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PHARMACOLOGICAL EVALUATION AND ROLE OF WATER EXTRACT OF RUMEX HASTATUS ROOTS IN OXIDATIVE STRESS AND INFLAMMATION

Dr. Jiwan P. Lavande[1], *Dr. Preeti Mangala[2], Dr. Mohammed Sofiqul Mollik[3], Gaurav Pratap Singh[4], Soumyarup Dutta[5], Dr. Rahul Shivajirao Solunke[6], Dr. Nayana Shivaji Baste[7], Dr. Shweta H Shahare[8]

[1]Professor, School of Pharmaceutical Sciences, Sanjay Ghodawat University, Kolhapur - Sangli Highway, Atigre - Hatkanangale, Kolhapur, Maharashtra. 416 118

[2*]Associate Professor and Head, Department of Chemistry, Jyoti Nivas College Autonomous, Koramangala, Hosur Road, Bangalore, Karnataka. 560 095

[3]Assistant Professor, School of pharmaceutical sciences, University of Science and Technology, Meghalaya, Ri-Bhoi, Techno City, Killing Road, Baridua, Meghalaya. 793101

[4]Assistant Professor, Dr. K. N. Modi Institute of Pharmaceutical Education and Reasearch, Kapra Mill, Opposite SBI Branch, Modinagar, Ghaziabad, Uttar Pradesh. 201204

[5]Anand College of Education, Department of Pharmacy, Kabilpur, Debra, Paschim Midnapur, West Bengal. 743422

[6]Principal, Godavari Institute of Pharmacy, Latur- Nanded Highway, Kolpa, Latur, Maharashtra. 413512.

[7]Associate Professor, SNJB's Shriman Sureshdada Jain College of Pharmacy, Chandwad, Nashik Maharashtra, India. 423101

[8]Associate Professor, KCT's, R. G. Sapkal Institute of Pharmacy, Anjaneri, Wadholi, Nashik, Maharashtra.

Corresponding Autor

***Dr. Preeti Mangala[2]**

[2*]Associate Professor and Head, Department of Chemistry, Jyoti Nivas College Autonomous, Koramangala, Hosur Road, Bangalore, Karnataka. 560 095

ABSTRACT

This present study aimed to evaluate and investigate the antioxidant, free radical scavenging activity and antiinflammatory mechanistic activity of the cold macerated water extract of leaves of *Rumex hastatus.* The plant leaves were extracted in cold maceration technique using water as extracting solvent for 7 days. The cold macerated water extract, RH-W was subjected to phytochemical screening using standard test. Further the extract was subjected to DPPH radical scavenging activity as well as total antioxidant activity was also evaluated. The antiinflammatory activity mechanistically evaluated in terms of the percentage inhibition of cyclooxygenase enzyme system including COX-1 and COX-2 enzymes. The results demonstrated significant free radical scavenging activity of the extract in terms of potent DPPH neutralizing efficacy. The total antioxidant capacity was also found to be significant. The extract (RH-W) demonstrated significant COX-1 and COX-2 inhibitory activity indicative of antiinflammatory activity. In conclusion the plant extract clearly indicated a significant antioxidant and anti-inflammatory profile suggestive of a potent role in treating and managing oxidative stress as well as inflammatory cascade.

Keywords: Oxidative stress, Inflammation, *Rumex hastatus,* Cold maceration, Antioxidant activity, Antiinflammatory activity

INTRODUCTION

Infectious microorganisms, including bacteria, viruses, and fungi, typically cause inflammation when they enter the body, settle in certain tissues, or move through the bloodstream. Inflammation can also occur as a result of events like degeneration, ischemia, malignancy, cell death, and tissue injury. Inflammation is primarily caused by both the innate immune response and the adaptive immunological response (Singh et al., 2010, Kataki et al., 2014, Landskron et al., 2014, Shabab et al., 2017). The primary defence mechanism against cancer cells and invasive microbes is the innate immune system, which is composed of mast cells, dendritic cells, and macrophages. More specialised cells called B and T cells work with the adaptive immune system to produce particular antibodies and receptors that kill cancer cells and other invasive invaders. Different forms of inflammatory responses result in the synthesis and secretion of numerous inflammatory mediators. Pro- and anti-inflammatory mediators are the two primary categories into which inflammatory chemicals are typically separated. However, some mediators have both pro- and anti-inflammatory qualities, such as interleukin (IL)-12 (Akcakavak et al., 2023, Al-Harbi et al., 2023, Anjum et al., 2023). A number of inflammatory mediators and cellular pathways, including cytokines (like interferons, interleukins, and tumour necrosis factor α), chemokines (like monocyte chemoattractant protein 1), eicosanoids (like prostaglandins and leukotrienes), and the powerful transcription

factor nuclear factor κ B, have been thoroughly studied in relation to human pathological conditions. Pro-inflammatory cytokine tumour necrosis factor (TNF)-α is secreted by different cells and has a wide range of cellular effects. TNF-α has been linked to a variety of human disease states, such as inflammatory and immunological illnesses, cancer, and mental health issues, among others. IL-1α is another cytokine that primarily promotes inflammation. It induces the release of pro-inflammatory cytokines like TNF-α and IL-1β (Ardizzone et al., 2023, Arega et al., 2023, Arjsri et al., 2023). But IL-1α has also been linked to anti-inflammatory properties. Like IL-1α, IL-6 mostly promotes inflammation but also possesses some anti-inflammatory properties. The cytokines in the IL-12 family, which includes IL-12, IL-23, IL-27, and IL-35, have been shown to have both pro- and anti-inflammatory properties. However, IL-10 is a strong anti-inflammatory cytokine whose effect inhibits that of numerous pro-inflammatory mediators (Chen et al., 2023, Chi et al., 2023, Ding et al., 2023). IL-10 serves to preserve tissue homeostasis and reduces the potential harm that can arise from an exacerbated inflammatory response by reducing and modulating the inflammatory response.

When it comes to PG and its relationships to human physiological and pathological situations, prostaglandin (PG) E2 has perhaps been studied the most. It regulates appropriate body temperature, maintains the integrity of the stomach mucosa, increases blood flow to the kidneys, and supports the function of the female reproductive system, among other physiological roles. Conversely, increases in PGE2 activity are linked to a number of pathological disorders, including colorectal cancer, aberrant body temperature fluctuations, and inflammatory diseases. Phospholipase A2 (PLA2) converts cell membrane phospholipids into arachidonic acid, which initiates the PG production pathway. Then, the enzyme cyclooxygenase (COX) transforms arachidonic acid into PGs (Kataki et al., 2014). When it comes to inflammatory processes, the inducible enzyme COX-2 is acknowledged as being the most active of the three known COX isoforms (COX-1, COX-2, and COX-3). LTB4 is one of the leukotrienes (LTs) that has been connected to human disease states such depression, asthma, and inflammation. 5-lipooxygenase (5-LOX) is the enzyme that produces LTs (Kataki et al., 2014, Choi et al., 2023, Mayangsari et al., 2023). The enzyme nitric oxide synthase (NOS), which generates nitric oxide (NO), is also closely linked to inflammatory disorders. Inducible NOS (iNOS) is the most pro-inflammatory NOS isoform, much like COX-2. The transcription factor known as nuclear factor κ B, or NFκB, plays a significant role in the pathophysiology of cancer and is a well-known regulator of inflammatory and immunological responses. The NFκB machinery in mammals is made up of a number of components, such as p50 and p65, who control both physiological and pathological processes. When the body is at rest or not under

stimulation, NFκB is found in the cytoplasm. After being activated by diverse infectious, inflammatory, or mitogenic stimuli, NFκB proteins relocate to the nucleus where they trigger the transcription of genes linked to inflammation (Choi et al., 2023, Mayangsari et al., 2023).

The imbalance between the ability of cells to scavenge reactive oxygen species (ROS) and the amount of ROS produced by the cells is known as oxidative stress (OS). The development of multiple diseases, including diabetes, heart disease, and cancer, has been linked to OS. Numerous cellular constituents, such as lipids, proteins, and nucleic acids, including DNA, are harmed by ROS, which ultimately results in cellular death through necrosis or apoptosis. Weakened cellular antioxidant defence systems may cause the damage to spread more widely. An antioxidant defence mechanism exists in all biological systems, which guards against oxidative damage and fixes enzymes to eliminate harmed molecules. It's crucial to consume antioxidant-rich foods because the body's built-in antioxidant system may not always function well. Eating fruits and vegetables is associated with a lower risk of a number of diseases, including cancer, cardiovascular disease, and stroke brought on by OS[3]. The main source of these health advantages is the phytochemicals found in fruits and vegetables, which include carotenoids, polyphenols, and vitamins E and C (Lee et al., 2006, Abbaszade-Cheragheali et al., 2023, Abd Elkader et al., 2023).

While phenolic compounds are frequently present in edible and inedible herbs, cereals, fruits, vegetables, oils, spices, and other plant materials, there is still a dearth of scientific data regarding the antioxidant capabilities of endemic plants—plants that are unique to a given region and are only known to the local population—in these areas. Thus, evaluating these qualities is still a worthwhile and fascinating undertaking, especially when it comes to discovering fresh, promising natural antioxidant sources for functional foods and/or nutraceuticals (Harborne and Harborne, 1973, Zheng and Wang, 2001). While it is still an unwritten science, herbal medicine is well-established in some countries and cultures, and nearly 80% of people living in rural regions consider it to be their way of life. Rheumatoid arthritis is one of the most common chronic anti-inflammatory illnesses affecting people worldwide. Even though synthetic medications currently rule the market, there is always a chance that they could be harmful. Prolonged usage of these medications may have serious side effects on long-term administration, the most prevalent of which being peptic ulcers and gastrointestinal bleeding (Zhang and Tsao, 2016). Thus, the creation of a novel anti-inflammatory drug with the fewest possible adverse effects is required. Research on safe and efficient anti-inflammatory drugs has taken precedence in scientific studies pertaining to herbal medicine (Zhang and Tsao, 2016). Since

ancient times, people have used plants, their parts, or their extracts as anti-inflammatory agents. The majority of the information about the use of plants or plant products for therapeutic purposes has been found in books and, more recently, a vast number of websites (some of which need to have their credibility thoroughly checked). The anti-inflammatory properties of plants have been the subject of hundreds of study and review articles published in recent decades. Therefore, this research work aimed to evaluate the antioxidant, free radical scavenging activity and antiinflammatory activity of the cold macerated leaf extract of Rumex hastatus.

MATERIAL AND METHODS

Drugs and chemicals

Enzymes and other crucial chemical reagents were obtained from Himedia Laboratories in Mumbai, India, along with butylated Hydroxy toluene. The suppliers of the remaining biochemical kits were R&D Systems in India and Sigma Aldrich in Mumbai, India, respectively. The remaining chemicals and reagents were all of high quality and analytical grade, and they were exclusively ordered from reliable and pre-verified suppliers.

Collection and authentication of the medicinal plant

Fresh *Rumex hastatus* leaves were gathered from their native environments in the Mandi area of Himachal Pradesh. The plants were selected using a combination of reports from local practicing herbalists and a thorough ethnomedical survey. An accredited taxonomist received the plant samples in order to verify their botanical identity. For future use, the voucher specimens (BKS-567-2023-67) were placed and kept.

Extraction of the medicinal plant

One kilogramme of fresh leaves of *Rumex hastatus* was gathered, shade-dried, and then ground into a 300 g dry sample using a mechanical grinder. This powdered form was then utilised for solvent extraction. The sample was prepared by extracting 300 g of dried sample twice (1000 ml for each extraction) using water at 25°C for 7 days using a cold maceration technique. The extracted material was then concentrated using a rotary evaporator at 45°C under decreased pressure to produce an extract (8.6%). The resulting water extract of Rumex hastatus leaves is known as RH-W.

Preliminary screen for phytochemicals

Using a variety of common tests that have been previously mentioned, a preliminary phytochemical screening was conducted on the extracts and the herbal blend (Harborne, 1973).

Role in oxidative stress: Antioxidant activity

Measurement of DPPH scavenging activity

The extract's ability to scavenge DPPH was assessed using the previously mentioned methodology, with minor adjustments (Zhao et al., 2014, Sarma Kataki et al., 2012). The working solution of DPPH in ethanol was combined with the diluted extract at different concentrations, and the volume of each mixture was adjusted to 1.0 mL. Before each combination was centrifuged at 3000g for 10 minutes, it was violently mixed and allowed to

sit at room temperature for 30 minutes in the dark. At 517 nm, the absorbance of every supernatant was calculated. Except for the extracts, every reagent was present in the control setup. Using the following formula, the percentage of DPPH scavenging was determined:

$$\%\ DPPH\ Scavenging\ activity = \frac{A_{control} - A_{Sample}}{A_{Control}} \times 100$$

where A sample and A control are the sample extract's and the control's absorbance values at 517 nm, respectively.

Calculating the overall antioxidant capacity (TAC)

To ascertain the extract's total antioxidant capacity, a TAC test kit was utilised (Zhao et al., 2014, Sarma Kataki et al., 2012). Using a spectrophotometer, the test detects the interaction between Fe2+ and phenanthroline at 520 nm to determine the total antioxidant capacity of the extracts. The results were given as U/g extract. The unit of total antioxidant capacity, or U, is the amount of antioxidants required to increase absorbance by 0.01 in 1 mL of reaction liquid at 37 ^{0}C. The total antioxidant capacity was calculated using the formula:

$$TAC = \frac{OD_U - OD_C}{0.01 \times 30} \times \frac{V_0}{V_1} \times N$$

where ODU and ODC are the absorbance values of the test sample and the reagent blank, respectively; V_0 is the total volume (in millilitres) of the reaction liquid; The volume of the extracts (mL) is V_1, and the sample's factor of dilution before testing is N. Thirty minutes is the reaction time.

Role in inflammation: Antiinflammatory activity using Cyclooxygenase (COX) assay

The somewhat altered assay created using a technique previously employed elsewhere was utilised (Landa et al., 2009, Kataki et al., 2014). In short, the COX-2 (0.88 µg protein) enzyme solution (10 µL) was activated on ice for 5 minutes using a 60 µL Tris–HCl (pH = 8.0) reaction mixture containing 1 mM L-epinephrine, 1 nM reduced glutathione, and 1 µM hematin. Ten microliters of the tested extract solution diluted in ten microliters of ethanol and [1-14C] AA were added to the enzyme combinations. The reaction was stopped by adding 40 µL of 4 M formic acid after it had been incubated at 37 °C for 15 minutes. After being separated using 200 µL of chloroform, dried under a N2 stream, diluted in 10 µL of ethanol, and examined, the metabolic products that resulted from AA were examined.

Statistcal Treatments

Every experiment was run through at least three times, using three duplicates each time. The means ± SD are used to express all values. For *post-hoc* comparisons, means were compared using a one-way analysis of variance and the Dunnet's multiple comparison test. When $P<0.05$, statistical significance is considered to have been reached.

RESULTS

Extraction of the plant

Water was used as a solvent in a cold maceration process to extract the ground-up,

shade-dried leaf powders over the course of seven days. Using this approach, the extract was effectively extracted with a noteworthy percent yield of 8.6%.

Preliminary phytochemical screening

The extract (RH-W) was subjected to standard phytochemical screening and results were presented in the following table 1.

Table 1. Results indicating presence of phytocompounds in the extract (RH-W)

Phytochemical Compound Group	**RH-W**
Phenols	+
Flavanoids	+
Saponins	+
Alkaloids	+
Fatty Acid	-
Phytosterols	+
Borntrager test	-

+: Presence of moderate active constituents, **-:** Absence of active constituents

Antioxidant activity

DPPH radical scavenging activity

The DPPH scavenging assay is widely employed to assess the plant extracts' ability to scavenge free radicals due to its straightforward, quick, accurate, and repeatable methodology. Figure 1 depicts the water extract's reported DPPH scavenging activity. As the extract's quantity increased, so did its ability to scavenge DPPH. The extract's capacity to scavenge DPPH was noticeably greater. At 500 µg/ml and 50 µg/ml, the measured DPPH scavenging capacity was as high as 98.85±1.669% and 20.936±1.40%, respectively. The extract's ability to scavenge DPPH was comparable to that of the benchmark, BHT. The greatest concentration of extract under study was shown to have the highest DPPH scavenging activity. The extract's plant origin, external circumstances, and the extraction solvent could be the cause of the variations in DPPH scavenging activity levels.

Total antioxidant capacity

The TAC value is calculated to be 80.75±2.23% with statistically significant differences, indicating a much-increased antioxidant capacity in the extract (Figure 2). After 36 hours, the extract exhibited a significant TAC value of 80.75±2.23%. The complex composition of the extract, which includes active polysaccharides, proteins, amino acids, vitamins, and microelements, may have an impact on its antioxidant activity. Different processing processes and tests for antioxidant capacity can result in variations in the extract's overall antioxidant activity. Because antioxidant capacity can be studied using a variety of extraction, estimate, and calculation techniques, it might be challenging to compare the current study's findings with those previously published in the literature.

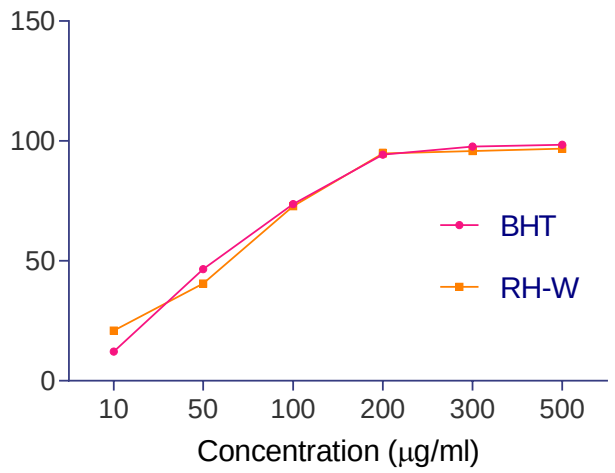

Figure 1. Free radical scavenging activity in terms of DPPH radical scavenging of RH-W

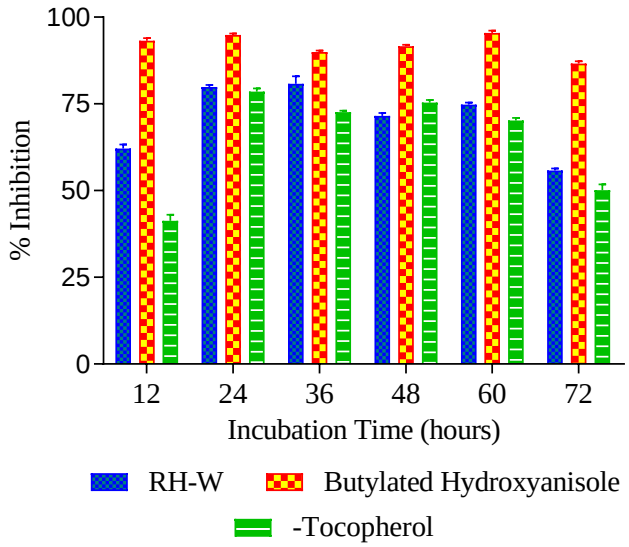

Figure 2. Total antioxidant capacity of the extract RH-W

Table 2. The calculated values of IC_{50} for DPPH scavenging activity of RH-W (mean ± SD, $n = 3$)

Drugs	**IC50 (µg·mL−1)**
	DPPH radical
RH-W	97.92 ± 0.98
Butylated	74.93 ± 0.96
Hydroxy Toluene (BHT)	

COX assay: Antiinflammatory activity using Cyclooxygenase (COX) assay

In vitro, the extract demonstrated a potent inhibitory effect on COX-1. At every measured concentration, the generation of PGE2 was considerably ($p<0.01$) lower than that of the untreated control. The observed inhibition was nearly identical to the reference inhibitors' indomethacin action (data not shown), and there was no statistically significant difference ($p<0.01$) in the variability between the RH-W and both positive controls. Significant differences were found in the IC50 values of every chemical tested. Our additional in vitro studies (Table 3) further supported this sort of activity by showing that the extract also strongly suppressed COX-2 (IC50 = 195.54 µg/mL). The aforementioned finding may provide an explanation for its biological actions associated with COX-1, including the inhibition of platelet aggregation induced by AA. Furthermore, our findings demonstrated that the extract may also efficiently block the inducible COX-2 isoform, which is important in the inflammatory process. Its effects are equivalent to those of the selective and nonselective inhibitors that were employed in this investigation as positive controls. Its capacity to block both isoforms of the enzyme approximately at the same rate suggests that it is a nonselective COX inhibitor, similar to other non-steroidal anti-inflammatory drugs

such as indomethacin or ibuprofen. However, it was demonstrated that COX-1 inhibition was marginally higher than COX-1. The extract's shown anti-inflammatory activity was comparable to the reference NSAIDs at the same dosage. Given that COX-2 is a key player in the regulation of the inflammatory process and a target for most antiphlogistic medications, the extract may have a possible mechanism of action in vivo based on its reported ability to suppress COX-2 in vitro. In conclusion, the extract's inhibitory effect against the COX-1 and COX-2 isoforms in vitro is demonstrated in this significant study. The results obtained show that the biological benefits of this natural component may be attributed to the suppression of prostaglandin production mediated by the AA route and may also have a role in the anti-inflammatory activity of this historically used medicinal plant.

Table 3. The antiinflammatory potential of RH-W in terms of percentage inhibition of the Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2).

Concentration (µg/mL)	**% Inhibition of Cyclooxygenase Enzyme System**	
	COX-1	**COX-2**
60	4.45±0.04	6.92±0.101
120	9.24±0.17	14.62±0.24
210	26.06±0.08	32.50±0.16
270	49.70±0.054	49.820±0.31
340	98.61±0.04	91.93±0.14
IC_{50}	187.82 µg/mL	195.54 µg/mL

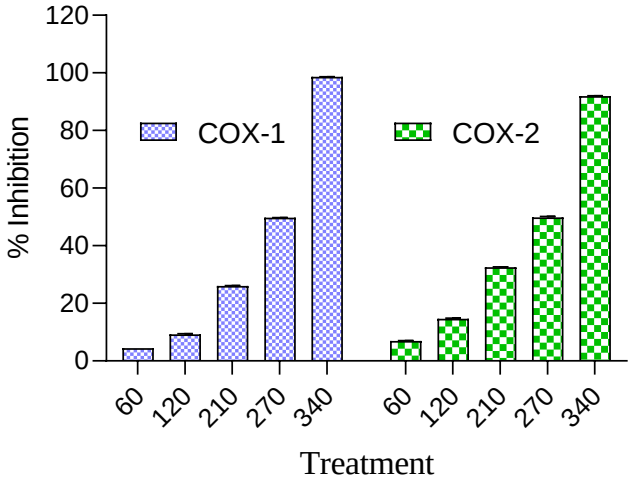

Figure 3. Cyclooxygenase enzyme system inhibitory activity as percentage inhibition

DISCUSSION

Traditional NSAIDs are among the most regularly recommended drugs for the management of pain and inflammation. Numerous negative symptoms are associated with the use of these drugs. Notwithstanding their potentially harmful side effects, NSAIDs are the recommended pharmaceutical option for treating pain and inflammation. A increasing body of research suggests a variety of novel approaches to treating pain and inflammation that lessen the possibility of side effects and other negative symptoms (Martínez-Cayuela, 1995, Hotter et al., 1997, Khodr and Khalil, 2001). Thus, it is increasingly evident that more effective anti-inflammatory drugs with fewer side effects must be created. Since COX inhibition is a well accepted, thoroughly studied, and useful concept, it is a very popular mechanistic approach for evaluating the

efficacy of anti-inflammatory drugs (Brito and Antonio, 1998). It is commonly recognised that the development of inflammation brought on by toxins and infections is linked to the synthesis of many inflammatory mediators, such as histamine, prostaglandins, leukotrienes, PAF (Platelet Activating Factor), and other cyclooxygenase and lipooxygenase products (Brito and Antonio, 1998). Leukocyte and neutrophil migration is also connected to the inflammatory process. Pathophysiology suggests that the process of inflammation may involve free radicals. The current data showed a considerable inhibitory impact, as indicated by the COX inhibition assays, and suggested that the principal mechanism of action of the RH-W may include the inhibition of products of the arachidonic acid pathway. When it comes to lowering inflammation, RH-W seems to be just as successful as NSAIDs. The COX inhibitory capability results showed that RH-W significantly inhibited COX (COX-1 and COX-2), with a somewhat higher level of inhibition of C0X-1. These results clearly demonstrated RH-W's anti-inflammatory mechanism.

Another theory about the pathophysiology of inflammation was the formation of free radicals and oxidative stress during leukocyte and macrophage migration to the site of injury (Khodr and Khalil, 2001, Coussens and Werb, 2002, Hussain et al., 2003). It has been demonstrated that the physiological system is very hazardous to different types of free radicals (Martínez-Cayuela, 1995). These radicals can also serve as second messengers, which can result in the production of other inflammatory mediators (Hotter et al., 1997, Khodr and Khalil, 2001, Hussain et al., 2003). Numerous in vitro assessment methods and models are accessible, including total antioxidant capacity estimation and DPPH radical scavenging. The current research showed significant concentration-dependent DPPH radical scavenging capabilities of the RH-W. It was also shown that the RH-W possesses potent overall antioxidant properties (Benzie and Strain, 1996, Benzie et al., 1999). It is hypothesised that the RH-W containing water extracts of Rumex hastatus leaves will improve oxidative stress, scavenge free radicals, and inhibit the COX enzyme systems of the arachidonic acid pathway, all of which will result in the anti-inflammatory and protective antioxidant effect.

CONCLUSION

The cold macerated extract of leaves of *Rumex hastatus* (RH-W) was found to possess the significant ability to scavenge DPPH radicals and a strong overall antioxidant as evidentb form this study. Mechanistically, the anti-inflammatory action was also found to be significant as evident from the % inhibition data of the cyclooxygenase enzyme system, which includes the COX-1 and COX-2 enzymes. In summary, the extract's strong ability to neutralise DPPH was shown by the results to have high free radical scavenging activity. Significant results were also obtained for the overall antioxidant capacity. Significant

COX-1 and COX-2 inhibitory activity, suggestive of anti-inflammatory action, was shown by the extract (RH-W). The extract was found to be a non-selective COX inhibitor as their mechanism of antiinflammatory action. However, COX-1 inhibition was highly pronounced as compared to COX-2 inhibition. Finally, it was evident from the plant extract that it had a strong antioxidant and anti-inflammatory profile, pointing to a potential function in the treatment and management of oxidative stress and the inflammatory cascade.

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