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# *In vitro* and *in vivo* pharmacological activities of the extracts of *Rheum nobile* Hook. f. & Thomson rhizomes

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*Rheum nobile* Hook. f. & Thomson (family Polygonaceae) is extraordinary, unexploited, and vulnerable Himalayan rhubarb with high ethnomedicinal values. Traditionally in Nepal, its rhizomes are used to relieve pain, swelling, and in bone fractures. In this study, several *in vitro* and *in vivo* assays of the rhizome extracts were carried out. The hexane, dichloromethane, ethyl acetate and butanolic extracts obtained after fractionation of 95 % ethanolic extract showed potentially cytotoxicity against *Artemia salina* nauplii and antibacterial effect. The 80 % ethanolic extract displayed sedative effect in mice ($ED_{50}$ = 100.32 mg/kg). The same extract inhibited 66.60 and 81.49 % of acetic acid-induced writhes in mice at doses 250 and 400 mg/kg, respectively ($p$ <0.0001). The extract could increase the reaction latency up to 774.59 % in the Eddy's hot plate assay ($p$ <0.0001). A high anti-inflammatory effect was observed in the Albumin-induced paw edema assay. The extract also exhibited hypoglycemic activity by lowering blood glucose level in normoglycemic rats, and antifertility activity in pregnant rats. CNS depressant and antifertility activities are observed for the first time in genus *Rheum*.

**Keyword:** Analgesic, Antibacterial, Antidiabetic, Antifertility, Anti-inflammatory, Himalayan rhubarb.

**IPC code; Int. cl. (2015.01)** – A61K 36/00, A61K 36/70

## Introduction

*Rheum nobile* Hook. f. & Thomson (family Polygonaceae) is a giant herbaceous plant (1-2 m tall), native to the Himalaya, distributed from North-Eastern Afghanistan, east through Northern Pakistan and India, Nepal, Sikkim (in India), Bhutan, and Tibet to Myanmar, occurring in the alpine zone at 3900-5000 m altitude[1-4]. It is a perennial herb with conical tower of delicate, straw-colored, shining, translucent, and regularly overlapping leafy bracts. Large, glossy and green radicle leaves, with red petioles and nerves, form a broad base to the plant. Thick rootstock and simple erect stem that stout to 1.5 m.

Leaf and stem of *R. nobile* are used as fodder and vegetable (either raw or cooked)[5,6]. Ethnomedicinally, rhizomes of *R. nobile* are considered as an astringent, carminative, depurative, diuretic, purgative, and tonic[7]. Small dose acts as an astringent tonic to the digestive system, whilst larger dose acts as a mild laxative[8]. It is used in the treatment of swelling, fullness of abdomen, and to rid the body from retained fluids. The stem is used in Tibetan medicine as a heating potent[9]. In Nepal, the rootstocks are boiled (sometimes with the rhizome of *Bergenia ciliata*) and used to make a paste with a little flour. The paste is then applied externally in cases of bone fractures and body ache, and the decoction is used for stomach and menstrual disorders[1]. Global status of the plant material is unknown; however, the species is vulnerable mainly because of its rare occurrence, livestock grazing, and over harvesting for local use. Isolation of flavonoid glycosides from the bracts[10], and quantitation of anthraquinone content in the rhizome are reported[11]. Besides these two, there is no other report on chemistry and pharmacology of the plant material.

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Available modern therapies are unable to control all the diseases. Furthermore, the allopathic drugs used for medical treatment are associated with side effects, enormous cost as well as poor in availability. At the same time, appearance of new diseases and drug resistance problems are emerging. These facts are making tremendous importance for searching of more efficacious and safe agents from the nature. Therefore, natural product chemists inherently focus towards traditional knowledge and medicinal plants. Recently, we have reported phytoconstituents and *in vitro* bioactivities of the rhizomes of *Rheum australe* D. Don (synonym *R. emodi* Wall. ex Meisn.)[12,13]. This Himalayan rhubarb is distributed at 3200-4200 m (beneath the natural habitat of *R. nobile*), and widely used in traditional herbal formulation by Amchis (traditional healers). Any adverse effect or toxicity by consuming *R. australe* is not evident[14]. The secondary metabolites it constitutes are: anthraquinones, anthrones, chromones, flavonoids, lignans, phenols, sterols and stilbenes. Anti-inflammatory[15] and antidiabetic[16-18] effects of the extracts of *R. australe* in experimental animals have been reported. In Nepal, both Himalayan rhubarbs *R. australe* and *R. nobile* are ethnically used for similar medicinal purposes and are collectively called as "Padamchal", despite they are two distinct species of the same genus *Rheum*[1]. Herein, we first time describe *in vitro* and *in vivo* studies of the bioactivities of the extracts of the rhizomes of *R. nobile*.

## Materials and Methods

### Plant material

Collection of the rhizomes of *R. nobile* was permitted by Department of National Parks and Wildlife Conservation, Ministry of Forests and Soil Conservation, Government of Nepal (Letter no. 2505-2071) for the scientific experiments. The rhizomes were collected from Topke Gola, Taplejung District, Eastern Nepal, at 4800 m altitude, in July 2014. The herbarium of the plant material was identified at the National Herbarium and Research Laboratory, Godawari, Lalitpur, Nepal, and a Voucher specimen (no. 20128) was deposited. The plant material was air dried at room temperature for several weeks and coarsely grounded using a blender machine.

### Chemicals and drugs

Chemicals and solvents were purchased from Merck, Qualigens, Sigma-Aldrich and Fisher Scientific companies, and were used as received. Antibiotic Amoxicillin (25 µg/disc) was purchased from HiMedia. Standard drugs *viz.* paracetamol (Supumol 500, Apple International Pharmaceutical Pvt. Ltd.), clonazepam (Clonaz 0.5, Nepal Pharmaceuticals Laboratory Pvt. Ltd.), acetyl salicylic acid (Ecosprin 75, USV Ltd.) and glipizide (Glipiz 5, Lapen) were obtained from Shree Ram Pharmacy, Tribhuvan University Teaching Hospital, Maharajgunj, Nepal. For estimation of blood glucose level, blood glucose meter and sticks were obtained from Life Scan Inc., Johnson and Johnson, China.

### Microorganisms and animals

Brine shrimp (*Artemia salina*) eggs were purchased from San Francisco Bay Brand Inc., USA. Brine shrimp nauplii were freshly hatched before the experiment. Bacterial strains *viz*. *Bacillus subtilis*, *Staphylococcus aureus* (ATCC 25952), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028) and *Serratia marcescens* were obtained from National Endemic Health Care Centre, Teku, Kathmandu, Nepal. The microorganisms were maintained on nutrient agar at 4 °C and subcultured before using.

Healthy Swiss albino mice (20-30 g) and rats (150-250 g) were obtained from the Animal House of the Department of Plant Resources, Thapathali, Kathmandu, Nepal. Care and use of laboratory animals were in accordance with international guidelines adopted by Department of Plant Resources, Ministry of Forests and Soil Conservation, Government of Nepal. The study was approved by the Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal (Reference no. 579/067/068) and the Dean Office, Tribhuvan University, Kirtipur, Kathmandu, Nepal (Reference no. 434/069/070). The animals were housed under standard conditions of temperature (22±1 °C), relative humidity (55±10 %) and 12 h light/dark cycle; and had free access to the standard pellet diet (Amrut Lab Animal Feed, Pranav Agro Industries Ltd., Sangli, India) and water *ad libitum*. The animals were fasted for 12 h prior to the experiments.

### Extraction and *in vitro* studies

Powder of *R. nobile* rhizome (100 g) was extracted with 95 % ethanol (1000 mL) using a Soxhlet extractor for 16 h followed by concentration. The concentrate was diluted with distilled water (500 mL) and then successively fractionated with hexane

(500 mL × 3), dichloromethane (500 mL × 3), ethyl acetate (500 mL × 3), and n-butanol (500 mL × 3) followed by concentration to obtain residues of the corresponding hexane extract (2.06 g), dichloromethane extract (1.23 g), ethyl acetate extract (13.63 g) and butanolic extract (8.43 g). These extracts were used in the following *in vitro* studies.

***Brine shrimp lethality assay***

Cytotoxicity of the extracts obtained above was evaluated using the Brine shrimp lethality assay as described previously[19]. Sample solutions were prepared by dissolving 100 mg of each plant extract in methanol up to the mark in 10 mL volumetric flasks. To calculated volume of the sample solution for 10, 100 and 1000 µg/mL dose levels in five replicates was introduced freshly hatched ten brine shrimp nauplii in artificial sea water (total volume 5 mL). After 24 h of incubation under illuminated light, the percentage death was determined and the $LC_{50}$ (Lethal concentration for 50 % mortality) value with 95 % CI (Confidence Interval) were calculated by regression analyses.

***Antibacterial susceptibility assay***

The Antibacterial susceptibility assay of the plants extracts was carried out following the Agar well diffusion method[20]. Stock solutions of the plant extracts with concentration 50 mg/mL in dimethyl sulphoxide (DMSO) were prepared. Thus prepared solution (50 µL) in triplicate was used to measure a zone of bacterial growth inhibition (ZOI) after 24 h of incubation at 37 °C. Amoxycillin (25 µg/disc) was used as positive control and DMSO (50 µL) as negative control. The Minimum Bactericidal Concentration (MBC) value was determined by standard two-fold micro dilution broth methodology[21]. The stock solution of different extracts was serially diluted with nutrient broth to obtain a concentration ranging from 25.0 to 0.78 mg/mL (total volume 1 mL). To this was added standard culture inoculum of each bacterial strain (25 µL) and then incubated at 37 °C for 24 h. The plates were observed for bacterial growth.

**Extraction and *in vivo* studies**

Soxhlet extraction of the rhizome powder (150 g) was carried out with 80 % ethanol (1000 mL) for 72 h. The extract was concentrated under reduced pressure, and then vacuum dried to obtain dark brown solid (34.9 g, 23.27 %). The plant extract as well as standard drugs used in following *in vivo* studies were dissolved in distilled water before administration to the experimental animals.

***Acute toxicity test***

The acute toxicity of the 80 % ethanolic extract of *R. nobile* rhizomes was determined by Lorke's method[22]. Thirty mice were divided into five groups of six mice each (n = 6). Graded doses of the extract (250, 400, 500, 750, and 1000 mg/kg) were orally (p.o.) given in a single dose to each mouse and then animals were housed under standard conditions. All the experimental animals were observed for mortality and any abnormal behavior for next seven days to evaluate the acute toxicity of the plant extract.

***Open field neurological activity test***

Mice were divided into six groups each consisting six animals (n = 6). The first group (control) received 0.9 % saline (10 mL/kg p.o.), the second group (positive control) received clonazepam (1 mg/kg p.o.), and the remaining groups (test) received the extract in different doses (62.5, 125, 250, and 400 mg/kg p.o.). After drug treatment, each mouse was placed in the middle of an open field (total size 50 × 50 cm) containing 25 squares of 10 × 10 cm. The number of squares crossed by the mouse over a period of 3 min was counted in 30 min intervals for 1.5 h. The percentage inhibition of locomotion due to sedation was calculated using the formula: % Inhibition = $(C_c - C_t)/C_c \times 100$, where $C_c$ is the mean of number of squares crossed in the control group and $C_t$ is the mean of number of squares crossed by the test group[23].

***Acetic acid-induced writhing syndrome test***

Six mice in each group were separated into four groups (n = 6): the first group served as control received 0.9 % saline (10 mL/kg p.o.), the second group as a positive control was given paracetamol (75 mg/kg p.o.), and the remaining as test groups received the extract in two different doses (250 and 400 mg/kg p.o.). The animals were given oral drug treatment 30 min prior to intraperitoneal (i.p.) administration of 0.6 % acetic acid solution (10 mL/kg). The animals were placed on an observation table and immediately after 5 min, the number of writhes in each mouse was observed for 20 min. Full writhing was not always accomplished; therefore, incomplete writhing was considered as half-writhing. Recordings were similarly made at 60, 90, and 120 min. The percentage inhibition of writhing due

to analgesia was calculated using the formula: % Inhibition = $(W_c - W_t)/W_c \times 100$, where $W_c$ is the mean of writhes in the control group and $W_t$ is the mean of writhes produced by the test group[24].

***Eddy's hot plate test***

Central analgesic action of the extract was studied by Eddy's hot plate method[25]. Mice were divided into four groups each consisting of six animals (n = 6). The first group (control) received 0.9 % saline (10 mL/kg p.o.), the second group (positive control) was given paracetamol (75 mg/kg p.o.), and the remaining groups (test) received the extract in two different doses (250 and 400 mg/kg p.o.). Immediately after drug treatment, the mouse was placed on a hot plate (55±1 °C) and the reaction time for licking of hind paw or jumping was noted. Recordings were taken at 0, 30, 60, 120, and 180 min. The % increase in reaction time due to analgesia was calculated using the formula: % Increase in reaction latency = $(I_t - I_0)/I_0 \times 100$, where $I_t$ is the mean of reaction time at time t and $I_0$ is the mean of reaction time at zero time.

***Albumin-induced paw edema test***

Rats were divided into four groups consisting six animals (n =6), and the paw circumference of right hind paw of each was measured. The first control group received 0.9 % saline (10 mL/kg p.o.), the second positive control group was subcutaneously administered acetyl salicylic acid (100 mg/kg), and the remaining test groups received the extract in two different doses (250 and 400 mg/kg p.o.). Paw edema in right hind paw of rats was induced by subcutaneous injection of 0.1 mL of fresh egg white. Thereafter, the paw circumference was measured after 30, 60, 120, and 180 min. The anti-inflammatory activity was evaluated using formula: % Inhibition = $(D_c - D_t)/D_c \times 100$, where $D_c$ is the change in paw circumference in control group and $D_t$ is the change in paw circumference in the treated group[26].

***Hypoglycemic activity test***

Rats were divided into four groups each consisting six animals (n =6). The first group (control) received 0.9 % saline (10 mL/kg p.o.), the second group (positive control) received glipizide (1 mg/kg p.o.), and the remaining groups (test) received the extract in two different doses (250 and 400 mg/kg p.o.). Blood sample was collected from the tail tip at 0, 30, 60, 120, and 180 min, and blood glucose level was determined. The percentage decrease in blood glucose level was calculated using the formula: % Decrease in glucose level = $(G_0 - G_t)/G_0 \times 100$, where $G_0$ is the mean of glucose level at zero time and $G_t$ is the mean of glucose level at time t[27].

***Antifertility test***

Female and male rats in ratio of 2:1 were caged overnight for mating. The presence of post-coital plug in the vaginal smear evidenced positive mating. The day of mating was designated as first day of pregnancy. The pregnant rats were divided into three groups (n =4). First group served as control, and received 0.5 % gum acacia mg/kg p.o., while the second and third groups received the extract at single p.o. doses 125 and 250 mg/kg respectively for the next 10 days. Free access to food and water was allowed. The animals were autopsied on the eleventh day of pregnancy under local anesthesia using chloroform and both the uterine horns were examined for the number of implantation sites and fetuses. % of fetal loss and % of inhibition of pregnancy were calculated to evaluate the antifertility activity of the extract[28].

**Statistical analysis**

Statistical analysis was carried out using software of Statistical Package for the Social Sciences (SPSS) and GraphPad Prism 6 for windows, version 6.05. Descriptive analysis was expressed as mean±standard error of the mean (SEM). Statistical differences between all the groups and between two groups were evaluated by one-way analysis of variance (ANOVA) followed by Student's *t*-test with Welch's correction.

**Qualitative phytochemical analysis**

Using standard methods[29], all the extracts used in the present study were used for preliminary qualitative phytochemical analysis to reveal the presence of alkaloids (Dragendorff's test), anthocyanosides (acid-base test), anthraquinones (Borntiager's test), cardiac glycosides (Kedde's test), carotenoids (sulfuric acid test), coumarins (fluorescence test), flavonoids (Shinoda's test), lactones (dinitrobenzoic acid test), reducing compounds (Fehling's test), saponins (froth test), steroids and terpenoids (Liebermann-Burchard's test), and tannins and polyphenols (Braymer's test).

## Results

**Brine shrimp cytotoxicity**

In the Brine shrimp lethality assay, hexane and butanolic extracts showed 100 % mortality at concentration of 1000 µg/mL with $LC_{50}$ values of

180.81 and 9.33 µg/mL, respectively (Table 1). Cytotoxicity toward brine-shrimp was also observed using dichloromethane extract ($LC_{50}$ = 147.90 µg/mL) and ethyl acetate extract ($LC_{50}$ = 53.70 µg/mL).

**Antibacterial activity**

The result of the antibacterial susceptibility assay of *R. nobile* rhizome extracts is shown in Table 2. Interestingly, all the extracts (dose = 2.5 mg/well) inhibited the growth of both gram-positive and gram-negative bacteria (ZOI = 12-29 mm), except *E. coli* and *S. marcescens* were unaffected using the hexane extract. Minimum bactericidal concentration (MBC) value of the plant extracts was then evaluated and the results are presented in Table 2.

**Acute toxicity in mice**

The 80 % ethanolic extract produced 100 % mortality in mice within 24 h, when administered at higher doses of 750 and 1000 mg/kg. The lethal dose ($LD_{50}$) was 500 mg/kg killing 50 % mice of the extract-treated group. When the mice were fed with 250-400 mg/kg of plant extract, then the animals were found clumsy for a couple of hours, but thereafter, there was no evidence for abnormal behavior (such as agility, tremor, convulsion, breathing problem, difficulty in consumption of food and water) or mortality for following seven days indicating no toxicity of the extract upon administration at dose up to 400 mg/kg, but efficient to produce sedative effect.

**CNS depressant activity**

Central nervous system (CNS) depressant effect of the plant material was studied by the Open field neurological activity test; in which induction of sleep in the experimental animals was observed after the sample administration. The experimental results are presented in Fig. 1 (one-way ANOVA, $p$ <0.001). Mice felt asleep within 30 min when the plant extract was administered at 250 and 400 mg/kg doses with effective dose ($ED_{50}$) of 100.32 mg/kg, and continued sleeping during observation period. A significant effect ($p$ <0.001) at a lower dose level was also observed as compared to the standard drug clonazepam. On next day, behavior of all the experimental mice was found normal. This experiment proved that the rhizome of *R. nobile* has strong neurological activity.

**Analgesic activity**

The analgesic activity of the extract was evaluated using the Acetic acid-induced writhing syndrome test and Eddy's hot plate method. Fig. 2a clearly shows

Table 1—Brine shrimp lethality assay of *R. nobile* rhizome extracts

| Plant extracts used | Percentage death at 24 h/dose | | | $LC_{50}$ (µg/mL) | 95 % CI (µg/mL) |
|---|---|---|---|---|---|
| | 10 (µg/mL) | 100 (µg/mL) | 1000 (µg/mL) | | |
| Hexane | 22 | 62 | 100 | 180.81 | 180.81±2.26 |
| Dichloromethane | 2 | 24 | 98 | 147.90 | 147.9±2.26 |
| Ethyl acetate | 14 | 90 | 98 | 53.70 | 53.7±2.26 |
| n-Butanol | 48 | 82 | 100 | 9.33 | 9.3±2.26 |

Table 2—Antibacterial activity of *R. nobile* rhizome extracts

| Pathogenic bacteria used | ZOI (MBC) of different extracts and standard drug[a] | | | | |
|---|---|---|---|---|---|
| | Hexane | Dichloromethane | Ethyl acetate | n-Butanol | Amoxycillin |
| *B. subtilis* | 12.33±0.58 (12.50) | 16.33±0.58 (12.50) | 17.33±1.15 (6.25) | 17.00±0 (12.50) | 11.67±0.58 |
| *S. aureus* | 15.00±0 (12.50) | 18.33±0.58 (6.25) | 20.67±1.15 (6.25) | 14.33±0.58 (12.50) | 42.33±0.58 |
| *E. coli* | – | 14.33±0.58 (12.50) | 15.67±1.15 (12.50) | 16.00±0 (12.50) | 22.67±0.58 |
| *K. pneumoniae* | 12.67±0.58 (12.50) | 16.33±0.58 (12.50) | 16.00±0 (12.50) | 16.67±0.58 (12.50) | 25.00±0 |
| *P. aeruginosa* | 16.33±0.58 (12.50) | 28.33±0.58 (3.12) | 24.00±0 (6.25) | 24.33±0.58 (6.25) | 32.33±0.58 |
| *S. typhimurium* | 13.67±0.58 (12.50) | 17.67±1.15 (6.25) | 19.33±0.58 (6.25) | 20.33±0.58 (6.25) | 44.67±2.89 |
| *S. marcescens* | – | 12.00±0 (12.50) | 16.67±0.58 (12.5) | 19.67±0.58 (6.25) | 8.67±0.58 |

[a]Values of ZOI (mm) are expressed as mean ± standard deviation (SD); MBC values (mg/mL) are presented in parenthesis. (–) = no significant result.

that both standard drug paracetamol and the plant extract significantly reduced the number of writhes induced by i.p. injection of acetic acid (one-way ANOVA, $p$ <0.001). Maximum analgesic effect was observed after 1.5 h of the drug treatment. The standard drug exhibited a writhing inhibition of 50.30 %, at the same time; the plant extract has shown more potential analgesic effect exhibiting 66.60 and 81.49 % inhibitions at doses 250 and 400 mg/kg, respectively. A high analgesic activity of the plant extract was also evidenced in the hot plate assay, and the results are depicted in Fig. 2b (one-way ANOVA, $p$ <0.001). By applying Student's $t$-test, significant effect of the plant extract ($p$ <0.005) in increasing the reaction time against hot plate was observed compared to paracetamol. Up to 774.59 % increase in the reaction latency was observed when the animals were given dose of 400 mg/kg. The results indicated an efficient analgesic activity of the rhizomes of *R. nobile*.

**Anti-inflammatory activity**

Anti-inflammatory activity of the extract was evaluated using the Albumin-induced paw edema test, and the results are depicted in Fig. 3 (one-way ANOVA, $p$ <0.001). Maximum edema was observed after 1 h in the control group, which persisted even after 3 h. The reduction of paw edema was observed throughout observation period after administration of both standard drug acetyl salicylic acid and the plant extract. Anti-inflammatory effect of the extract at dose 250 mg/kg was comparable to that of acetyl salicylic acid given at dose of 100 mg/kg.

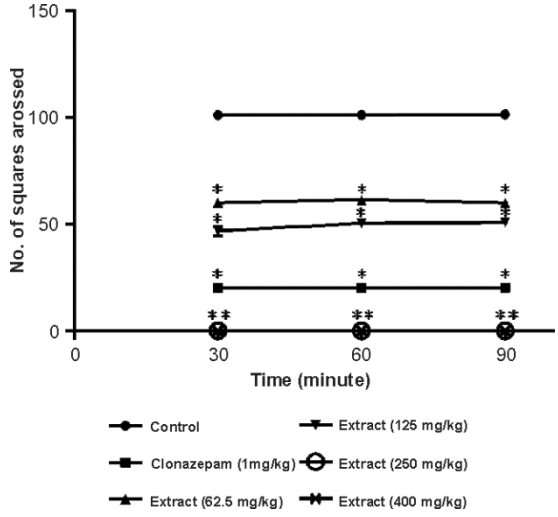


Fig. 1—Effects of clonazepam and the plant extract in the Open field neurological activity test in mice. * $p$ <0.0001 compared with control group.

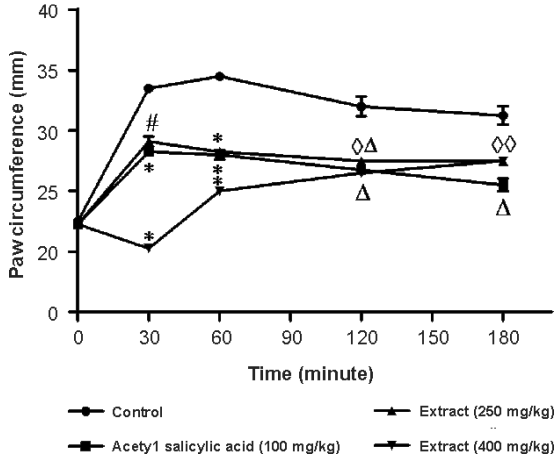


Fig. 3—Effects of acetyl salicylic acid and the plant extract in the Albumin-induced paw edema test in rats. * $p$ <0.0001, # $p$ <0.0005, Δ $p$ <0.005 and Δ $p$ <0.02 compared with control group.

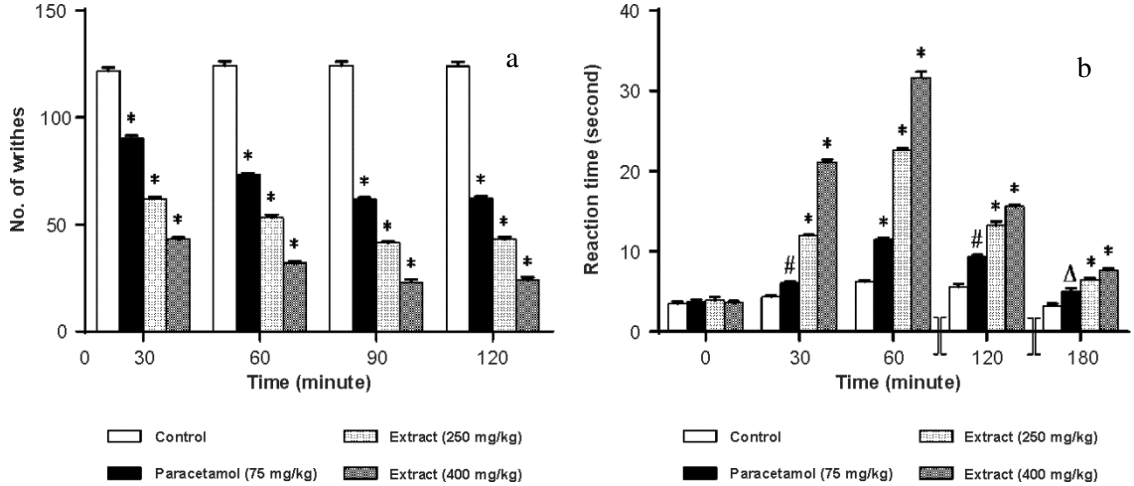


Fig. 2—Effects of paracetamol and the plant extract on (a) Acetic acid-induced writhing and (b) Eddy's hot plate assays in mice. * $p$ <0.0001, # $p$ <0.0005 and Δ $p$ <0.005 compared with control group.

Surprisingly, a very quick effect of the plant extract in shrinking of paw volume was observed upon administration of increased dose of 400 mg/kg ($p$ <0.0001). When the animals were given dose of 400 mg/kg, the paw was found to shrink more than normal volume during the initial 30 min and then gradually swelled. As compared to the control, significant inhibitions of paw edema by administration of acetyl salicylic acid (% inhibition = 62.90 % after 180 min), extract 250 mg/kg (% inhibition = 50.04 % after 60 min), and extract 400 mg/kg (% inhibition = 118.16 % after 60 min) were observed. In contrast to the standard drug, anti-inflammatory effect of the plant extract was found to gradually diminish after 60 min.

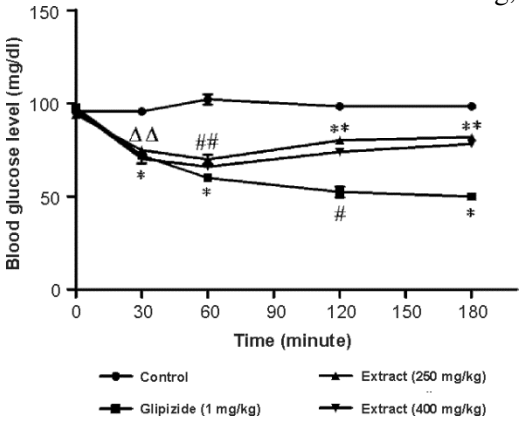


Fig. 4—Effects of glipizide and the plant extract in the blood glucose level in normoglycemic rats. * $p$ <0.0001, # $p$ <0.0005 and Δ $p$ <0.005 compared with control group.

**Hypoglycemic activity**

Fig. 4 shows the hypoglycemic effect of single dose administration of the plant extract on blood glucose level in normoglycemic rats and the results were found significant (one-way ANOVA, $p$ <0.001). At 60 min, glipizide (dose 1 mg/kg) and the extract (250 and 400 mg/kg doses) reduced blood glucose level by 38.78 % ($p$ <0.0001), 25.53 % ($p$ 0.0002) and 31.43 % ($p$ 0.0005), respectively. The hypoglycemic effect of the crude extract in the tested doses was inferior to the reference drug glipizide. However, these results apparently evidenced that the rhizomes of *R. nobile* bear hypoglycemic agents, and possess antidiabetic activity.

**Antifertility activity**

In antifertility activity test, all the mated female rats were found pregnant in the control group (Table 3). On the other hand, the fertility index was found to be declined to 75 and 50 % in rats receiving doses of 125 and 250 mg/kg extract per day, respectively. The number of total uterine implantation sites and viable fetuses showed a dose-dependent fetal loss percentage.

**Phytochemical analysis**

The result of preliminary phytochemical analysis is shown in Table 4, which revealed that the rhizome of

Table 3 — Antifertility activity of the *R. nobile* rhizome extract in female rats

| Group (n = 4) | Dose | No. of animals (pregnant/treated) | % Fertility index | % Inhibition of pregnancy | % Fetal loss |
|---|---|---|---|---|---|
| 0.5% Gum acacia | 10 mL/kg | 4/4 | 100 | 0 | 0.06 |
| Extract 1 | 125 mg/kg | 3/4 | 75 | 25 | 37.70 |
| Extract 2 | 250 mg/kg | 2/4 | 50 | 50 | 63.04 |

Table 4—Qualitative phytochemical screening of *R. nobile* rhizome extracts

| Phytochemicals | Various extracts | | | | |
|---|---|---|---|---|---|
| | Hexane[a] | Dichloromethane[a] | Ethyl acetate[a] | n-Butanol[a] | Ethanol[b] |
| Alkaloids | – | – | – | – | – |
| Anthocyanosides | – | – | – | – | – |
| Anthraquinones | + | + | + | + | + |
| Cardiac glycosides | – | – | – | – | – |
| Carotenoids | – | – | – | – | – |
| Coumarins | – | + | + | – | + |
| Flavonoids | + | + | + | + | + |
| Lactones | – | – | – | – | – |
| Reducing compounds | – | + | + | + | + |
| Saponins | – | – | – | + | + |
| Steroids and terpenoids | + | + | – | – | + |
| Tannins and polyphenols | + | + | + | + | + |

[a]Extract was used in *in vitro* studies; [b]Extract was used in *in vivo* studies; (+) = presence and (–) = absence.

*R. nobile* rhizome constituted of anthraquinones, coumarins, flavonoids, reducing compounds, saponins, steroids and terpenoids, and tannins and polyphenols.

## Discussion

Rhubarbs are considered as king of herbs[30]. Despite folkloric usefulness of *R. nobile* being mentioned elsewhere, scientific evaluation for its medicinal potentiality has not been examined (rare occurrence of the plant species could be a major reason). Considering popular uses of rhubarbs and in continuation to study activities of different rhubarb species, pharmacological properties of rarely explored *R. nobile* have been investigated in the present study.

The Brine shrimp lethality assay is considered as a simple, safe, and economic method, which correlates with *in vitro* growth inhibition of cancer cell lines and therefore, used as a tool for prescreening assay in anticancer drug research[31,32]. The plant extract displaying $LC_{50}$ value less than 1000 is considered as pharmacologically active and is potentially anticarcinogenic. All the fractionated rhizome extracts displayed cytotoxicity against brine shrimp nauplii (Table 1); therefore, rhizome of *R. nobile* is considered to be constituted of anticarcinogenic ingredients. As depicted in Table 2, antibacterial activity of all the extracts used was evidenced, which may be due to the presence of anthraquinones such as chrysophanol and emodin[11]. Antibacterial activity of these compounds has already been reported[33,34].

Oral administration of the 80 % ethanolic extract of *R. nobile* rhizome up to 400 mg/kg body weight to mice produced no acute toxic effect except onset of sleep due to sedative activity and after being awake, they behaved normally for an observation period of seven days. A higher dose treatment caused death of mice with $LD_{50}$= 500 mg/kg. It is well established that bioactive extract/ compound possess some toxicity, but when administered, it should not produce fatality. Therefore, in the present study, the maximum dose was set at 400 mg/kg.

Palmitic, oleic and omega-3 fatty acids functioning as precursors of sleep inducing oleamide were shown to be effective for depression[35-37]. Psychomotor stimulants enhance locomotor activity, and produce a false positive antidepressant affect. Decrease of locomotor activity is related to sedation resulting from depression of CNS[38]. γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the CNS. Anxiolytic, analgesic and hypnotic drugs activate GABA receptors. In the Open field test, the extract of *R. nobile* rhizomes showed significant CNS depressant effect inducing asleep in mice within 30 min when administered 250 and 400 mg/kg doses. During observation period, the number of squares they have crossed was zero with 100 % inhibition (Fig. 1). Therefore, the extract was possibly acted through direct activation of GABA receptor. Plants containing flavonoids, saponins and tannins were found useful in many CNS disorders[39]. In phytochemical analysis, flavonoids, saponins and tannins were detected in the plant extract.

Pain is sensed as a result of interaction of peripheral and central structures through a complex mechanism that modulated *via* peripheral mechanisms involving inhibition of prostaglandins and leukotrienes or *via* central processes of opiate, dopaminergic descending noradrenergic and serotonergic pathways[40-42]. To evaluate peripherally acting analgesics, the abdominal constriction response induced by acetic acid is a sensitive method. Intraperitoneal injection of acetic acid stimulates nociceptive neurons, and causes acute inflammatory reaction due to releasing of endogenous substances such as serotonin, histamine, bradykinins, prostaglandins $E_2$ and $F_{2\alpha}$ as well as lipoxygenase products in peritoneal fluids[43,44]. Non-steroid anti-inflammatory agents, non-narcotic analgesics and some antioxidants prevent synthesis of prostaglandins in neutrophil polynuclear cells. In the present study, *R. nobile* rhizome extract decreased the mean number of abdominal constrictions or writhes significantly ($p$ <0.0001) (Fig. 2a). The analgesic effect was found more prone than standard drug paracetamol, which is used to treat pain by blocking cyclooxygenase-2 in the CNS. The hot plate test is considered to be selective for centrally acting analgesics that focuses on changes above the spinal cord. As compared to the control group, significant increase in pain threshold in treated groups suggests involvement of central pain pathways (Fig. 2b). Thus, analgesic potentiality of the extract of *R. nobile* rhizomes may acting through both peripheral and central mechanisms of pain inhibition.

Inflammation is a complex biological response of vascular tissues to harmful stimuli that leads vasodilation, exudation of plasma proteins, tissue injury, and eventually leading edema at the site. Histamine and other mediators such as bradykinin increase vascular permeability leading hyperalgesia. In the Albumin-induced paw edema test, the extract showed dose-dependent inhibitory effect on edema

formation in early stage. Compared to both control and positive control groups, the group treated with 400 mg/kg showed a highly significant ($p$ <0.0001), and strongest inhibition of edema in 30 min (Fig. 3). At this time, the paw was found to be shrunk than the normal paw volume. Compared to acetyl salicylic acid, data obtained in 2 h observation were no significant at dose 250 mg/kg, while a significant inhibition was found at 400 mg/kg. Similar extent of significant inhibitions was observed in 3 h at doses 250 and 400 mg/kg ($p$ <0.027). The results suggest that the extract may effectively suppress the exudative phase of acute inflammation. Further detailed investigation is required.

Study on human diabetics and experimental animal models showed that diabetes causes oxidative stress, which results in depletion of the antioxidant defense system, and enhancement of free radical generation[45]. Polyphenols as well as anthraquinones are known to exhibit antidiabetic activity[17,46-48]. To evaluate acute effect of the extract on fasting blood glucose levels in normoglycemic rats, a single p.o. dose of the extract was administered and found that the extract produced a significant reduction in the blood glucose level (Fig. 4). Recently, Radhika *et al.* have reported antidiabetic activity of 70 % ethanolic extract of *R. emodi* rhizome, which corrects impaired liver and kidney by limiting gluconeogenesis similar to insulin[18].

In the present study, oral administration of the extract at doses 125 and 250 mg/kg to pregnant rats from day 1 to 10 produced a dose-dependent adverse effect on fertility index with decrease in the number of implantation sites and viable fetuses in the uterine horns indicating antifertility potentiality of *R. nobile* rhizome (Table 3). Effect of the extract on male rats was not studied in the present study. Effects of the extract on sperm motility, density, and concentration of spermatozoa as well as testosterone level that are responsible for ova fertilization should be studied. Further investigations of hematological and serological parameters after extract treatment and isolation of bioactive chemical constituents form the plant material are in progress.

## Conclusion

We have reported for the first time that the extracts of *R. nobile* rhizomes possess a number of pharmacological activities (i.e. cytotoxic, antibacterial, CNS depressant, analgesic, anti-inflammatory, hypoglycemic, and antifertility activities) in *in vitro* and *in vivo* studies. To the best of the author's knowledge, CNS depressant and antifertility activities of genus *Rheum* are not reported before. This study scientifically supports the folkloric usage of *R. nobile* to treat pain and inflammation in Nepal. Considering several biological activities of *R. nobile* evidenced, this extraordinary plant material could be a promising source for discovery of new drugs.

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