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Phytochemical Screening and Pharmacological Activities of *Gentiana ornata* (Wall. ex G. Don) Griseb from Lauribinayak, Rasuwa, Nepal

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Abstract

Gentiana ornata (Wall. ex G. Don) Griseb, also called decorated gentian or showy gentian, has been used as a decoction plant in temperate regions of Nepal. This work aims to evaluate the phytochemical properties, antioxidant values, and pharmacological properties of this plant collected from Lauribinayak, Gosainkunda Rural Municipality, Rasuwa, Nepal. The extract of whole plant was prepared by cold extraction process at 100% ethanol. Then, the qualitative preliminary phytochemical test of plant extract was performed to know its chemical constituents. The total phenolic content using Folin-Ciocalteu method, total flavonoid content using aluminium chloride colorimetric assay and antioxidant potential of the plant extract were also determined by using diphenylpicrylhydrazyl (DPPH) reagent. The pharmacological activities like oral acute toxicity test, antidiarrheal test and anti-ulcer test were also carried out in laboratory animals. The qualitative phytochemical screening showed the presence of flavonoids, steroids, glycosides, anthocyanin, alkaloids, terpenoids and phenolic constituents. The total phenolic content, total flavonoid content and antioxidant assay value of this plant extract were 242.64 ± 0.280 mg GAE/g, 39.23 ± 0.005 mg QE/g and 42 ± 0.072 µg/mL respectively. The extract showed no morbidity, mortality, toxicity signs, or symptoms in the oral acute toxicity test. Though it could not show considerable antidiarrheal effect by reducing gastrointestinal motility when compared to the standard drug, loperamide, it showed significant anti-ulcer effects by reducing gastric lesions when compared to the standard drug, sucralfate, which may be due to its mucosal protecting nature. Therefore, isolation and purification of phytochemicals responsible for the pharmacological activity of this plant have been recommended.

Keywords; Acute toxicity, Antioxidant, Anti-ulcer, Gentiopicroside, Medicinal plant

Introduction

Nepal, being rich in biodiversity has about 1515 to 2331 types of precious medicinal and aromatic plants among the 7000 species of medicinal plants recognized all over the world (Chhetri et al., 1970; Karki et al., 2023; Lamichhane et al., 2023; Shrestha et al., 2015;). Among the various medicinal plants, *Gentiana* is the largest genus of the Gentianaceae family with more than 400 species and is distributed at higher altitudes around the Himalayan foothills (Mirzaee et al., 2017; Wai et al., 2024). Nepal consists of 49 species of *Gentiana* together with three endemic species and it has been recently recorded that *Gentiana* is the fifth most diversified genera in the flora of Nepal (Rajbhandari, 2023; Shakya et al., 2023).

Gentiana ornata (Wall. ex G. Don) Griseb also called decorated gentian or showy gentian is native to Bhutan, Eastern Nepal and Sikkim (Mariana et al., 2013). It is found in temperate regions of Nepal having stony pastures and grassland slopes from 3300-5000 m above sea level (eFlora, 2024). It is a perennial ornamental herb of 4-7 cm tall, having solitary deep blue flowers in the form of a trumpet, with semi perdurable rosette, with basal arranged leaves of 3-4 cm length, lance width (eFlora, 2024; Mariana et al., 2013; World Flora Online [WFO], 2024).

Numerous species of *Gentiana* genus have ornamental value but its pharmaceutical interest is increasing due to its rich bioactive phytochemical properties like bitter iridoid glycosides (gentiopicroside, gentianin) and alkaloids (gentiomarin) (Baba & Malik, 2014; Ifrim & Mardari, 2014; Jiang et al., 2021; Mirzaee et al., 2017). It has been widely used in treatment of human disorders. It is good for treating digestion

problems like loss of appetite, bloating, diarrhea and heartburn and is hence used as an ingredient in foods and beverages (Capasso et al., 2003; Manish, 2018). It is also used against fever and to prevent muscle spasms (Prakash et al., 2017). It has been reported that it possesses significant anti-inflammatory, analgesic, anti-asthmatic, anticonvulsant, antihistaminic, antimalarial, antiamoebic, cytoprotective, diuretic, hepatoprotective and hypoglycemic properties (Li et al., 2023; Mirzaee et al., 2017; Singh, 2008). It is also effective for treating skin wounds and cancer. It has also been documented for colds, coughs and headaches (Chakraborty et al., 2017; Shakya et al., 2023).

Though *G. ornata* has numerous biological values, it is an underutilized medicinal herb whose very rare research has been conducted for its phytochemical analysis and no *in-vivo* test has been performed to date in Nepal. This work aims to evaluate the phytochemical preliminary screening and antioxidant values and first attempt to perform *in-vivo* tests like oral acute toxicity, anti-diarrheal and anti-ulcer tests of this plant collected from Nepal.

The present work aims to discover the phytochemical properties and bioactivity of selected species of *Gentiana* L. from Nepal. It also estimates major phytochemicals like amarogentin, mangiferin and swertiamarin semi-quantitatively using thin-layer chromatograph Very less research has been done on gentian ornata globally. This work aims to discover the phytochemical preliminary screening and the antioxidant value of gentian ornata from Nepal. Also, this paper represents the first attempt at pharmacological in-vivo tests like oral acute toxicity, anti-diarrhoeal, and anti-ulcer test of this plant.

Materials and Methods

Collection and extraction of plant materials

The plants *Gentiana ornata* was collected from wild habitants of the Lauribinayak area having latitude 28°5'29.45" N and longitude 85°22'58.100" E with 3964 meter elevation from Gosainkunda Rural Municipality, Rasuwa District of Bagmati Province, Nepal. The plant was identified and authenticated from National Herbarium and Plant Laboratories (KATH) with voucher code no. GK-011 for future reference. All laboratory works were carried out in the Natural Products Research Laboratory (NPRL), Thapathali, Kathmandu, Nepal. The whole plant material was cleaned and dried under shade to avoid decomposition. The dried plant was then powdered using electric blender and about 20g of the powdered plant was soaked in 100 ml of ethanol. It was left for 3 days with intermittent agitation at room temperature so that alkaloids, terpenoids and other constituents if present get dissolved. Then, the soaked sample was later filtered through Whatman grade 1 filter paper. The solvent was allowed to evaporate from the filtrate in rotary vacuum evaporator under reduced pressure at 40-45°C temperature to yield dark brown semi solid extract, which was stored at 4ºC in the refrigerator until further use (Harborne, 1998; Nortjie et al. 2022; Seyfe et al. 2017).

Phytochemical screening

The qualitative phytochemical screening for the presence of alkaloids, glycosides, flavonoids, tannins, phenols, saponins, carbohydrates and steroids was identified for qualitative screening of phytochemicals using the standard method as given by Harborne (1998) and Mishra & Tripathi (2015).

Determination of total phenolic contents (TPC), total flavonoid contents (TFC) and IC_{50} value

The total phenolic content was determined by using the Folin-Ciocalteu method taking gallic acid as standard for the calibration curve as described by Singleton & Rossi (1965) with a slight modification. Briefly, 20 µL triplicate of 1 mg/mL plant sample solution was loaded in 96-well plate. Gallic acid of different concentration of 10, 20, 30, 40, 50, 60, 70 and 80 µg/mg was loaded in triplicate; used as standard control. 100 µL of the Folin-Ciocalteu (FC) reagent was added in each well containing gallic acid and plant sample. An initial reading of the plate was taken at 765 nm using a microplate reader. After initial reading 80 µL of Na_2CO_3 was added separately to each well and incubated for 15 minutes. The final absorbance was taken in 765 nm (Epoch2, BioTek, Instruments, Inc, USA) microplate reader

(Fombang & Mbofung, 2015; Mujic et al., 2009). Then, the standard curve of gallic acid was plotted and the TPC content in the extract was calculated using the following formula:

$C = cV/m$

Where, C = Total phenolic content in milligrams per gram (mg/g) of dry plant material, in gallic acid equivalent (GAE),

c = Concentration of gallic acid established from the calibration curve in milligrams per milliliter (mg/mL),

V = Volume of the extract solution in milliliters (mL), m = Weight of the extract in grams (gm).

Similarly total flavonoid contents were determined by aluminium chloride colorimetric assay as per the method given by Fombang & Mbofung (2015) and Nobossé et al. (2018) using quercetin as standard. 130 µL of different concentration i.e., 10, 20, 40, 60, 80, 100 µg/ ml of quercetin was loaded triplicate in 96 well plate. Similarly, 20 µL of the plant sample (1000 µg/ml) was loaded in triplicate and 110 µL of distilled water was added in each well-containing plant sample. 60 µL of ethanol was added to each well containing plant extract and quercetin. The initial reading was taken at wavelength 415 nm in a microplate reader. Then, 5 µL of $AlCl_3$ and 5 µL of Potasium acetate were added to each plate, incubated in dark for 30 minutes and the final reading of the plate was taken at the same wavelength i.e., 415 nm. The total flavonoid content in the extract was calculated using the following formula:

$C = cV/m$

Where, C = Total flavonoid content in milligrams per gram (mg/g) of dry plant material, in quercetin equivalent (QE),

c = Concentration of quercetin established from the calibration curve in milligrams per milliliter (mg/mL),

V = Volume of the extract solution in milliliters (mL), m = Weight of the extract in grams (gm)

For anti-oxidant activity, method developed by Band-Williams et al. (1995) was used with some modifications. Briefly, 100 µL quercetin of different concentrations (20, 10, 5, 2.5, 1.25, and 0.625 µg/mL) was loaded in triplicate as a positive control, and 50% DMSO was loaded in triplicate as a negative control. Plant extracts of different concentrations of 500, 250, 125, 62.5, and 31.25 µg/mL were prepared from a stock solution of 5000 µg/mL solution in 50% DMSO. 100 µL of different concentrations of plant samples were loaded to each well and initial absorbance was measured at 517 nm. After the initial reading, 100 µL of 0.1 mM DPPH solution was added to each well and incubated for 30 minutes in the dark. After incubation, the final reading was taken and the percentage inhibition of DPPH radicals by the plant extract was calculated as (Nobossé et al., 2018):

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} * 100$$

Whereas, Ac= absorbance of control, As = absorbance of sample extract

Acute toxicity test

All *in-vivo* tests were performed as per the animal ethical guidelines in Natural Products Research Laboratory, Thapathali, Kathmandu. Instructions regarding the care and uses of laboratory animals were completely followed. They were kept in a room under standard conditions having 25±5°C room temperature and natural light-dark cycle. All of the animals were given standard rodent diets with free access to water.

According to Bruce (1985), the acute oral toxicity test was carried out using the up-and-down method. Healthy female, overnight fasted, non-pregnant mice of 25 to 35 grams weight were randomly selected and divided into two groups containing five mice in each group (n=5), Group I: the control group, received only distilled water at 1ml per 100 gm body weight, and Group II: the experimental group, received plant extract at 2000 mg/kg body weight dissolved in distilled water. Then, the animals were observed for mortality, neurological and any other behavioral abnormalities for 14 days (periodically first 24 hours). Also, LD50 was expressed by observing

the toxicological effects exhibited by extract (if any) according to the Organization for Economic Co-operation and Development (OECD, 2022) guidelines for testing of chemical, test guidelines no. 425 (Bruce, 1985) The extract was also classified into various toxicity categories as criteria given by Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nations [UN], 2011). If the extract showed any mortality at 2000 mg/kg, LD50 would be calculated by the linear regression of the constructed curves as cited in Randhawa (2009).

Anti-diarrheal test

It was carried out using a gastrointestinal motility test as given by Fokam Tagne et al. (2019) with randomly selected 24-hour fastened albino mice with three animals in each three groups (n=3); Group I: the negative control group, received only normal saline at 1 ml per 100 gm weight mouse; Group II: the experimental group, received plant extract at 500 mg/kg dissolved in distilled water and group III: the standard group, received loperamide at 2 mg/kg body weight Then charcoal meal was prepared by dissolving 10% charcoal and 5% gum acacia in distilled water and orally administered to each mice at the dose of 0.3 ml per mouse. After 30 minutes, the animals were euthanized using chloroform. Then, a small incision was made on the abdomen and the entire small intestine was removed from the duodenum to end of ileum with the help of focep. After that, the distance covered by the charcoal meal in the small intestine (DCCM) was measured along with the total length of the small intestine (TLSI). The peristaltic index (PI) was calculated by the following formula (Fokam Tagne et al., 2019; René et al., 2015).

$$PI = \frac{DCCM}{TLSI} * 100$$

Similarly, the inhibition of gastrointestinal motility was calculated with the following formula:

$$I\% = \frac{(PIc - PIt)}{PIc} * 100$$

Where, PIc: Peristaltic index in the control group; PIt: Peristaltic index in the experimental group.

Ethanol induced gastric ulcer test

The test was performed as given by Robert (1979) to induce ulcers using ethanol in albino rats. Healthy, 24-hour fastened albino rats were divided into three groups with three animals in each, the control group: received only normal saline at 1 ml per 100 gram weight rat, the test group: received plant extract at 500 mg/kg dissolved in distilled water and the standard group: received sucralfate at 100 mg/kg After 60 minutes, 100% ethanol was administered orally to each animal at 1 ml per 200 gram weight. Then the animals were sacrificed using an overdose of ether after an hour of ethanol administration. After that, a midline incision was made; the stomach was removed and excised along the greater curvature. The stomach was grossly evaluated with the help of 10× magnifier lens and the ulcer was scored as the normal colored stomach (0), red coloration (0.5), spot ulcer (1), hemorrhagic streak (1.5), deep ulcers (2), and perforation (3) (Dashputre & Naikwade, 2011; Kulkarni, 1987; Sahoo et al.,2016; Süleyman et al., 2002). Ulcer index (UI) was then calculated as given by Deshpande & Balekar (2018) and Roy et al. (2013).

Ulcer index (UI) = Mean sum of the score of ulceration

Similarly, the percentage inhibition of gastric ulceration was calculated as below:

$$\%\ inhibiton\ of\ ulceration = \frac{(ulcer\ index\ of\ control\ group - ulcer\ index\ of\ test\ group)}{ulcer\ index\ of\ control\ group} \times 100$$

Statistical analysis

The overall data were expressed in mean±SEM in Microsoft Excel 2021 and one-way ANOVA (Analysis of Variance) test was used to calculate the overall mean differences among treatment groups at 0.05 level of significance (±) and significant ANOVA was followed by Post- hoc (Dunnett's 2 sided) test was done using IBM SPSS (Statistical Package for Social Sciences) version 26 at 95 % of confidence level.

Results and Discussion

Preliminary phytochemical test

The ethanolic extract of *Gentiana ornata* was found to have the presence of flavonoids, steroids, glycosides, anthocyanin, terpenoids, alkaloids and phenolic content and the absence of fixed oil, saponin, carbohydrate, and protein. Shakya et al. (2023) found the methanolic and aqueous extracts of this plant had alkaloids, phenols, glycosides, diterpenes, and tannin along with resin, flavonoids, and phytosterols present only in aqueous extract (Shakya et al., 2023). Our findings are in agreement with Pan et al., (2016) and Shakya et al. (2023).

Total phenolic contents and total flavonoid contents

In this experiment, the TPC and TFC of *G. ornata* were found to be 242.64±0.280 mg GAE/g and 39.23±0.005 mg QE/g respectively. However, Shakya et al. (2023) found the TPC and TFC of 78±0.37 mg QE/g and 11.31±0.49 mg QE/g in methanolic extracts respectively which are lesser than in this study. It may be due to seasonal variation of the plant collected and type of solvent used during the extraction process.

Antioxidant activity (DPPH Assay)

The IC_{50} value of ethanolic extract of *G. ornata* was found to be 44.51 ± 0.82 µg/mL compared to 16.4 ± 1.22 µg/mL of ascorbic acid, which is more than twice that of our standard Vitamin C. But, Shakya et al. (2023) found the IC_{50} value of 42 ± 0.72 µg/mL in methanolic extract which is slightly lower than the finding in this study. A higher anti-oxidant value shows it has higher hydroxyl radical scavenging activity to inhibit lipid peroxidation as explained by Niiforovi et al., (2010).

Pharmacological tests

Acute toxicity test: The extract of *G. ornata* showed no morbidity mortality, toxicity signs and symptoms at 2000 mg/kg. As a result, the LD_{50} of the extract is greater than 2000 mg/kg body weight and hence can be categorized as "may be harmful if swallowed" as per Globally Harmonized System classification criteria for acute toxicity (GHS, 2018). Jiang et al., 2021 also found no major toxic effects in *in-vivo* experimental studies of the *Gentiana* genus even at elevated doses which is in agreement with our findings.

Anti-diarrheal test: The extract of *G. ornata* could not reduce the gastrointestinal distance traveled by the charcoal meal in mice noticeably compared with the control group and the standard group as shown in Table 1. Hence, it had been found to have no antidiarrheal properties by inhibiting gastrointestinal motility in mice. But Abdurasulovna & Ernazarov, (2024) has recommended *Gentiana olivieri* Griseb for the creation of anti-diarrheal drug in the Republic of Uzbekistan.

Anti-ulcer test: The gastric organs treated with *G. ornata* were found to be normal grossly with no any hemorrhage and ulcer, and seemed to be even clearer than the gastric organ treated with the standard drug, sucralfate whereas gastric organs treated with only normal saline had so many hemorrhagic streaks with deep ulcers shown in Figure 1. Also, the mean±SEM of ulcer index of the control group, the experimental group, and the standard group were found to be 8.00 ± 0.00, 0.167 ± 0.28 and 1.50 ± 0.00 respectively. Figure 2 shows that the *G. ornata* treated group had a lesser ulcer index than that of the standard and control groups. Figure 3 depicts that *G. ornata* was found to have a higher ulcer inhibition percentage i.e. 97.91%

Table 1: Anti- diarrheal effect of *G. ornata* extract

Treatment	Dose (mg/kg, p.o)	Mean length of small intestinal (cm)	Mean distance traveled by charcoal (cm)	Peristatic index	% of inhibition of gastrointestinal motility
Group I: Control	10 ml/kg	42.00±4.00	39.50±3.50	94.05	
Group II: *Gentiana ornata*	500mg/kg	46.00±4.93	46.00±4.93	100.00	0.00
Group III: Standard Drug (Loperamide)	2 mg/kg	43.33±1.86	9.00±0.58	20.77	73.27

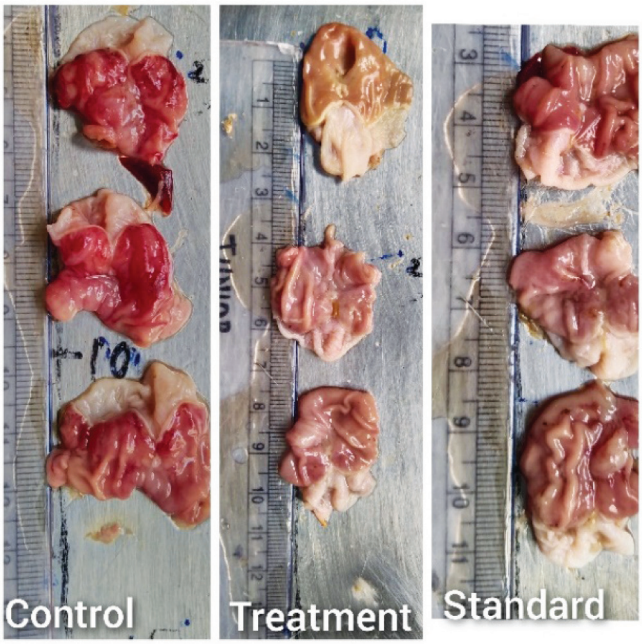

Figure 1: Gross lesion of the stomach of control, treatment and standard group of rats

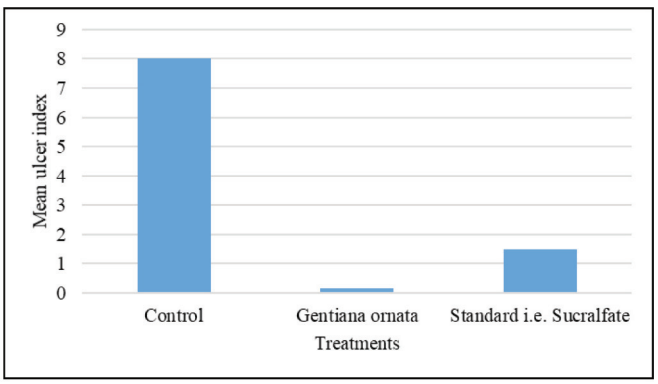

Figure 2: Mean ulcer index of ethanolic extract of *G. ornata*

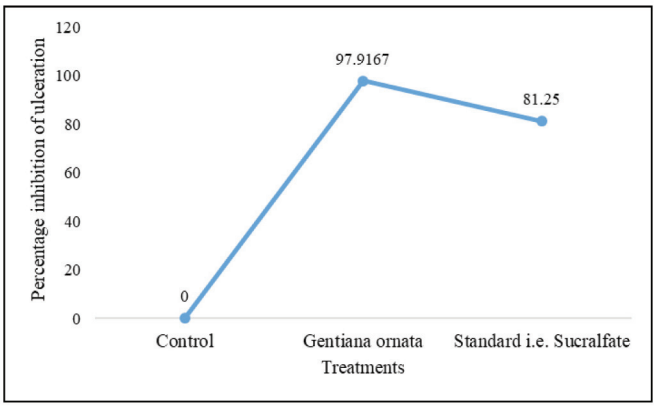

Figure 3: Percentage of gastric protection by *G. ornata*

in comparison to the sucralfate group (81.25%) and the control group (0%). Moreover, ethanolic extract of *G. ornata* was found to reduce gastric lesions significantly in ethanol-induced ulcer model when compared to standard i.e. sucralfate with *p*- value 0.00 (i.e. $P < 0.05$) at 95 % level of significance. The plant have showed anti-gastric ulceration by protecting mucosal barriers of the stomach as cytoprotective agents in peptic ulcer management (Sharifi-Rad et al., 2018). This protective property may be due the presence of higher anti-oxidant with anti-inflammatory effects. Yang et al. (2017) also found that gentiopicroside, iridoid glycosides isolated from *Gentiana macrophylla* Pall. had significantly positive protective effect against 70% ethanol- induced gastric mucosal injury in mice which is in agreement with our findings (Yang et al., 2018).

Conclusion

Gentiana ornata is rich in flavonoids, steroids, glycosides, anthocyanin, terpenoids and phenolic content. Its lethal dose $(LD)_{50}$ was greater than 2000 mg/kg signifying it can be considered relatively safe on acute exposure. The results of the present study have indicated that the ethanolic extract of *G. ornata* does not have any anti-diarrheal effects but possesses significant antiulcer properties by protecting mucosal barriers of the stomach as cytoprotective agents in peptic ulcer management, which may be due to its richness in antioxidant value and gentiopicroside. Therefore, isolation and purification of phytochemicals showing the pharmacological activity of this plant is recommended shortly for the development of novel antigastric ulcer agents.

Author Contributions

All authors have contributed equally to bringing the manuscript to this form.

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