nociceptors when released, facilitating pain transmission and peripheral inflammatory response (Wright 1999). Continuously opposing many medicines of plant source had been used subsequently eons without any adverse effects. It is therefore essential that efforts should be made to introduce new medicinal plants to develop more effective and cheaper drugs. Plant represents a large natural source of useful compounds that might serve as lead for the development of novel drugs with lower cost and minimum of side effects.

Rivea hypocrateriformis is a robust woody climbing shrub belonging to the family Convolvulaceae is found in subtropical forests of India. The plant is medicinally used by the indigenous population of Karnataka to cure various types of diseases, such as malaria, and to relieve pain. Even though the plant is known for a large number of biological activities such as antidiabetic, antiimplantation, in treatment of burns and piles, pregnancy, antidepressant, anticancer and analgesic properties (Kirtikar and Basu 1935; Dhavan et al. 1980; Shivalingappa et al. 1999). A phytochemical survey revealed that *R. hypocrateriformis* contains alkaloids, glycosides tannins saponins, phenolic compounds, steroids and flavonoids. The great interest in these chemically varied compounds has resulted in the development of novel therapeutic agents for the various treatments of diseases aggrieved by human beings. Literature survey reveals lack of any systematic biological and biochemical investigation of this plant for its medicinal properties. Recent studies demonstrated that plant phenolic compounds and flavonoids act as reducing agents, which explains their antioxidant activities (Cicco ET al.2009). Therefore, the present work aims at further investigating their antioxidant activity through the use of several in vitro assay systems, analgesic and anti-inflammatory activities of the EERH aerial parts, administered orally, in animal models of pain and inflammation. This will provide insight information with reference to their medicinal values.

MATERIALS AND METHODS

Collection of plant material

The aerial parts of *R. hypocrateriformis* were collected during flowering stage in August-September from the field in and around Belur village, Basavakalyan taluka, Bidar district, Karnataka. Plant material was authenticated at the herbarium of the Laboratory of Botany, Gulbarga University, and Gulbarga with code number HGUG 90 voucher specimen of the titled plant has been deposited in the herbarium of the Department of Botany, Gulbarga University, and Gulbarga. Herbariums are made and their voucher specimen retained in the Department for the future reference.

Preparation of extracts

The aerial parts of *R. hypocrateriformis* were dried under the shade and powdered in a grinder. The powdered material was packed in paper bags and stored in air tight container until use. The dried powder material (400 g) of *R. hypocrateriformis* was extracted successively by ethanol for 48 h using Soxhlet apparatus. The extract was concentrated under reduced pressure using rotary flash evaporator to obtain crude extract. The extract was stored in refrigerator for phytochemical and pharmacological studies.

Chemicals

All chemicals used in this study were of analytical grade, purchased from Merck and Sigma Aldrich, chemical company and purified whenever necessary.

Phytochemical investigation:

The extracts obtained were subjected to preliminary phytochemical screening, to identify the chemical constituents. The methods of analysis employed were those described by (Trease and Evans 1983; Harbone and Baxter 1993). The plant extracts were screened for the presence of biologically active compounds like alkaloids, glycosides, saponins, terpenoides, tannins, phytosterols, flavonoids and phenolic compounds *Determination of total phenolic compounds* the total phenolic content was determined by Folin ciocalteu's colorimetric method (Cicco et al.2009). 50 µL of sample and 50 µL of Folin ciocalteu's phenol reagent were pipetted into test tube. The contents were vortexed for 10 s and then left at room temperature for 2 minute. After 2 minute 500 µL of 5% (w/v) sodium carbonate solution was added to stop the reaction, and then 400 µL of distilled water was added to make up to 5 mL. The vortexed reaction mixture was heated in a water bath at 45^0 C for 30 minute and then cooled rapidly in an ice bath. Absorbance was measured at 725 nm. Total phenol content was expressed in terms of tannic acid equivalent (milligrams per gram of extracted compounds). The samples were analysed in duplicate.

Determination of Flavonoid Content :

Total flavonoids were estimated according to the aluminium chloride method (Zhishen et al. 1999). An aliquot of 0.5 mL extract was mixed with 1.9 mL of distilled water and subsequently with 300 µL of $NaNO_2$ (1:20 w/v) that was pipetted into a test tube. After 6 min, 300 µL of $AlCl_3$ (1:10 w/v) solution was added and allowed to stand for 6 min and then 2 mL of NaOH (1:25 w/v) solution was added to the mixture. Immediately distilled water was added to bring the final volume to 5 mL, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. the absorbance for each sample was measured at 424 nm. The flavonoid content was expressed in terms of standard rutin equivalent (milligrams per gram of extracted compounds).The sample were analysed in duplicate.

Animals:

Swiss albino mice (20-25 g) and Wistar albino rats (180-220 g) of either sex were obtained from the Central animal house M. R. Medical College, Gulbarga. The mice and rats were divided into four groups of five animals per group. The animals were randomly housed at 22 $\pm 2^0$C under standard laboratory conditions and were fed with commercial rat feed with water *ad libitum* and kept in laboratory environment. They were fasted for 18 h before the experiment. All experimental protocols were pre-approved by the institutional Animal Ethics Committee (HKES COP/IAEC/2012/54) for conduction of experiments.

Table 1. Analgesic activity of the EERH on the hot-plate test in mice.

Group	Dose mg/kg	Pain latency after administration (s)				
		0 h	1 h	2 h	4 h	6 h
Control	------	12.09±1.32	13.05±0.86	13.65±0.82	13.46±0.82	9.81±1.12
Diclofenac sodium	0.13	10.58±0.97	11.45±1.21	08.86±1.06**	10.84±0.99*	10.60±0.77
EERH	200	11.25±0.74	10.81±1.02	11.67±0.71*	10.91±0.84	09.63±1.05
EERH	400	08.30±0.80	07.49±0.36**	07.46±1.02	07.22±0.76*	08.10±0.63*

Values are expressed as mean±SEM (n=5), *P<0.05, ** P<0.01, compared with standard (ANOVA followed by Student's t-test)

Table 2. Analgesic activity of the EERH by the tail-flick test in rats.

Group	Dose mg/kg	Pain threshold after administration (s)				
		0 h	1 h	2 h	4 h	6 h
Control	------	6.14±0.72	5.4±0.98	7.45±0.58	6.87±0.54	7.43±0.77
Diclofenac sodium	0.9	8.81±0.46*	9.02±0.84*	8.67±0.77*	9.01±0.50	8.63±0.62
EERH	200	7.47±0.55	6.52±0.31*	5.85±0.52**	7.04±0.34**	7.08±0.59
EERH	400	6.79±0.46*	5.60±0.50**	5.67±0.42**	6.26±0.46***	7.10±0.57*

Values are expressed as mean±SEM (n=5), *P<0.05, ** P<0.01, compared with standard

Table 3. *In vitro* anti-inflammatory activity of EERH aerial parts by HRBC method

Treatments	Concentration (µg/ml)	Mean absorbance ± SEM	Percentage protection
Control	------	2.43±1.40	------
Diclofenac sodium	025	1.18±0.75*	51.44
	50	2.67±1.80	09.80
	100	2.38±1.44	02.07
EERH	150	2.42±1.52	00.40
	200	3.94±3.02*	62.13
	250	3.95±3.01*	62.55

Values are expressed as mean±SEM (n=3), *P<0.05 compared with standard

Table 4. In vivo anti-inflammatory activity of diclofenac sodium and the EERH against carrageenan-induced paw edema in rats.

Group	Dose mg/kg	Carrageenan-induced paw edema in rats (Volume in ml)				
		0 h	1 h	2 h	4 h	6 h
Control	------	0.02±0.03	0.04±0.02	0.06±0.02	0.07±0.02	0.06±0.01
Diclofenac sodium	0.9	0.1±0.02*	0.11±0.02*	0.13±0.04	0.13±0.02*	0.1±0.03
EERH	200	0.09±0.03	0.08±0.02*	0.06±0.02**	0.05±0.02**	0.04±0.01
EERH	400	0.08±0.03*	0.1±0.03**	0.03±0.02**	0.02±0.03***	0.06±0.03

Values are expressed as Mean±SEM, (n=5), *P<0.05, ** P<0.01, ***P<0.001 compared with standard

Blood sample:
Fresh whole blood (5 ml) was collected intravenously from healthy human volunteers into heparinised tubes to prevent coagulation following IEC guideline.

Acute Toxicity:
Swiss albino mice (20-25 g) and Wistar albino rats (180-220 g) of either sex were used for acute oral toxicity study. The study was carried out as per the guideline set by OECD and no adverse effects or mortality was detected in the mice and rats up to 5000 mg/kg, p.o., during the 24 h observation period. Based on the results obtained from this study, the dose for anti-inflammatory and analgesic activities were fixed to be 200 and 400 mg/kg b.w. for dose dependent study, hourly for 6 hours after induction of inflammation.

Evaluation of the antioxidant effect (In vitro)

Determination of total antioxidant activity (TAA) :
The total antioxidant activity of extracts was determined using the phosphomolybdenum method according to the procedure described by (Prieto et al. 1999).Each sample solution (0.1 mL, 0.5 mg/mL) was combined with 0.3 mL of reagent solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The reaction mixture was incubated at 95 0C for 90 min. After the mixture had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank using a spectrophotometer. The antioxidant

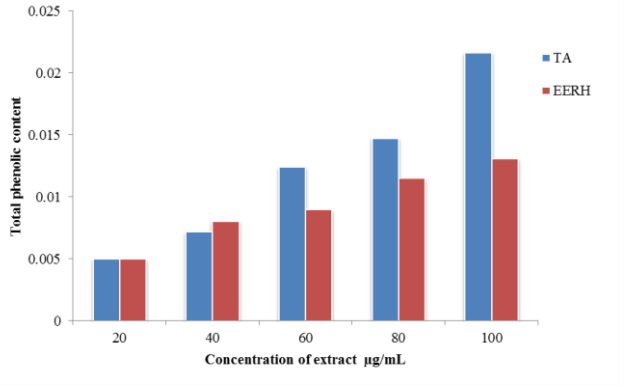

Figure 1: Total phenolic content of ethanolic extract of *R.hypocrateriformis* (EERH).Values expressed are mean ± standard deviation (n=3). Total phenol contents are expressed as mg of tannic acid equivalent (TAE) per gm of extract.

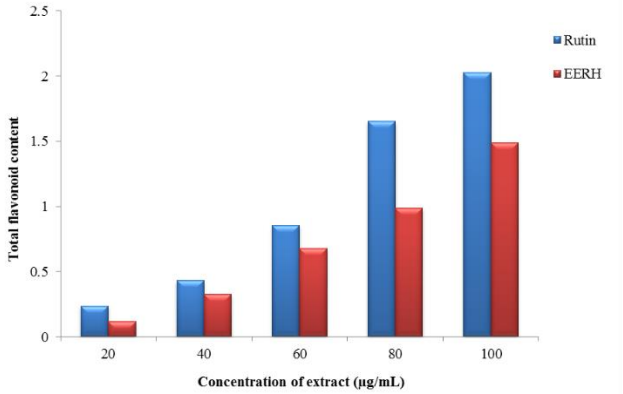

Figure 2: Total flavonoid content of ethanolic extract of *R.hypocrateriformis* (EERH). Values expressed are mean ± standard deviation (n=3). Total flavonoid content is expressed as mg of rutin equivalent (RE) per gm of extract.

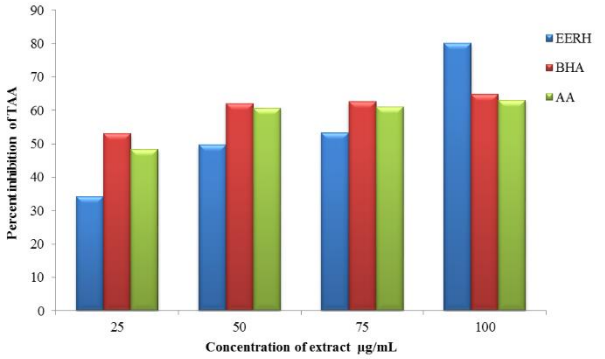

Figure 3: Percent inhibition of total antioxidant activity of different ethanolic extract *R.hypocrateriformis* (EERH). Values expressed are mean ± standard deviation (n=3). BHA-Butylated hydroxyl anisole; AA- Ascorbic acid

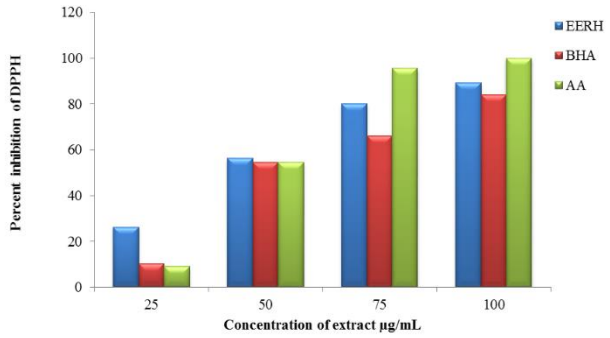

Figure 4: Percent inhibition of DPPH free radical scavenging activity of ethanolic extract of *R.hypocrateriformis*.

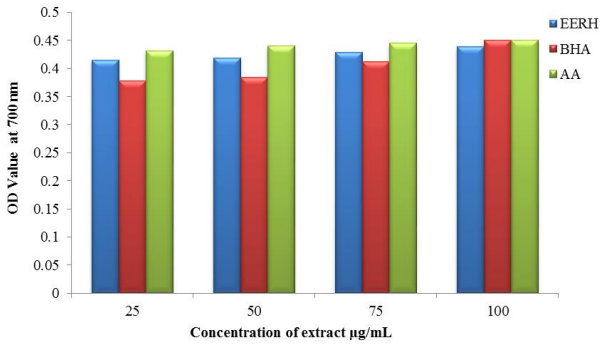

Figure 5: Reducing power activity of ethanolic extract of *R.hypocrateriformis*. Values expressed are mean ± standard deviation (n=3).

activity was expressed as (l mol/g) of ascorbic acid. Ascorbic acid and Butylated hydroxyanisole (BHA), (0.5 mg/mL) were used as reference compounds.

DPPH free radical scavenging assay:

The free radical scavenging activity of the extract was measured by using DPPH by the modified method (Mc Cune and Johns 2002). The reaction mixture (3.0 ml) consisted of 1.0 ml DPPH in ethanol (0.1 mM), 1.0 ml ethanol, and 1.0 ml of different concentrations (25 to 100 µg mL^{-1}) of different solvent extracts diluted in ethanol, was incubated for 30 min at 27º C, in dark, after which the absorbance was measured at 517 nm using a UV–VIS Spectrophotometer against a blank sample. Controls were prepared in a similar way as for the test group except for the replacement of the antioxidant solution with the corresponding extraction solvent. The inhibition of the DPPH radical by the sample was calculated according to the following formula:

% Inhibition = [1- (B/A)] X 100

Where, A is the absorbance of the blank (DPPH plus ethanol) and B is absorbance of the sample (DPPH, ethanol, plus sample).The scavenging activity was expressed as IC_{50}, which is the inhibitory concentration of the test samples that inhibits 50% substrate. Ascorbic acid and Butylated hydroxyanisole (BHA) were used to compare the DPPH radical scavenging activity.

Reducing Power assay :

The reducing power activity of the extract was evaluated by Oyaizu method (Oyaizu 1986).Different concentration of EERH, ascorbic acid and BHA as standard (50-250 µg/mL) were taken in test tube, 0.25 mL methanol were mixed with phosphate buffer (0.625 mL, 0.2 M ,in pH 6.6) and potassium ferricyanide (0.625 mL, 1% w/v). The mixture was vortexed and incubated at 50º C for 20 min. After the incubation, 0.625 mL of 10% TCA was added and the content was centrifuged at 3,000 rpm for 10 min. The upper layer of the supernatant (1.8 mL) was mixed with equal volume distilled water and 0.36 mL of ferric chloride (0.1%) solution and the absorbance was measured at 700 nm. The reducing power of the extract was linearly proportional to the concentration of the sample. Phosphate buffer (PH 6.6) was used as blank solution.

Pharmacological studies

Analgesic effect on the hot plate test:

The hot plate test was executed to measure response latencies according to the method described by (Eddy and Leimback 1953).Swiss albino mice (20-25 g) were randomly assigned to four groups of five mice in each. The group I and II were given 2% acacia suspension (control) and standard group received the diclofenac sodium (0.13 mg/25 g/mice, i.p) and group III and IV were orally administered EERH at doses of 200 and 400 mg/kg. Analgesic activity was measured 1 h after administration of extracts and standard drugs. Mice were placed on the hot plate at 55±0.5° C (Analgesiometer Eddy's hot plate, Dolphin Mumbai, India) and the analgesic activity was estimated by measuring the latency period preceding the animal reaction of paw licking and jumping. Animals showing a reaction time greater than 20 second were removed to avoid lesions to the animals' paws.

Analgesic effect by the tail flick method:

The analgesic activity of the extracts was done by employing tail-flick method (D' Amour and Smith 1941).The pre-screened rats (reaction time: 3-5 sec) were divided into control, Standard and Test group with five rats in each group. Diclofenac sodium was used as the standard drug. The drugs were administered orally with saline, diclofenac sodium (0.9 mg/200 g/rat body weight i.p) EERH (200 and 400 mg/kg). The dose of diclofenac administered was extension of human dose to rat based on body surface area. The distal part of the tails of the animals was assessed for analgesic activity by using (Analgesiometer Dolphin Mumbai, India), maintained at 55±0.5º C. The time taken to withdraw the tail was noted as reaction time. A cut off time of 12 second was maintained at 55º C to prevent tissue damage. The reaction time was measured at 0,1,2,4 and 6th h after treatment.

Anti-inflammatory activity (*in vitro)*:

Human red blood cell membrane Stabilization method (HRBC method) (Gandhidasan and Thamaraichelvan Baburaj 1991) was used for the estimation of anti-inflammatory activity *in vitro*. Blood was collected from healthy volunteers and it was mixed with equal volume of sterilized Alsevers solution. This blood solution was centrifuged at 3000 rpm and the packed cells were separated. The packed cells were washed with isosaline solution and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property. Different concentrations of extract, reference sample and control were separately mixed with 1ml of phosphate buffer, 2 mL of hypo saline and 0.5mL of HRBC suspension. All the assay mixtures were incubated at 37º C for 30 minute and centrifuged at 3000 rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a Spectrophotometer at 560 nm. The percentage haemolysis was estimated by assuming the haemolysis produced in the control as 100%. Percentage protection = [1- OD sample/ OD control] x 100.

Anti-inflammatory activity (in vivo) :

The anti-inflammatory activity of the tested extracts was evaluated in Wistar albino rats by the carrageenan-induced paw edema method (Winter et al. 1962). For this purpose, Wistar albino rats of either sex weighing 180-220 g body weight were used. Rats were divided into four groups of five rats each. The I and II group were given 2% acacia suspension (control) and standard group received the diclofenac sodium (0.9 mg/200 g/rat, i.p), and group III and IV were orally administered EERH at doses of 200 and 400 mg/kg, 1 h before the injection of carrageenan. Thirty minute before the injection carrageenan (0.1 ml of 2% w/v) subcutaneously in the plantar region of the rats right hind paw for the induction of edema. In the left hind paw used as a control 0.1 ml of the sterile saline was injected. The paw edema volume of the rats was measured plethysmographically immediately after the injection of carrageenan at 0, 1, 2, 4 and 6th h. The difference between the initial and subsequent values gave the actual edema volume which was compared with standard and the percent inhibition of edema was determined using the formula. Percent inhibition= 1-Vt/Vc X 100, Where 'Vc' represents edema volume in control and 'Vt' edema volume in group treated with test extract.

Statistical analysis: The data were expressed as mean±SEM and the statistical significance was determined using analysis of variance (ANOVA) followed by Student's t-test. Values were considered to be significant at $P<0.001$.

RESULTS AND DISCUSSION

Phytochemical investigation :

The qualitative phytochemical composition of ethanolic extracts of aerial parts of *R. hypocrateriformis* showed relatively high concentration of bioactive compounds such as alkaloids, glycosides, steroids, tannins, flavonoids, saponins and phenolics compounds .

Total Phenolic compounds and Flavonoids Content:

Phenolic compounds are the most active antioxidant derivatives in plants (Bors et al. 2001). They are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because they are also stable radical intermediates (Maillard et al. 1996). Flavonoids and phenolic acids are important contributing factors to the antioxidant and neuronal cell protective effects of the human diet. However, almost phenolics, suffer extensive biotransformation before absorption in the human body. The ethanolic extract had highest total phenol content in *R. hypocrateriformis* by successively Soxhlet extraction method. Flavonoid content was considerably less than that of total phenol content. Ethanolic extracts has highest phenol content, which support the results of the present study as shown in figure 1 and 2. Therefore, the high content of phenolics and flavonoids in extracts of the *R. hypocrateriformis* might indicate the strong antioxidant properties. The results of the present study strongly suggest that phenolics are important components of these plants and some of their pharmacological effects could be attributed to the presence of these constituents.

Total antioxidant activity (TAA):

The total antioxidant activities of *R. hypocrateriformis* extracts were determined using the phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. High absorbance values indicate that the sample has a significant antioxidant activity. In the present work, the total antioxidant activities of *R. hypocrateriformis* extracts were measured and compared with that of butylated hydroxyanisole (BHA) and ascorbic acid as shown in the figure 3. From these results of the present study suggest that the extracts have the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo (V) compounds and could serve as good total antioxidant activity. The variation of the total antioxidant activity as affected by the extraction solvent used has been reported in many previous studies (Hayouni et al. 2007; Pinelo et al. 2004; Mohdaly et al.2010).

DPPH free radical scavenging activity:

The DPPH is a stable free radical with a maximum absorbance at 517 nm it can readily undergo scavenging by an antioxidant and gets converted into 2, 2-diphenyl-1-picrylhydrazine. The degree of decolouration indicates the scavenging potentials of the antioxidant extract (Chi et al. 2003). In the present study, the free radical scavenging potential of the extracts at different concentrations was tested. The concentrations of extract necessary to decrease the initial concentrations of DPPH free radical by 50% (IC_{50}) under the specified experimental condition was calculated and the results of IC_{50} value of EERH is compared with a standard ascorbic acid and Butylated hydroxyanisole as shown in figure 4. From these results suggest that the extracts have proton donating ability and could serve as good DPPH free radical inhibitors or scavengers.

Reducing power activity:

The ability of EERH to reduce iron (III) to iron (II) was determined at different extract concentrations compared to ascorbic acid and BHA used as reference is shown in figure 5. The EERH showed different capacities for electron donation which was found to be proportionally related to the extract concentration however, the activities were inferior to that of AA and BHA. The results of reducing power demonstrated the electron donor properties of the different concentration of EERH extracts by neutralizing free radicals and forming stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging. Antioxidant can be explained as reductants and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other (Gulcina et al., 2010) observed that evaluation of the total antioxidant capacity of fruits, vegetables and other plant products cannot be performed accurately by any single method due to the complex nature of phytochemicals. Because of involvement of multiple reaction characteristics and mechanisms, no single assay could accurately reflect all antioxidants in a mixed or complex system (Du et al. 2009). Therefore, to characterize a substance as an antioxidant, it is necessary to assess its interaction against a wide range of species directly responsible for oxidative damage (Yamaguchi et al. 1999). Interestingly, the extracts from aerial parts of the *R. hypocrateriformis* have the ability to scavenge almost all free radicals and metal ions.

Acute toxicity:

The toxicity study of EERH indicated, there was no adverse effect or mortality detected in Swiss albino mice and Wistar albino rats that were administered up to 4000 mg/kg, orally of EERH. They have not shown any adverse effect or mortality that was observed during 24 h observation period and the extract was found to be safe at the given dose.

Analgesic activity :

The analgesic effect of EERH using two pain models (on the hot-plate and tail flick models for peripheral and central activity).The analgesic effect of EERH demonstrated using a novel and objective behavioural test in which quantitation is independent of the observer and is sensitive to all classes of analgesics (Tonussi, and Ferreira 1992). Oral administration of the EERH (200 and 400 mg/kg) was statistically significant ($P<0.001$) on the hot-plate and tail flick models. The latency time 0, 1, 2, 4, and 6^{th} h after the administration of extract which was comparable with the effect of standard drug diclofenac sodium (0.13 mg/25 g, i.p.) was significantly higher in Table 1. Moreover the extract by radiant heat on tail flick model has shown increased pain threshold significantly during the period of observation as shown in Table 2. This indicates the involvement of a higher centre and ethanolic extract was the most active. The activity was comparable to that of diclofenac sodium (0.9 mg/200 g, i.p) that was used as a reference drug. The extract showed very good peripheral and central analgesic activities as evident from the hot plate and tail flick methods. The hot plate test is a classical approach widely applied in the analgesic

investigations for several decades. When male mature animals touch the heated plate, they will lick their scrotums against thermal injury. In this case, we often record a false reaction time of licking the hind paws. Therefore, only female animals are used on the hot plate test. Our results showed that EERH significantly prolonged potential increased pain threshold period of mice and rats on the hot plate and tail flick tests, suggesting that this antinociceptive effect is carried out mainly via the participation of central nerve.

Anti-inflammatory activity (*in vitro)* :

The *in vitro* anti-inflammatory effects of EERH at different concentrations (50- 250 µg/ml) showed that the extract exhibited statistically significant ($P<0.05$) stabilization towards HRBC membranes. The percentage protections at concentration 200 and 250 µg/ml are 100% when compared to diclofenac sodium. However on higher concentration the percentage protection was found to be increased they are shown in Table 3. Inflammation is common phenomenon and it is a reaction of living tissues towards injury. Steroidal anti-inflammatory agents will lyse and possibly induce the redistribution of lymphocytes causing rapid and transient decrease in peripheral blood lymphocyte counts to effect longer term response. HRBC method was selected for the *in vitro* evaluation of anti-inflammatory property because the erythrocyte membrane is analogous to the lysosomal membrane (Vadivu and Lakshmi 1992) and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing 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. The result indicated that the EERH at various concentrations had significant anti-inflammatory property.

Anti-inflammatory activity (in vivo):

The effect of EERH on carrageenan-induced rat paw edema at different hours of study was compared to that of control for the evaluation of anti-inflammatory activity on the basis of percent inhibition of paw edema volume. The experiment showed that the extract exhibited statistically significant ($p<0.001$) inhibition of paw volume in a dose-dependent manner. Significant inhibition of paw edema was observed with both doses tested till the sixth hour. However, maximum inhibition of paw edema were found to be 28.57% and 71.42% at fourth hour at doses of 200 mg/kg and 400 mg/kg body weight when compared with standard drug 85.71%. Although the inhibition of paw edema with the extract were more than that found with the standard drug diclofenac sodium at a dose of 0.9 mg/kg body weight, the duration of action were found to be comparable to that of diclofenac sodium till the sixth hour during investigation. The effect of EERH in carrageenan induced paw edema in rats is shown in Table 4. Carrageenan-induced paw edema is a well-established animal model to assess the anti-inflammatory effect of natural products as well as synthetic chemical compounds (Winter et al. 1962). Edema formation due to carrageenan in paw is a biphasic event, during 0, 1, 2, 4 and 6^{th} h; the initial phase (0-6 h) is predominately a non-phagocytic edema followed by a second phase (2–6 h) with increased edema formation that remained up to 6 h (Khan et al. 2009; Khan et al. 2009). The initial phase has been induced due to the action of mediators such as histamine, serotonin and bradykinin on vascular permeability (Maity et al. 1998). The late phase or second phase edema has been shown to be the result of overproduction of prostaglandins (Perez-Guerrero et al. 2001). The result of pre-treatment of EERH demonstrated that the extract (200 and 400 mg/kg i.p.) is effective in the early phase of inflammation, which is due to release of histamine and serotonin primarily. The anti-inflammatory effect of the extract remains significant up to 6th h of the experiment. The EERH showed significant activity against histamine induced edema in both phases. Based on those reports it is possible that the inhibitory effect of the EERH produced dose dependent and significant inhibition of carrageenan-induced paw edema. Significant inhibition of paw edema in the first hour of study could be attributed to the inhibition of histamine and/or serotonin release. These activities affirm the presence of biologically active compounds in the plant. Further investigations are in progress for the isolation of bioactive molecules and the establishment of the mechanism of actions to produce potential bioactive molecules from this plant.

CONCLUSION

The aerial parts of *R. hypocrateriformis* possess very good antioxidant, analgesic and anti-inflammatory activities, ascribable to high level of phenolic compound and flavonoids in the extract. The EERH was proved a natural safe remedy for the treatment of algesia and inflammation. Our current findings demonstrated superior antioxidant and free radical scavenging activities and scientific rationale for the traditional use of the plant as analgesic and anti-inflammatory. Interestingly the *R. hypocrateriformis* exhibited both peripheral as well as central analgesic effect which might have been attributed to the presence of such active principles, due to which it has proven traditional use in various treating disorders and increases the interest and potential use of this sample as neutralceutical and pharmacological agent. Nevertheless, the isolation of pure secondary metabolites from the plant will help us further in understanding the mechanism of action and identification of lead compounds of clinical utility.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to the Chairman, Department of Chemistry, Gulbarga University, and Gulbarga for providing facility to carry out research work. Also we thank to University Grants Commission (F.No.40-58/2011 (SR)), New Delhi, India for providing financial support as Junior Research Fellow and authorities of HKES Matoshree Taradevi Rampure Institute of Pharmaceutical Science, Gulbarga for the facilities for testing.

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