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An investigation on antioxidant and anti-arthritic activity of *Rheum nobile* Hook.f. & Thomson from Sikkim Himalayan region

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The present study was carried out to determine the phytochemical constituents, physicochemical values, antioxidant activity and *in-vitro* anti-inflammatory activity of *Rheum nobile* Hook.f & Thomson according to the standard pharmacopoeial method, which is traditionally used by the indigenous communities of the Sikkim Himalayan region for the treatment of rheumatoid arthritis. The phytochemical investigation revealed the presence of various secondary metabolites such as flavonoids, tannins, phenols, steroids, phlobatannins, saponins, anthraquinones and glycosides. The *in-vitro* anti-inflammatory activity of the plant extract assessed by HRBC membrane stabilisation and protein denaturation methods is found to effectively inhibit HRBC haemolysis and protein denaturation in a dose-dependent manner. The present finding provides evidence for the antioxidant and anti-inflammatory activity of the plant extract, which approves the traditional statements of *Rheum nobile* as an anti-arthritic agent.

Keywords: Anti-arthritic, Antioxidant, Phytochemicals, *Rheum nobile*, Sikkim Himalayan region

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease associated with the progressive destruction of multiple synovial joints, affecting people from all walks of life and causing a major health problem globally[1]. Even though there are various available modern medicines for the management of RA, like NSAIDs (non-steroidal anti-inflammatory drugs) or DMARDs (disease-modifying antirheumatic drugs), corticosteroids and biological agents, their continuous use may result in extreme side effects, including gastrointestinal problem along with peptic ulcers[2]. So, there is a critical need to develop novel anti-RA agents without side effects. T-cells, cytokines and reactive oxygen species (ROS) like superoxide anions and hydrogen peroxide released by activated macrophages play an important role in developing RA[3]. Earlier studies confirm the role of ROS in the development of rheumatoid arthritis[4]. A proper supply of dietary antioxidant nutraceuticals may reduce free radical development and improve the level of antioxidants in RA patients[5]. In this way, antioxidants are related to RA, and medicinal plants having various phytochemicals with potent antioxidant properties may help manage clinical outcomes and oxidative stress in RA patients. Numerous studies have been conducted to analyse antioxidant and anti-inflammatory activity from medicinal plants[6]. Medicinal plants are known to have various phytochemicals with potent antioxidant and anti-inflammatory properties[7]. Thus, they may help improve the lifestyle of clinical patients of RA by acting as adjuvant in the treatment regime.

Sikkim is a mountain state of India located at 27˚04'46" to 28˚07'48" N latitude and 88˚00'58 "to 88˚55'25" E longitudes having a geographical area of 7096 sq. km. In this state, most of the population in the villages and far-flung areas still rely on traditional medicine systems prescribed by traditional herbal healers, and this practice has existed since time immemorial. However, traditional herbal healers are unaware of different phytochemicals present in the plants being used by them to treat various diseases, and very few studies have been conducted to scientifically validate traditional healing systems[7]. The development of experimental data to validate the use of medicinal plants by traditional herbal healers is very important for various reasons.

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In Sikkim Himalayan region, *Rheum nobile* Hook.f. & Thomson, locally known as *Kenju/Chuba* (Bhutia), *Padamchal/Padmaguru* (Nepali), and *Sikkim Sundari* in general. It is a monocarpic perennial herb belonging to the family Polygonaceae and confined to the alpine region of Hindukush Himalaya. It prefers open areas at the altitudinal ranges of 3700–5200 m above sea level and attains a height of about 1–1.5 meters on maturity[8]. The plant has showy translucent bracts covering the towering inflorescence as a greenhouse, protecting it from extreme cold conditions in the Himalayan region. It has been documented that *R. nobile* is preferably used in traditional herbal medicine to treat several inflammatory diseases such as ulcers, bronchitis and rheumatoid arthritis[9]. However, no validation of the antioxidant and anti-inflammatory activities has been carried out to date. Therefore, the present investigation aims to study the *in-vitro* antioxidant and anti-inflammatory activity of *R. nobile* to understand its anti-arthritic efficacy and to validate the traditional knowledge of herbal healers about the use of this plant as an anti-arthritic agent.

Materials and Methods

Collection of plant samples

R. nobile was collected from Gnathang Valley, East district of Sikkim, in August 2019, located at 3,872 m above sea level with prior permission from the Sikkim Biodiversity Board, Government of Sikkim. The plant is identified and authenticated by the Botanical Survey of India, Eastern Himalayan Circle (Sikkim), with voucher No. BHSC0214.

Sample preparation and extraction

About 300 g of fresh rhizome was taken from the collected rhizome plant and thoroughly washed with tap water. The cleaned rhizome was chopped into pieces and dried at 40ºC in a thermostatically controlled oven until they attained a constant weight. The dried plant sample was then crushed into a fine powder using a mechanical grinding machine. About 10 g of plant sample from the stock powder was used for plant extraction in 100 mL of methanol using the Soxhlet apparatus for 24 h at 30°C. After 24 h, the obtained extract was filtered and concentrated under a vacuum using a rotary evaporator (Heidolph, Schwabach, Germany) and dried further in the vacuum desiccators. After complete dryness of the extract, the yield value was calculated and kept in the refrigerator at 4°C until further study.

Physicochemical analysis

The rhizomatic powder of the plant sample was evaluated for physicochemical analysis such as total ash content, water-soluble ash content, acid-insoluble ash content, loss on drying and extractive value in methanol as per the standard method[10].

Phytochemical analysis

Qualitative phytochemical screening of *R. nobile* was done according to standard procedures[11] to determine the presence or absence of various phytochemical constituents, viz. alkaloids, flavonoids, phenols, tannins, steroids, terpenoids, saponins, phlobatannins, anthraquinones, carbohydrates, glycosides and proteins.

Quantitative phytochemical estimation

Total phenolic content (TPC) was determined following the standard method and gallic acid as standard[12]. Exactly 1 mL methanol extract of the plant sample was mixed with 5 mL of 10-time diluted Folin-Ciocalteu reagent and 4 mL of 7.5 % sodium carbonate. The mixture was allowed to stand for 90 min at room temperature, and the absorbance was measured at 760 nm using a UV-visible spectrophotometer. A standard curve was prepared using gallic acid by serial dilution from 10-100 µg/mL. Values are expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g).

Total flavonoid content (TFC) was measured by a modified colourimetric method[12]. About 5 mL of methanol extract from the plant sample was mixed with 0.3 mL of 5% sodium nitrite for 5 min in a test tube. Then 0.3 mL of 10% aluminium chloride was added; after 6 min, 2 mL of sodium hydroxide was added to stop the reaction. The mixture was further diluted with distilled water up to 10 mL. The absorbance was immediately measured at 510 nm using a UV-visible spectrophotometer. A standard curve was prepared using a 10–100 µg/mL rutin concentration. The TFC was expressed as milligrams of rutin equivalents per gram of dry extract (mg of RtE/g).

TFC was determined using the standard method with some modification and rutin as standard[13]. Exactly 2 mL of 2% aluminium trichloride and 3 mL sodium acetate solution were added to 2 mL of methanol extract of the plant sample. The solutions were kept at 20°C for 2.5 h, and the absorbance was measured at 440 nm using a UV-visible spectrophotometer. Results were expressed as milligrams of rutin equivalent per gram of dry extract (mg RtE/g).

Tannin content was measured by using the standard method with slight modifications[14]. Exactly 0.1 mL of each extract was taken in a test tube containing 7.5 mL of distilled water, followed by adding 0.5 mL of Folin-Ciocalteu reagent and 1 mL of 35% Na_2CO_3 and diluted to 10 mL with distilled water. The mixture was shaken well and kept at room temperature for 30 min. Absorbance was measured at 725 nm using a UV-visible spectrophotometer. Results were expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g).

Evaluation of antioxidant activities

DPPH free radical scavenging assay

The free radical scavenging activities of the methanol extract of the plant were determined based on their scavenging activity of the stable DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical as per the standard method with slight modifications[15]. Exactly 1 mL of each solution of different concentrations (100, 200, 300, 400, and 500 µg/mL) of methanol extract of the plant was added to 3 mL of 0.04% methanolic DPPH free radical solution. After 30 min, the absorbance of the solution was measured at 517 nm using a UV-visible spectrophotometer, which was compared with the absorbance of standard ascorbic acid concentrations (100–500 µg/mL). Then, the percentage inhibition was measured by using the following formula.

$$\% \text{ inhibition} = \frac{\text{Absorbance of blank} - \text{absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

The IC50 (inhibitory concentration 50%) was determined from the calibration curves obtained from different plant extract concentrations. IC_{50} value denotes the concentration of plant sample that inhibits the DPPH free radical by 50%.

Ferrous chelating assay

The chelating activity of the extract for ferrous ion (Fe^{2+}) was measured according to the standard method with slight modifications[16]. To 0.4 mL of plant extract of different concentrations (200, 400, 600, 800, and 1000 µg/mL), 1.6 mL of methanol was diluted and mixed with 0.04 mL of $FeCl_3$ (2 mM), followed by the addition of 0.8 mL of ferrozine (5 mM). The mixture was shaken well and incubated at room temperature for 10 min. The absorbance of the mixture was measured at 562 nm against a blank. The EDTA (Ethylene Diamine Tetraacetic Acid) was used as the positive control. The ability of chelating activity was calculated using the following formula.

$$\% \text{ inhibition} = \frac{\text{Absorbance of blank} - \text{absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

From the calibration curve obtained, the IC_{50} (µg/mL) value was determined.

Ferric ion-reducing assay

The reducing activity of the methanol extract of the plant for ferric ion (Fe^{3+}) was measured according to the standard method with minor modifications[17]. 1 mL of different concentrations of the sample (200, 400, 600, 800, 1000 µg/mL) was taken and mixed with 2.5 mL of phosphate buffer saline solution (pH 6.6) and 2.5 mL of potassium ferricyanide solution followed by incubation at 50°C in the water bath for 20 min. The reaction was stopped by adding 2.5 mL of 10% trichloroacetic acid and allowed to stand at room temperature for 10 min. Exactly 2.5 mL of the upper portion of the solution was taken, and the same volume of distilled water and 0.5 mL of 0.1% $FeCl_3$ were added and left for 20 min at room temperature, and absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used for standard control.

In vitro anti-inflammatory activity

The Human Red Blood Corpuscle (HRBC) membrane is similar to the membrane of the lysosome, and stabilising it alleviates inflammation. Therefore, the anti-inflammatory activity of the methanolic extract of *R. nobile* was determined by Human Red Blood Cell (HRBC) membrane stabilisation and inhibition of protein denaturation methods.

HRBC membrane stabilisation method

In the present investigation, the anti-arthritic activity of *R. nobile* was evaluated through the HRBC membrane stabilisation method using methanolic extract[18]. For this test, the method was followed with some modifications. Diclofenac Sodium was used as the positive control. Fresh blood, about 5 mL, was collected intravenously from one of the investigators who had not taken any dose of non-steroidal anti-inflammatory drug (NSAID) for 30 days before the experiment. The collected blood sample was mixed with an equal volume of Alsever's solution (dextrose 2%, sodium citrate 0.8%, citric acid 0.05%, NaCl 0.42%) and centrifuged at 3000 rpm for 10 min. Then, the packed cells were washed three times with

isosaline (0.8%, pH 7.2) and reconstituted as 10% v/v suspension by using isosaline and 10 mL each of different concentration gradients of methanolic extract of both plant and standard drug (diclofenac sodium) were prepared (1000-5000 µg/mL).

The reaction mixture (4.5 mL) contained 2 mL hyposaline, 1 mL phosphate buffer (0.15 M, pH 7.4), 1 mL extract of different concentrations (1000-5000 µg/mL) and 0.5 mL of 10% HRBC suspension. Similarly, control was also prepared by replacing the extract with distilled water. Then, the reaction mixtures were incubated for 30 min at 37°C and centrifuged at 2500 rpm for 5 min. The supernatant was collected carefully, and the haemoglobin content in the supernatant was measured using a spectrophotometer at 560 nm. The haemolysis percentage in control was assumed to be 100%. The percentage of HRBC membrane stabilisation or protection was calculated by

$$\text{Protection \%} = \frac{100-OD_1}{OD_2} \times 100$$

where OD_1= optical density of the test sample, OD_2= optical density of the control sample.

Protein denaturation assay

The anti-arthritic activity of the plant extract was examined through the protein denaturation method[19] with minor modifications.

Plant extract of different concentrations ranging from 1000-5000 µg/mL was prepared in methanol. Exactly 0.5 mL of each solution of different concentrations (1000, 2000, 3000, 4000, and 5000 µg/mL) of methanol extract was transferred to an Eppendorf tube with the help of a micropipette followed by the addition of 5 mL 0.2% bovine serum albumin solution (BSA in Tris-buffer saline, pH=6.8). Similarly, the standard solution of Diclofenac sodium with different concentrations ranging from 1000-5000 µg/mL was also prepared. A mixture of 0.5 mL of methanol and 5 mL of 0.2% BSA were used as a control. Then, the solution mixtures were incubated at 37°C for 20 min and then at 72°C for 5 min. Finally, sample extract, standard and control were cooled for 5 min, and the absorbance of these solutions was measured using a UV visible spectrophotometer at 660 nm. The percentage inhibition was calculated by

$$\text{\% inhibition} = \frac{\text{Absorbance of blank} - \text{absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

The IC_{50} was calculated from the graph of inhibition against different concentrations of extract.

Statistical analysis

All the measurements were made in triplicate, and the results obtained were expressed as the mean±standard deviation (SD). Statistical analysis was done by SPSS version 23, IBM Crop. A T-test was performed to compare the mean difference. P-value ≤0.05 was considered significant. To calculate the IC_{50} values, linear regression analysis was carried out using Microsoft Office Excel 2010 (Microsoft Corporation, USA).

Results

Physicochemical analysis

Ash value is the amount of inorganic content in drug material, which is used to determine the purity and quality of the drug. Extractive yield decides the number of important phytoconstituents extracted by a particular solvent from medicinal plants, which is useful in evaluating drug adulteration. The results of the physicochemical analysis and extractive yield of the plant sample are given in Table 1. The percentage yield is based on the solubility of the phytoconstituents present in the plant sample into a desirable solvent. The extractive value was 31.7% in 10 g of sample extracted in 100 mL of methanol.

Phytochemical analysis

Phytochemical estimation of medicinal plants in terms of qualitative and quantitative is regarded as an important step in the field related to medicinal plant research. The preliminary phytochemical analysis carried out on the methanol extract of *R. nobile* shows the presence of a wide range of phytoconstituents (such as Alkaloids, flavonoids, phenols, tannins, anthraquinones) that are presented in Table 2.

Quantitative estimation was carried out for four major phytoconstituents: phenol, flavonoid, flavonol and tannin. Phenolic compounds are one of the most predominant plant secondary metabolites which play an active role in the antioxidant capacity of medicinal plants and also contribute many benefits to human

Table 1 — Physicochemical evaluation of rhizomatic powder of *R.nobile*

Physicochemical parameters	Observed value (% W/W)
Total ash	9.35
Water soluble ash	4.1
Acid insoluble ash	2.05
Extractive yield	31.7

Table 2 — Preliminary phytochemical analysis of *R. nobile* in methanol extract

Phytochemicals	Methanolic extracts
Alkaloids	+
Flavonoids	+
Tannins	+
Phenols	+
Steroids	+
Phlobatannins	+
Terpenoids	-
Saponins	+
Anthraquinones	+
Proteins	-
Glycosides	+
Carbohydrates	+

Note: + (presence), - (absence)

Table 3 — Results of the quantitative phytochemical content in methanolic extract of *R. nobile*

Total phenolic content (GAE mg/g)	39.2±0.072
Total flavonoid content (RE mg/g)	80.17±0.099
Total flavonol content (RE mg/g)	81.95±0.084
Total tannin content (GAE mg/g)	58.17±0.049

Values are expressed as mean±SD, n=3

health as free radical scavengers. The antioxidant activity of plants may be due to the presence of phenolic compounds. Therefore, it is required to examine the phenolic compounds (qualitatively and quantitatively). The total phenolic content of a methanolic extract is 39.2±0.072 mg GAE/g (Table 3).

Flavonoids are a group of polyphenolic compounds that show good antioxidant properties due to various mechanisms, including direct scavenging of reactive oxygen species, metal chelation, inhibition of oxidases, activation of antioxidant enzymes and anti-inflammatory action. The total flavonoid content in the methanolic extract is 80.17±0.099 mg RtE/g (Table 3).

Flavonols are the class of flavonoids which have 3-hydroxyflavone backbone. Flavonols are present in almost all plants and act as potent antioxidants, protecting plants from reactive oxygen species. In the present study, the total flavonol content is 81.95±0.084 mg RtE/g in the methanolic extract (Table 3).

Tannins are naturally occurring polyphenols and the most abundant antioxidants in human diets that protect against oxidative stresses and degenerative diseases. Tannin not only functions as a primary antioxidant (i.e., acts as a Hydrogen donor) but also functions as a secondary antioxidant, which is able to chelate metal ions like Fe (II) and prevent one of the reaction steps in the Fenton reaction that hinder the progression of oxidation. In the present study, the total tannin content of the methanol extract is 58.17±0.049 mg GAE/g (Table 3).

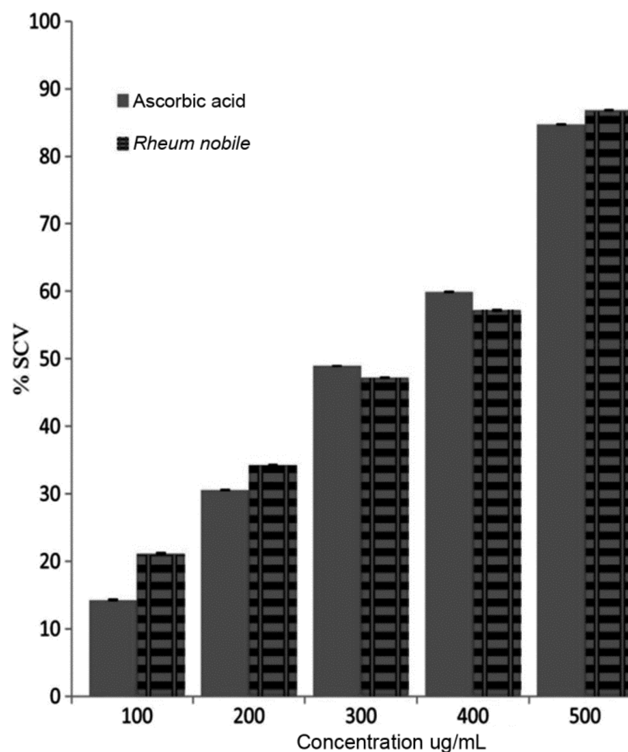

Fig. 1 — DPPH free radical scavenging activity of *R. nobile* in comparison with standard Ascorbic acid. Values are expressed as mean±standard deviation (n=3), and the concentration of the extract is provided in terms of μg/mL.

Antioxidant activity

The total antioxidant activity of the methanolic extract of *R. nobile* was tested *in-vitro* by using three different assays, namely DPPH radical scavenging assay, ferrous chelating assay and ferric ion reducing assay, in order to support the medicinal properties of this plant.

DPPH scavenging assay

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay is one of the most commonly used methods for calculating free radical scavenging activity and antioxidant activity of medicinal plants. The concentration-dependent curve of the DPPH free radical scavenging activity of methanolic extract is presented in Fig. 1, which shows an increase in scavenging activity with increasing concentration. Antioxidant activity in terms of IC_{50} value was also calculated (Table 4), corresponding to the concentration of plant samples that can scavenge 50% of free radicals in the reaction mixtures.

Table 4 — DPPH activity of *R. nobile* and standard ascorbic acid with their respective IC_{50} value

Concentration (µg/mL)	*R.nobile*	IC_{50} value (µg/mL)	Ascorbic acid	IC_{50} value (µg/mL)
100	21.15±0.0712		14.26 ±0.105	
200	34.23±0.103		30.54±0.0245	
300	47.2±0.0216	305.05	48.94±0.0246	274.04
400	57.18±0.0756		59.9±0.04	
500	86.84±0.0469		84.72± 0.025	

Values are expressed as mean±SD, n=3 in each concentration

Table 5 —Ferrous ion chelating assay of *R. nobile* and EDTA with their respective IC_{50} value

Concentration (µg/mL)	*R. nobile*	IC_{50} Value (µg/mL)	EDTA	IC_{50} value (µg/mL)
200	12.5±0.084	619.4	13.7±0.091	524.3
400	34.2±0.109		35.8±0.063	
600	45.5±0.057		48.6±0.033	
800	70.3±0.055		67.7±0.043	
1000	82.6±0.058		78.4±0.057	

Values are expressed as mean ± SD, n=3 in each concentration

High IC_{50} values signify low antioxidant activity. At 500 µg/mL, the scavenging activity percentages were 86.84% for *R. nobile* and 84.62% for ascorbic acid, respectively. The calculated IC_{50} value of *R. nobile* was 305.05 µg/mL, which is found to be closer to the reference compound ascorbic acid (IC_{50} 274.04 µg/mL), indicating the sample contains a potent scavenging activity. The methanolic extract exhibited good antioxidant properties and a high content of phenol, flavonoid, flavonol and tannin, confirming the positive correlation between antioxidant activity and total phenolic, flavonoid, flavonol, and tannin content.

Ferrous chelating assay

The ferrous chelating assay is also used as a significant method for the determination of antioxidant activity by its metal chelating power. The chelating activity of the methanolic extract of *R. nobile* by ferrous ion binding ability is given in Fig. 2. It shows that the chelating ability increases with the increase in concentration. The IC_{50} values of the plant extract and EDTA were 619.4 and 524.3 µg/mL, respectively (Table 5). At 1000 µg/mL, the percentage inhibition of the plant extract was 82.6%, whereas that of the standard EDTA was 78.4%. Phytoconstituents like phenolic groups, including flavonoids, can chelate the metal ions, inhibiting free radical production. Results showed a strong correlation between antioxidant activity and the total content of major phytoconstituents (phenol, flavonoid, flavonol and tannin).

Ferric ion-reducing assay

The reducing assay is based on the concept that substances having reduction ability react with

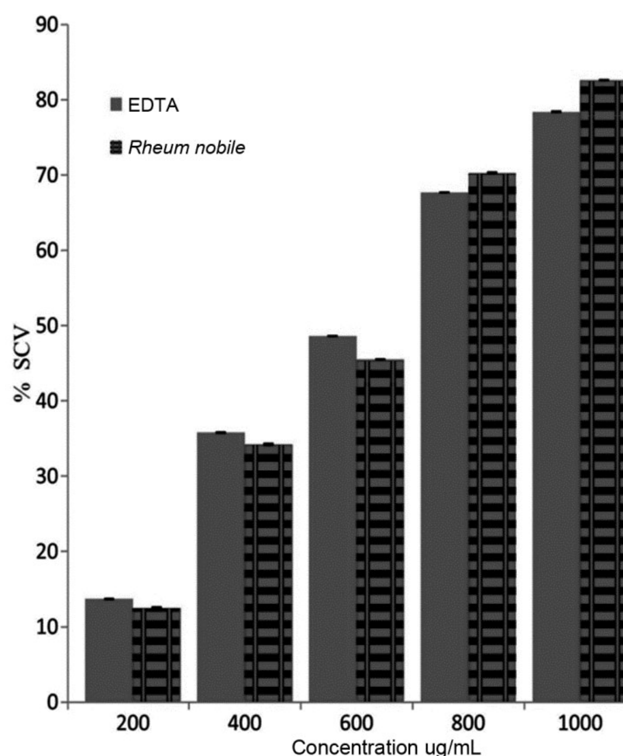

Fig. 2 — Ferrous chelating free radical scavenging activity of *R. nobile* in comparison with standard EDTA. Values are expressed as mean±standard deviation (n=3), and the concentration of the extract is provided in terms of µg/mL.

potassium ferricyanide to form potassium ferrocyanide, further it reacts with ferric chloride to form a ferric-ferrous complex in the presence of plant extract or compound, which shows maximum absorption at 700 nm. This reducing ability is based on the electron-donating capacity of medicinal plant extract and is considered one of the indicators of antioxidant activity. Increased

absorbance of the reaction solution indicated the high reducing activity of the plant extract. In the present study, methanolic extract showed concentration-dependent reducing ability, indicating that the plant extract can donate electrons. The concentration-dependent reducing activity of *R. nobile* extract compared to that of ascorbic acid is given in Fig. 3. At 200 µg/mL, the absorbance of the plant extract and ascorbic acid were 1.141±0.031 and 1.326±0.0314 respectively, while at 1000 µg/mL, the absorbance of the plant extract was 2.927±0.022 whereas that of ascorbic acid was 2.869±0.029. This result indicates that the plant extract possesses high reducing power compared to standard ascorbic acid.

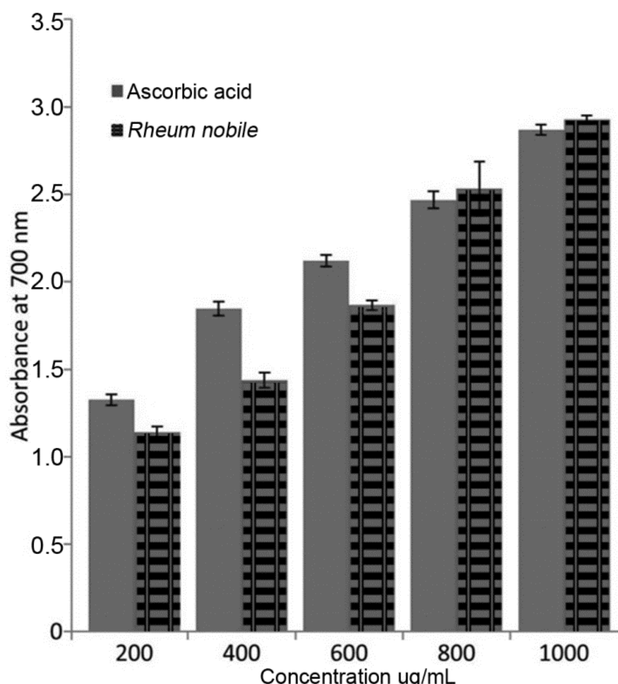

Fig. 3 — Ferric ion reducing ability of *R. nobile* in comparison with standard Ascorbic acid. Values are expressed as mean value ± standard deviation (n=3), and the concentration of the extract is provided in terms of µg/mL.

***In vitro* Anti-inflammatory property**

HRBC membrane stabilisation

The effects of the methanolic extract of *R. nobile* on the stabilisation of the HRBC membrane are shown in Table 6. The result shows that the plant extract showed a stabilising effect on the HRBC membrane, comparable with that of the standard drug (Diclofenac sodium). The membrane stabilising effect of the plant extract was found to be increased with an increase in concentration (Fig. 4a). Thus, the maximum percentage of stabilisation (73.12%) was found at the concentration of 5000 µg/mL, which was comparable to the standard drug (77.98%).

Protein denaturation assay

The effects of the methanolic extract of *R. nobile* and standard drug (Diclofenac sodium) on the inhibition of protein denaturation are presented in Table 6. The result shows a protective effect of plant extract on protein denaturation in a dose-dependent manner with the highest inhibition at the dose of 5000 µg/mL (79.98%) (Fig. 4b).

Discussion

In the traditional medicinal system, various plants are used to treat RA, and various studies have been performed to scientifically validate the potency of plants exhibiting anti-RA activity[20,21]. The present study investigated the *in-vitro* anti-arthritic and antioxidant activity of methanolic extract of *R. nobile*. Furthermore, qualitative phytochemical screening and quantitative phytochemical estimation were done for total phenolics, flavonoids, flavonols, and tannins content. This study shows evidence for the antioxidant and anti-inflammatory activity of *R. nobile*. Qualitative phytochemical analysis showed the presence of numerous phyconstituents (alkaloids, flavonoids, phenols, tannins, anthraquinones, terpenoids, etc.) along with significant content of four phytochemicals (total phenols, flavonoid, flavonol, and tannin).

Table 6 — *In-vitro* anti-arthritic activity of *R. nobile* in comparison with standard drug (Diclofenac sodium)

Concentration (µg/mL)	HRBC Membrane stabilisation		Protein denaturation	
	Protection % of *R. nobile*	Protection % of diclofenac sodium	Inhibition % of *R. nobile*	Inhibition % of diclofenac sodium
1000	54.87±0.08	63.18±0.13	21.8±0.03	25.76±0.04
2000	59.37±0.03	65.26±0.15	32.49±0.07	34.78±0.05
3000	63.25±0.06	71.18±0.14	41.06±0.05	45.1±0.07
4000	67.87±0.03	74.43±0.1	57.61±0.07	64.2±0.05
5000	73.12±0.06	77.98±0.08	79.98±0.03	83.8±0.06

Values are expressed as mean±SD, n=3 in each concentration

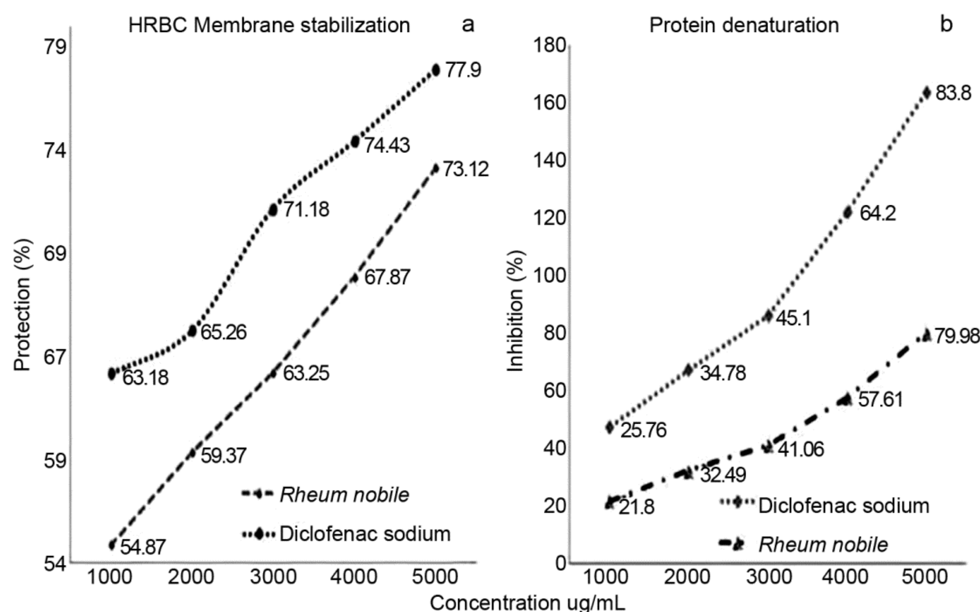

Fig. 4 — *In vitro* anti-inflammatory activity of *R. nobile* in comparison with standard drug (Diclofenac sodium). a) Percentage of HRBC haemolysis; and b) Inhibition of protein denaturation by methanolic extracts of *R. nobile* and standard drug Diclofenac sodium in various concentrations. Values are expressed as mean±standard deviation (n=3).

Oxidative stress is one of the factors causing rheumatoid arthritis[22]. Antioxidants are the most important substances which are able to stabilise free radicals and help to inhibit the imbalance developed during oxidative stress. At the same time, plant products are considered as significant source of natural antioxidants[23]. There are numerous studies on antioxidants from medicinal plants with antioxidant and anti-inflammatory properties, which can scavenge oxidising species[24,25]. Various phytochemicals derived from medicinal plants, like alkaloids, terpenoids, flavonoids, phenolic compounds, lignans, tannins, quinones, and coumarins, have significant antioxidant and other therapeutic activities[26]. Results of the present study revealed that *R. nobile* exhibited preeminent antioxidant activity, which can knock off free radicals in three scavenging assays (Fig. 1-3). It may be due to the presence of numerous phytochemicals which are able to neutralise the free radicals.

The membrane of the RBC is comparable to the membrane of the lysosome. The lysosomal degranulation is a major step in triggering an inflammatory response. Therefore, homology can be drawn if a plant extract has a stabilising effect on the RBC membrane. It may also stabilise the lysosomal membrane, thus inhibiting degranulation and, in turn, limiting inflammatory response[27]. Since arthritis is an inflammatory disorder, inhibiting RBC hemolysis in the hypotonic medium may provide evidence for the anti-arthritic effect of the plant extract. Present results suggested that *R. nobile* acquired a protective effect on erythrocyte lysis in a dose-dependent manner (Table 6), and the activity of the plant extract was not significantly different ($t=1.59$, $df=8$, $p=0.15$) to that of standard drug, signifying that the activity of plant extract is equivalent to that of the standard drug. The results of the present study provide evidence for the anti-inflammatory properties of *R. nobile*. However, in comparison to standard drugs, *R. nobile* was found to have lower HRBC membrane stabilising potential (Fig. 4a). Moreover, *R. nobile* was observed to inhibit the protein denaturation induced by heat, which suggests that it can prevent the loss of biological function, of proteins (Fig. 4b). The available literature shows that this is the first investigation to study the anti-inflammatory property of *R. nobile.* However, some plants of Sikkim Himalaya were also found to have anti-inflammatory property[28,29].

In the present study, the protein denaturation method was used for investigating the anti-arthritic activity of *R. nobile* using bovine serum albumin (BSA). When BSA is heated, it undergoes denaturation. It expresses antigens associated with type-III hypersensitivity reactions related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus

erythematosus[30]. This protein denaturation is likely to be related to the breakdown of hydrogen, electrostatic, disulphide and hydrophobic bonds of protein structures[31], and it is very much recorded as a factors for inflammatory conditions in rheumatoid arthritis, cancer, and diabetes[32]. Protein denaturation is one factor causing rheumatoid arthritis and autoantigen production in certain arthritic diseases[33]. Therefore, the inhibition of protein denaturation method was used to study the anti-arthritic activity of *R. nobile*. The results show that *R. nobile* can effectively inhibit protein denaturation in a dose-dependent manner (Table 6). The t-test revealed that the activity of plant extract was not significantly different ($t=0.28$, $df=8$, $p=0.786$) from that of the standard drug, which suggests that the activity of *R. nobile* is comparable to that of the standard drug used. The results suggest the potential of *R. nobile* in controlling the production of auto antigen and inhibiting the protein denaturation in arthritic disease.

The anti-arthritis activity of *R. nobile* in methanolic extract is possibly due to diverse phytochemical constituents (Phenol, terpenoids, flavonoids, flavonols, tannin, etc.). Phenolic compounds work in a comparable way to NSAIDs; some inhibit other pro-inflammatory mediators by inhibiting their activity[34]. The flavonoids smoothly inhibit the production of free radicals and prevent the development of pro-inflammatory mediators[35]. Plants containing flavonoids or polyphenols are good choices for anti-RA treatment based on their three properties: antioxidant, anti-apoptotic and anti-inflammatory[36]. Terpene can be useful in managing chronic pain mainly due to inflammation, and it may adjust the immune system and destructive tissues that cause the clinical appearance and development of arthritis[37]. The present findings on *R. nobile* in terms of the presence of numerous phytoconstituents and antioxidant activity and *in-vitro* anti-RA activity justified the earlier findings of bioactive components with anti-RA effects.

The present investigation has certain limitations. First, the antioxidant and anti-inflammatory properties of *R. nobile* were studied only by the *in-vitro* method. Secondly, only a few qualitative and quantitative phytochemical screenings were performed with the plant extract. Thirdly, the specific molecule(s) responsible for the antioxidant and anti-inflammatory activity of *R. nobile* was not identified. Considering the above limitations, the study provides evidence for the antioxidant and anti-inflammatory properties of *R. nobile*.

Conclusion

Himalayan plants have been known as one of the major sources of phytochemicals used to treat various diseases since the ancient period in India. Scientific approaches to investigate their bioactive principles are meagre. The present investigation attempted to validate the traditional knowledge-based healthcare system used by the ethnic people of the Sikkim Himalayan region.

The present study reveals the antioxidant and anti-inflammatory properties of *R. nobile*, which may be due to the presence of diverse phytochemicals, further suggesting its potential use in inflammatory diseases such as arthritis, for which the people of Sikkim Himalayan region extensively use it. Further studies are warranted in the *in-vivo* model to understand the mechanism of antioxidant, anti-inflammatory and anti-arthritic properties.

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Conflict of interest

The authors declare that there is no conflict of interest.

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