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In vivo pharmacological assay, antioxidant activity and metal ions analysis of *Thymus linearis* Benth collected from Nepal

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The aim of the study was to study the metal ion content, anti-diarrheal, anti-ulcer, antioxidants and antibacterial activities of the *Thymus linearis* Benth crude extract collected from Nepal. The *T. linearis* crude methanol extract revealed highest amount of total phenolic content (TPC) 211.709±2.51 mg GAE/g DW and total flavonoid content (TFC) 29.12±0.65 mg QUE/g DW, respectively. Furthermore, highest antioxidant activities were also recorded in methanol extract with IC_{50} value of 80.77±0.99 µg/ml. Our result also revealed that the methanol crude extract showed the highest antibacterial and antioxidant activities followed by acetone, water and hexane extract. The crude water extracts revealed highest anti-diarrheal and anti-ulcer properties with 68.75% and 66% of inhibition, respectively in the mouse model. The metal ion analysis of the plant extracts was carried out using AAS. Results revealed that *T. linearis* is a rich source of iron, sodium, and potassium. In conclusion *T. linearis* Benth possess potential compounds like polyphenols, flavonoid, tannins, quinone, cardioglycosides as well as the rich source of essential elements which are the reasons behind the anti-ulcer, anti-diarrheal, antioxidant and anti-microbial property of the herbal extract.

Keywords: AAS, TPC, TFC, DPPH, *Thymus linearis*, pharmacological assay.

Introduction

Thymus linearis, also known as Himalayan Thyme, belong to the Lamiaceae family is a pleasant smell containing perennial shrub. Mostly found in Mediterranean region, Asia, Southern Europe and North Africa and comprises of 350 species[1,2]. In Nepal it is widely distributed in western region and commonly known as Godhamarcha. Himalayan thyme is found in the rocky slope at the altitude of 1500–4300 m from Afghanistan to China. Furthermore, *Thymus linearis* a small shrubby plant with a strong spicy taste also used in culinary is also known as creeping thyme, mountain thyme, and wild thyme[3]. In western region of Nepal, people used to drink the leaves of *T. linearis* with hot water. It is believed to be function for the treatment of several ailments like high blood pressure, mouth infection, cold, fever as well as liver diseases[4]. The use of *T. linearis* as the herbal medicine for various disease and conditions in day to day life makes it inquisitive to learn more about its biological activities.

Medicinal plants are rich source of secondary metabolites, micro and macronutrients. Although the elemental compositions of many of the plant's materials are known, it varies from plants to plants and climatic conditions. Medicinal plants are able to accumulate both essential as well as toxic metals ions. Some of the elements like Fe, Cu, Co, Ni, Zn, Mg, Mn, Cr, V plays important role in improving human health, however metals like Pb, Cd and Hg are considered to be toxic even at lower concentration[5]. Many of these medicinal plants and herbs in many developing countries are used in day to day life. Hence, long term consumption without knowing the actual composition of metal ions might have adverse effect in human health. Thus, proper quantification and identification of the micronutrients, metallic and metalloids elements in the herbal extract is important not only due to its nutritional importance but also to assess the quality of the herbal extract for potential risk to human health. Furthermore, phytochemicals like flavonoids, phenolic compounds, tannins, saponins, terpenoids have a diverse biological activity such as antioxidant, anti-ulcer, anti-cancer, anti-inflammatory as well as several other pharmacological activities[6]. Among these phytonutrients, flavonoids and phenolic com-

pounds are reported to have antioxidant, anticancer, anti-inflammatory activities as well as function for lowering the risk of cardiovascular diseases as well. Thus, proper quantification of total flavonoids and total phenolic content helps understanding the medicinal importance of herbal extract.

In this present study, the anti-bacterial, antioxidant, anti-ulcer, anti-diarrheal as well as the elemental composition of *T. linearis* crude extract collected from Mugu district of Nepal have been investigated. Result revealed that of *T. linearis* have several health benefits and are the potential source of bioactive compounds and are also the rich source of essential metal ions.

Experimental

Plant materials collection and extraction of metabolites:

The *T. linearis* Benth was obtained from Mugu, Nepal and was authenticated by Binod Kumar Basnet (Assistant Research Officer), National Herbarium and Plant Laboratories (KATH). For further reference, a voucher specimen number "KATH027901" of the plant has been deposited in National Herbarium and Plant Laboratories, Nepal. The whole plant extract of *T. linearis* were grinded and 100 g of the crude extract is extracted with each of 1000 ml methanol, acetone, water, and hexane. The extract was then concentrated and evaporated in a Rota evaporator (HS-2005V).

Mouse model used:

Swiss albino mice weighing 20–40 g were obtained from the Natural Products Research Laboratory (NPRL), Department of Plant Resources (DPR), and Government of Nepal. Animal model experiments were carried out at Department of Plant Resources, Government of Nepal following the standard guideline published by NHRC. All the animals used in this experiments were housed in environmentally controlled conditions (23±2°C, 12-h light-dark cycle), and provided standard diet and water. Animals were kept 12 h in fasting prior to the experiments with only access to water.

Phytochemical screening:

The phytochemical analysis such as carbohydrate, Xantho-protein, cardiac glycoside, saponin, phenol, flavonoid, terpenoids, tannins, sterols, quinone, coumarin and resin was conducted using a standard protocol[7].

Total phenolic content:

The total phenolic content was determined by the spectrophotometric method using Folin-Ciocalteu's methods following the standard protocol[8]. In general, 0.5 ml of sample was mixed with 0.5 ml of Folin-Ciocalteu's phenol reagent, after 5 min, 2 ml of a 7% Na_2CO_3 solution was added to the mixture followed by the addition of 2.25 ml of deionized distilled water and mixed well. In this way prepared mixtures were kept in the dark for 30 min at 23°C and absorbance was recorded at 760 nm. The TPC was measured using gallic acid as a standard and result were expressed milligrams of gallic acid equivalents (GAE) per g of dried sample (mg GAE/g DW). Each experiments were carried out in triplicate.

Total flavonoid content:

Total flavonoid content was analyzed using aluminum chloride colorimetric assay following a standard method[9]. The reaction mixture consists of 1 ml of plant extracts, 2.7 ml 30% methanol, 0.15 ml of $NaNO_2$ (0.5 *M*) and 0.15 ml of $AlCl_3.6H_2O$ (0.3 *M*). The solution was mixed together and kept for about 5 min and 1 ml of NaOH (1 *M*) were added. In this way prepared solution were mixed well and absorbance were recorded at 506 nm using UV-Vis spectrophotometer. Quercetin were used as a standard and results were expressed as milligrams of quercetin equivalents per gram of dry weight (mg QUE/g DW) of triplicate experiments.

Antioxidant assay:

The antioxidant activity was performed using DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay[10]. The test solution was prepared in methanol at various concentrations ranging from 20–100 μg/ml. The 1 ml of plant extracts were mixed with 1 ml of DPPH (100 μM) as a control and 1 ml of methanol were mixed with 1 ml of DPPH separately. The resultant mixtures were stored in dark conditions for 30 min and absorbance was recorded at 517 nm. The DPPH radical scavenging activities were calculated using the formula:

$$\% \text{ Scavenging} = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 = absorbance of control, A_1 = absorbance of different concentration of extracts.

The IC_{50} value (extract concentration providing 50% inhibition) was calculated using plot of percentage inhibition using GraphPad Prism. Each experiment was performed in triplicates and results were expressed as mean ±SD.

Anti-microbial activity:

The agar plate overlay technique was used for the determination of antibacterial activity following the standard protocol with slight modification[11]. Bacterial strains were grown overnight on LB broth at 37°C and subculture into LB broth until OD_{600} reach to 0.6. In this way culture broth were diluted to 1/10 and 1 ml of the culture broth is mixed with 9 ml of soft agar (0.75% agar) and top layer of agar plate was overlain with the soft agar consisting. The disc size of 6 mm was dipped into the plant extract (5 to 0.625 mg/ml) and placed into the agar plate and cultured for 24 h at 37°C. Bacterial growth inhibition was measured and compared with standard antibiotics.

Acute toxicity analysis:

Toxicity test was performed following the OECD guideline for testing of chemicals. Limit test was performed at 2000 mg/kg as single dose with some modification. The dose was first administered to a single mouse according to the body weight and were observed closely for 3–4 h without food. If mouse survived, additional four mice were administered with the same does under the similar condition and observed for additional 48 h intervals. In total five mouse were observed for the toxicity test. As a control, distilled water was administered to five mouse and were observed. The LD_{50} value considered to be greater than 2000 mg/kg if three or more animals survived[12].

Anti-diarrheal activity:

Anti-diarrheal activity was measured using charcoal movement following the standard protocol[12,13]. Mice were separated into seven groups. Each group consists of 3 mice. Group I: All mice were given distilled water (0.10 ml per body weight), Group II: Loperamide (standard drug) at the concentration of 2 mg/kg, Group III: Loperamide (standard drug) at the concentration of 1 mg/kg, Group IV: Loperamide (standard drug) at the concentration of 0.5 mg/kg, Group V: Extract of concentration 1000 mg/kg, Group VI: Extract of concentration 800 mg/kg, Group VII: Extract of concentration 600 mg/kg.

Distilled water, loperamide, and extract were given to respective groups orally. After 15 min 10% activated charcoal was given orally. Thirty minutes later mouse was sacrificed by cervical dislocation/chloroform. The small intestine was removed and movement of charcoal in the small intestine was calculated using formula:

$$\% \text{ Mobility} = \frac{\text{Length of charcoal movement}}{\text{Total length of intestine}} \times 100$$

Anti-ulcer activity:

The anti-ulcer activity was measured using standard protocol[14]. Mice were separated into five groups. Each group consists of three mice. Group I: All mice were given distilled water (0.10 mL per body weight), Group II: Standard drug (ranitidine) used at the concentration of 50 mg/kg, Group III: Extract of concentration 1000 mg/kg, Group IV: Extract of concentration 800 mg/kg, Group V: Extract of concentration 600 mg/kg.

Distilled water, standard drug (ranitidine), and the extract were given orally according to the weight of the mouse. Sixty min later 1 mL/kg of 80% ethanol (ulcer inducer) was given orally. The mice were sacrificed by cervical dislocation/chloroform (Sigma-Aldrich, Germany) after 60 min and stomachs were isolated, washed with clean water and cut open along the greater curvature. The stomachs were then fixed in 10% formalin and craters observed and ulcer scores were measured macroscopically using magnifier glass lens and ulcer score were recorded.

Ulcer index were measured using the codes given below:

Code	Extent of ulcer activity
0	Normal stomach
1	Red coloration
1	Spot ulcers
1	Hemaorrhagic streaks
1	Ulcer > 3 mm < 5 mm
1	Ulcer > 5 mm

The percentage protection was calculated using the formula:

$$\% \text{ Protection} = 100 - \frac{U_t}{U_c} \times 100$$

where U_t = ulcer index of treated group and U_c = ulcer index of the control group.

$$\% \text{ Inhibition of ulcer index} = \frac{\text{Control mean ulcer index} - \text{Test mean ulcer index}}{\text{Control mean ulcer index}} \times 100$$

Metal ion analysis:

Approximately 1.0 g powder form of *T. linearis* was digested with 10 mL of concentrated HNO_3 using the wet digestion method by slowly heating on a hot plate until a clear solution was obtained[15]. The digested sample was allowed to cool down at the room. The final volume of the solution was adjusted to 100 mL with deionized water. Copper, zinc, iron, manganese, chromium and nickel were analyzed by air-acetylene flame method using Atomic Absorption Spectrophotometer. Boron, cobalt and molybdenum were analyzed by nitrous oxide – air-acetylene flame method using Atomic Absorption Spectrophotometer. Sodium and potassium were analyzed by flame emission method. Arsenic and selenium were analyzed by hydride generation method in hot vapor system whereas mercury was determined by hydride generation method in cold vapor system.

Table 1. Phytochemical analysis of *T. linearis*

Phytonutrients	Solvent extract			
	Acetone	Methanol	Water	Hexane
Carbohydrate	–	–	–	–
Xantho-protein	–	+	–	–
Cardiac glycoside	+	+	+	–
Saponin	–	–	+	–
Phenol	+	+	+	+
Flavonoids	+	+	+	–
Terpenoids	+	+	+	+
Tannin	+	+	+	–
Sterols	+	–	–	+
Quinone	+	+	+	+
Coumarin	–	–	–	–
Resin	+	+	+	–

where, (+) = present and (–) not present.

Results and discussion

Traditional healing system using the medicinal plants material and its extract are still prevalent in different part of the world. Although modern drugs are easily available, herbal medicine are gaining popularity day by day because of its natural origin and less side effect. It has been estimated that around one quarter of the globally prescribed drugs are herbal based. Hence, continuous screening of medicinal plants materials for biological activities are the starting point of drug discovery. Most of the herbal medicine available in the market are used for the treatment of several human ailments such as diabetes, inflammation, microbial infection, ulcer, diarrhea as well as cancer. The phytochemicals present in the herbal extract is mainly responsible for the biological activities (Table 1). Our present study aims to evaluate the biological activities *Thymus linearis* collected from Mugu district of Nepal. The phytochemical analysis of a crude extract of *T. linearis* shows the presence of tannins, resins, quinone, cardiac glycosides, phenolic compounds, and Xantho-proteins. The phytochemical analysis of *T. linearis* is summarized in Table 1. Furthermore, the crude methanol extract showed the highest TPC and TFC, followed by acetone and water extract. The *T. linearis* crude methanol extract revealed 211.70±2.51 mg GAE/g DW and 29.12±0.65 mg QUE/g DW of TPC and TFC, respectively. Similarly, hexane extract revealed the lowest amount of TPC and TFC i.e. 13.44±1.26 mg GAE/g DW and 9.40±0.87 mg QUE/g DW, respectively. The TPC and TFC content of crude *T. linearis* extract is summarized in Table 2.

Table 2. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activities of *Thymus linearis* Benth

Solvent extract	TPC (mg GAE/g DW)	TFC (mg QUE/g DW)	DPPH Assay IC_{50} value (μg/ml)
Methanol	211.70±2.51	29.12±0.65	80.77±0.99
Acetone	186.18±0.94	17.83±0.86	87.62±1.74
Water	69.86±0.88	15.40±0.50	139.31±2.60
Hexane	13.44±1.26	9.40±0.87	281.2±3.24

The solubility of the compounds to be extracted and solvent polarity plays important role in the extraction of the metabolites. Hence, the phytochemical extraction was carried out in four different solvents i.e. methanol, acetone, water, and hexane. *T. linearis* showed wide varieties of phytochemicals like tannins, terpenoids, resins, quinone, cardiac glycosides and phenolic compounds. The quantitative analysis was performed for the determination of TPC and TFC as well as antioxidant activity. The phenolic compounds are associated with several health benefits such as antioxidant, anticancer and anti-inflammatory activities and are comprehensively researched and quantified[16]. Methanol extract of thymus showed highest phenolic and flavonoid content. Similarly, hexane extracts showed lowest phenolic and flavonoid con-

tent. The highest amount of TFC is observed in methanol extract while the lowest is reported in hexane extract. It might be due to the poor extraction efficiencies of the phenolic compounds by a non-polar solvent like hexane. Furthermore, higher amounts of TPC and TFC signifies its medicinal importance for the treatment of several diseases[17].

Furthermore, antioxidant activities of the crude extract in four different solvents were analyzed by measuring DPPH scavenging activity. DPPH is used for the measurement of the free radical scavenging capacities of the extract through the colorimetric assay. The extract having high antioxidant capacities changes color of DPPH solution. The degree of color change is proportional to the concentration and potency of the antioxidants. Furthermore, absorbance of the reaction mixture measured using UV-Visible spectrophotometer enable us to calculate the free radical scavenging activity of the extract. The antioxidant capacities of the crude extract expressed as IC_{50} value (i.e. 50% inhibition of the free radicals). Our results revealed that crude methanol extract showed significant antioxidant tendencies with IC_{50} value 80.77±0.99 μg/ml. On the other hand, hexane crude extract has significantly lowest IC_{50} i.e. 281.2±3.24 μg/ml (Table 2). The antioxidant tendencies of *T. linearis* in four different solvents are in the order of methanol > acetone > water > hexane. The presence of high amounts of polyphenols and flavonoid in the extract might be responsible for the antioxidant activity.

Pharmacological activity analyses for anti-ulcer and anti-diarrheal were performed in mice model. Our acute toxicity test revealed that LD_{50} value is greater than 2000 mg/kg body weight for each extract. Diarrhea is one of the major problem is developing countries and causes frequent defecation, leading to dehydration and malabsorption. The plant and plant derived products have proven to be effective in controlling the diarrhea[18,19]. Our study suggests that overall anti-diarrheal activity reveals dose-dependent activity, as extract of 1 g/kg of all extracts showed higher percent inhibition compared to 0.6 g/kg. The Thymus water extracts showed 68.75% inhibition at 1 g/kg concentration while the acetone extract of Thymus showed inhibition of 59.84% at 1 g/kg concentration (Table 3). Thymus seems to possess high anti-diarrheal activity mostly because of the presence of diverse secondary metabolites. The compounds like polyphenols and flavonoid might contribute to some extent for the better anti-diarrheal property of Thymus species[20].

Gastric ulcers also known as gastric ulcer causes the painful sores in the stomach affecting the vast majority of the people worldwide. In traditional healing system, natural remedies derived from medicinal herbs and plants were used for the treatment of ulcer, which is still in practice. Scientific evidence also supports the facts. In this study, the anti-ulcer effect of *T. linearis* crude extract in HCl/ethanol-induced mice models were investigated. The free radicals such as superoxide anion, hydroperoxy-free radicals increase the lipid peroxidation[21,22]. Our result revealed that water extract was found to significantly inhibit HCl/ethanol-induced ulcer by 66%

Table 3. Anti-diarrheal activity of *T. linearis* Benth

Test extract	Concentration	Average distance travelled by charcoal (Inch±S.D)	Percentage mobility	Percentage inhibition
Blank		17.83±0.7	69.52	30.47
loperamide	2 mg/kg	8.0±0.5	33.31	66.69
loperamide	1 mg/kg	11.0±0.5	43.71	56.28
loperamide	0.5 mg/kg	15.3±0.5	60.92	39.08
Methanol	1 g/kg	11.0±0.8	44.57	55.43
Methanol	0.8 g/kg	13.3±1.0	55.16	44.84
Methanol	0.6 g/kg	14.6±0.28	58.29	41.71
Acetone	1 g/kg	9.16±0.28	40.16	59.84
Acetone	0.8 g/kg	13.16±0.76	57.24	42.75
Acetone	0.6 g/kg	15.0±0.5	63.38	36.62
Water	1 g/kg	6.66±0.28	31.25	68.75
Water	0.8 g/kg	8.5±0.5	42.85	57.14
Water	0.6 g/kg	11.66±0.28	55.13	44.87

Table 4. Anti-ulcer activity of *T. linearis* Benth

Test extract	Concentration	Ulcer score	Ulcer index	Percentage ulcer inhibition
Control		4.0±0.0	1.33±0.0	–
Ranitidine	50 mg/kg	1.0±0.0	0.33±0.0	75.00
Methanol	1 g/kg	2.0±0.0	0.66±0.0	50.0
Methanol	0.8 g/kg	2.66±0.57	0.88±0.19	33.33
Methanol	0.6 g/kg	3.0±1	1.0±0.33	25.0
Acetone	1 g/kg	1.66±0.57	0.55±0.19	58.33
Acetone	0.8 g/kg	2.0±0.0	0.66±0.0	50.0
Acetone	0.6 g/kg	2.33±0.57	0.77±0.19	41.66
Water	1 g/kg	1.33±0.57	0.44±0.19	66.66
Water	0.8 g/kg	2.33±0.57	0.77±0.19	41.66
Water	0.6 g/kg	2.66±0.57	0.88±0.23	33.33

and 58.33% inhibition by acetone extract. The activity was determined to be dose dependent as 1 g/kg showed higher inhibition than 0.6 g/kg (Table 4). The oxidative stress in the body is mostly responsible for digestive system diseases, including stomach ulcers and gastric carcinoma.

Furthermore, anti-microbial tests were performed in four different ATCC strains that are *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 700603, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The *T. linearis* acetone extract showed inhibition at a concentration of 1.25 mg/ml in *S. aureus* and *E. coli* but showed inhibition in *K. pneumonia* at 5 mg/ml. While the methanol extract showed inhibition at a concentration of 1.25 mg/kg in *S. aureus* and *K. pneumonia* while *E. coli* was inhibited at 5 mg/ml. In our observation, *P. aeruginosa* was not inhibited by any of the crude extracts at the given concentration range of 0.625 mg/ml to 5 mg/ml (Table 5). The thymols that are present in Thymus have the potential anti-microbial activity. As thymols are slightly soluble in water, water extract was not capable enough to inhibit any of the four microbes[23]. However, we firmly believe that separation and purification of the metabolites might increase the anti-microbial activities of the extract.

Scientific evidence support that plant materials are often contaminated with toxic heavy metals, depending on the environment and nature or origin. Such toxic heavy metals have adverse health effect to the consumer. It is very important to properly identify and quantified the metal ions in the herbal extract to protect consumers from contamination. The

Table 5. Anti-microbial activities of *T. linearis* Benth Extract

Solvent extract	Concentration (mg/ml)	Zone of microbial growth inhibition (mm)			
		S. aureus ATCC 25923	*K. pneumoniae* ATCC 700603	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
Acetone	5	11	12	13	ND
	2.5	10	ND	10	ND
	1.25	10	ND	10	ND
	0.625	ND	ND	ND	ND
Methanol	5	12	11	10	ND
	2.5	11	10	ND	ND
	1.25	11	10	ND	ND
	0.625	ND	ND	ND	ND
Water	5	ND	ND	ND	ND
	2.5	ND	ND	ND	ND
	1.25	ND	ND	ND	ND
	0.625	ND	ND	ND	ND
Hexane	5	ND	ND	ND	ND
	2.5	ND	ND	ND	ND
	1.25	ND	ND	ND	ND
	0.625	ND	ND	ND	ND
Ampicillin	100 μg/ml	15	15	13	14

Where, ND = not detected.

purpose of the present work was to quantify the concentration of some of the macronutrient, micronutrients and trace toxic metals in the *T. linearis*. If the metal ions exceed the permissible range than it causes several toxic effects on human health. An excess amount of cadmium causes renal dysfunction, lungs disease. Similarly, an excess amount of chromium causes damage to nervous system. Hence, proper quantification of the metal ions in herbs is important, as many of these herbal extracts is used for the treatment of several diseases. The concentration of Fe, Zn, Cu, Mn, Ni and Cr were measured as 399.64, 44.12, 12.97, 56.8, 3.2 and 19.96 mg/kg respectively. Whereas, Na and K are found to have 741.29 and 3682.75 mg/kg in the *T. linearis* (Table 6). Concentration pattern of metals in *T. linearis* in decreasing pattern is K > Na > Fe > Mn > Zn > Cr > Cu > Cd. The analyzed metal ions are in the range of recommended daily intake[24]. Result revealed that *T. linearis* is a huge source of several metal ions essential for human health.

Table 6. Atomic absorption spectroscopy metal ion concentration in *T. linearis* extract

Metal ions	Amounts (mg/kg)
Mercury	< 0.1
Lead	< 0.2
Cadmium	0.12
Copper	12.97
Zinc	44.12
Iron	399.64
Manganese	56.8
Boron	< 0.19
Cobalt	< 0.19
Nickel	3.2
Chromium	19.96
Sodium	741.29
Potassium	3682.76
Arsenic	< 0.49
Selenium	< 0.49

Conclusion

In conclusion, *T. linearis* crude extract possesses the antioxidant, anti-microbial, anti-diarrheal, anti-ulcer. Further, *T. linearis* is also the source of several essential metal ions like Fe, Zn, Cu, Cr, Mn, Na and K. Hence consumption of the crude extract of *T. linearis* are associated with several health benefits.

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