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In-vitro antioxidant and antidiabetic potential of *Ephedra gerardiana* stem extract

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ABSTRACT

Introduction: Gymnosperms hold significant economic and ethnomedicinal importance with diverse bioactivities, including antimicrobial, antioxidant, and anticancer properties. *Ephedra gerardiana* is traditionally used as an antidiabetic remedy. This study aimed to estimate the total phenolic and flavonoid contents, antioxidant activity, and antidiabetic potential of *E. gerardiana*.
Methods: The plant's stem powder was extracted in methanol and fractionated using hexane, dichloromethane, and aqueous. The extracts' total phenolic and flavonoid contents were determined using Folin-Ciocalteu's reagent and aluminium chloride method, respectively. The antioxidant and antidiabetic potential were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and α-glucosidase inhibition assays. The toxicity of the fraction was evaluated in brine shrimp larvae.
Results: The aqueous fraction contained a high phenolic content of 123.27 µg GAE (gallic acid equivalent), while the dichloromethane fraction had a high flavonoid content of 35.43 µg QE (quercetin equivalent). The aqueous fraction exhibited high antioxidant activity by scavenging DPPH with a half inhibitory concentration (IC_{50}) value of 25.04 ± 1.57 µg/mL. Additionally, it showed strong antidiabetic activity by inhibiting α-glucosidase with an IC_{50} value of 4.33 ± 0.11 µg/mL. It was non-toxic to the brine shrimp nauplii.
Conclusion: *Ephedra gerardiana* aqueous extract is rich in phenolic and flavonoid compounds and possesses strong antioxidant and antidiabetic properties. Thus, this plant might be used to prepare antidiabetic drugs after complementary trials.

Implication for health policy/practice/research/medical education:
Ephedra gerardiana showed potent antioxidant and antidiabetic properties and no toxicity, which may have implications for medical practices. Further research validation may solidify its potential role in diabetes management.
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Introduction

Plants and their derived compounds have long captivated researchers due to their potential health benefits (1). Ethnomedicine, still prevalent in many developing countries and expanding rapidly, underscores the enduring relevance of traditional herbal remedies (2). Traditional herbal medicines constitute a substantial portion, ranging from 30% to 50%, of medical consumption in China alone (3). Moreover, a notable statistic highlights that approximately 57% of the top 150 patented drugs globally originate from plant-derived active components (4).
Among the plant kingdoms, gymnosperms, a distinct group of plants, hold particular significance owing to their economic and traditional medicinal implications (5). Abundant in secondary metabolites such as tannins, flavonoids, alkaloids, terpenoids, and phenols, gymnosperms serve as a rich reservoir of bioactive compounds (6). These phytochemicals, endowed with antibacterial, antifungal, antiviral, and anthelminthic properties, act as a formidable defence mechanism against invading pathogens (7,8). These activities have been related to gymnosperm-derived compounds like isorhapontigenin, piceatannol, and gnetol (6,7). Terpenoids such as limonene, α-phellandrene, and β-caryophyllene oxide, are also found abundantly in gymnosperms, which exhibit notable anticancer

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properties. Additionally, compounds like leelamine and α-pinene have shown potential in inhibiting cancer cell growth (8).

Ephedra (family: Ephedraceae) has 68 species in the world and 2 species in Nepal (*E. gerardiana* and *E. pachyclada*) occur in arid and semi-arid zones. *E. gerardiana* is a perennial shrub, evergreen, profusely branched with dense slender joined branches arising from the woody base; xerophyte grows from 3700 to 5200 m altitude (9,10). It is listed in the Vulnerable category of the IUCN red list (10). This genus is well-known in traditional medicines for allergies, bronchial asthma, fever, cough, headaches, oedema, and nasal congestion (11). A variety of phytochemicals belonging to alkaloids, flavonoids, phenolics, tannins, and terpenoids were isolated from the different species of *Ephedra* (12). The major compounds in the plants are ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine, ephedradine, ephedrannin, ephedroxane, vicenin etc. isolated from *E. alata*, *E. campylopoda*, *E. foeminea*, *E. foliata*, *E. fragilis*, *E. major*, *E. nebrodensis*, *E. pachyclada*, *E. sinica* (13-19). Different species of *Ephedra* have been tested for their antioxidant activity (*E. alata*, *E. aphylla*, *E. foeminea*, *E. gerardiana*, *E. intermedia*, *E. nebrodensis* and *E. sarcocarpa*) and antidiabetic activity (*E. foeminea*, *E. pachyclada*) (20-28). *E. gerardiana* can protect against arthritis (29), antioxidant and antimicrobial activity (26).

Phenolic and flavonoid derivatives present in the *Ephedra* species exhibit potent antioxidant and antidiabetic effects (30,31). These compounds play pivotal roles in regulating insulin secretion, glucose uptake, and combating oxidative stress, potentially mitigating conditions like hypertension (31). Hence, exploration of *E. gerardiana*, reveals a treasure trove of bioactive compounds with immense therapeutic potential in antidiabetic and antioxidant activities. The integration of traditional knowledge with scientific inquiry paves the way for harnessing plant resources to address contemporary health challenges and develop novel antidiabetic interventions. Therefore, the present research's objectives are to explore the total phenolic and flavonoid contents, and antioxidant and antidiabetic activities of *E. gerardiana*.

Materials and Methods

Plant material

The stems of *E. gerardiana* (Figure 1) were collected from its natural habitat in Luza, Khumbu Valley, Sagarmatha National Park, an altitude range of 4000 to 4400 m (coordinates latitude 27°49'1.9" and longitude 86°42'34.7"). The plant species was authenticated by Prof. Dr. Suresh Kumar Ghimire of the Central Department of Botany, Tribhuvan University. A voucher herbarium specimen (No.: UGC-2019-04) of this plant was thoughtfully deposited at the Tribhuvan University Central Herbarium (TUCH) of Central Department of Botany, Tribhuvan University, Kathmandu, Nepal.

Extraction of plant materials

A cold maceration process was employed for extraction involving immersion of 100 g of powdered stem in 1000 mL of 90% methanol for three days. The resulting filtrate underwent evaporation at 37 °C using a rotary evaporator. Subsequently, the crude extract was dissolved in 100 mL of distilled water, and 300 mL of hexane was added to the mixture in a separating funnel. The hexane layer was meticulously separated into a vial. Following this, the aqueous layer was combined with 300 mL of dichloromethane, and the dichloromethane and aqueous fractions were separated. These fractions underwent vaporization in the rotary evaporator, with the resulting dried fractions stored at 4 °C.

Determination of total phenolic and flavonoid contents

The total phenolic content was determined using the Folin-Ciocalteu's reagent method (32). Briefly, 20 µL of the fraction (1 mg/mL) was gently mixed with 100 µL of 10% Folin-Ciocalteu's reagent and 100 µL of 7.5% $NaHCO_3$. The mixture was then incubated at room temperature for 45 minutes, and its absorbance was measured at 765 nm. The total phenolic content was quantified and expressed as gallic acid equivalent (µg GAE/mg of extract's fraction). Similarly, the total flavonoid content was quantified using the aluminium chloride method (32). Briefly, 25 µL of the fraction (1 mg/mL) was mixed with 25 µL of 2% $AlCl_3$. After a one-hour incubation at room temperature, absorbance readings were recorded at 420 nm. The flavonoid content was expressed as quercetin equivalent (µg QE/mg of extract's fraction).

Evaluation of antioxidant activity

The antioxidant activity of the fraction was determined using the DPPH free radical scavenging assay (33). Briefly, 100 µL of the fraction, with concentrations ranging from 1.95 µg/mL to 1000 µg/mL, was mixed with 100 µL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution.

Figure 1. *Ephedra gerardiana* on natural habitat at Luza.

After a 30-minute incubation in the dark at room temperature, the absorbance of the reaction mixture was measured at 517 nm. The same procedure was employed for ascorbic acid used as the control. The antioxidant activity of the fraction was quantified by determining the concentration required to achieve 50% inhibition of the DPPH radical (IC_{50}) using a regression equation.

Evaluation of antidiabetic activity

The antidiabetic activity of the fraction was evaluated using an α-glucosidase enzyme inhibition assay (34). To assess the antidiabetic potential, 20 µL of the fraction, with concentrations ranging from 1.95 µg/mL to 1000 µg/mL, was combined with 20 µL of α-glucosidase enzyme (1 unit) and 60 µL of phosphate buffer. After incubation at 37°C for 15 minutes, 40 µL of p-nitrophenyl-α-D-glucopyranoside (PNPG) was added, and the mixture was incubated at room temperature for another 15 minutes. The absorbance was then measured at 405 nm. To halt enzymatic activity, 40 µL of Na_2CO_3 was introduced, and the absorbance was measured again at 405 nm. The same procedure was employed using acarbose as a control. The antidiabetic activity of the fraction was quantified by determining the concentration required to achieve 50% inhibition of α-glucosidase (IC_{50}) using a regression equation.

Toxicity of the fraction

The toxicity of the fraction was evaluated using brine shrimp larvae (*Artemia salina*) (35). Briefly, approximately 10 larvae reared in artificial salt water were treated with 100 µL of the fraction, with concentrations ranging from 62.5 µg/mL to 1000 µg/mL, for 24 hours under continuous light. After treatment, the number of living and dead larvae was counted.

Statistical analysis

Each experiment was conducted in triplicate, and subsequent data analysis was performed. The results were expressed as mean values accompanied by standard errors. The half inhibitory concentration (IC_{50}) values for antioxidant and antidiabetic activities were determined using linear regression equations. A paired t-test was also conducted to compare the sample with the positive control.

Results

Total phenolic and flavonoid content

The total phenol content was determined through the linear equation y = 0.0061x + 0.0224 obtained for gallic acid with an R^2 value of 0.9975 (Figure 2a). The total phenolic content was found to be 123.27 ± 2.27 µg GAE/mg in the aqueous fraction, 70.48 ± 2.87 µg GAE/mg in the dichloromethane fraction, and 23.49 ± 3.33 µg GAE/mg in the hexane fraction (Table 1). Similarly, the total flavonoid content was determined through the linear equation y = 0.0031x−0.0195 obtained for quercetin with an R^2 value of 0.9942 (Figure 2b). The total flavonoid content in the dichloromethane fraction was 35.43±3.67 µg QE/mg, in the aqueous fraction it was 31.67±3.56 µg QE/mg, and in the hexane fraction it was 30.48±1.21 µg QE/mg (Table 1).

Antioxidant activity

The half inhibitory concentration (IC_{50}) for antioxidant activity was also determined using a linear regression equation obtained from a percentage of DPPH scavenged by the concentration of a sample. The IC_{50} value of the reference compound, ascorbic acid, was found to be 3.52 µg/mL which is significantly different than fractions. Among the fractions, the aqueous fraction exhibited high antioxidant activity with an IC_{50} value of 25.04 ± 1.57 µg/mL, while the dichloromethane fraction demonstrated low activity with an IC_{50} value of 86.41 ± 6.21 µg/mL (Table 2). However, the hexane fraction couldn't inhibit the DPPH radicals.

Antidiabetic activity

The IC_{50} value for antidiabetic activity was calculated using linear regression equations obtained from a percentage of

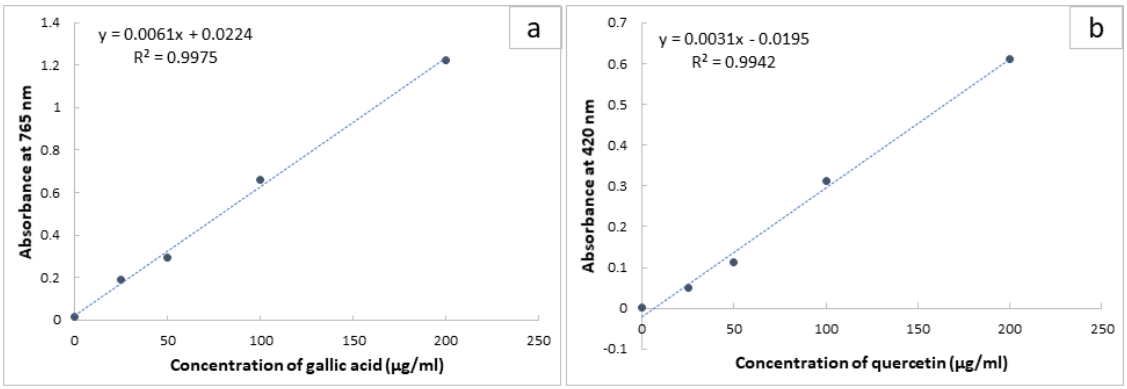

Figure 2. Linear regression equation of gallic acid (a) and quercetin (b) for calculation of total phenolic and flavonoid contents.

Table 1. Total phenolic content (TPC) and total flavonoid content (TFC) in different fractions of *Ephedra gerardiana* stems

Extract's fraction	TPC (µg GAE/mg extract's fraction)	TFC (µg QE/mg extract's fraction)
Hexane	23.49 ± 3.33	30.48 ± 1.21
Dichloromethane	70.48 ± 2.87	35.43 ± 3.67
Aqueous	123.27 ± 2.27*	31.67 ± 3.56

Values are means ± standard error of three replicates; Value with an asterisk (*) indicates a significant difference at $P \leq 0.05$ compared between the fractions for TPC and TFC; GAE: gallic acid equivalent; QE: quercetin equivalent.

α-glucosidase inhibition by the concentration of a sample. The aqueous fraction has shown significantly stronger antidiabetic activity with an IC_{50} for α-glucosidase inhibition of 4.33 ± 0.11 µg/mL than acarbose which had an IC_{50} of 220 ± 15.2 µg/mL (Table 3). However, hexane and dichloromethane fractions had no activity for α-glucosidase inhibition.

Toxicity of the fraction

Significantly, α-glucosidase inhibitor aqueous fraction up to 1000 µg/mL showed viability of nauplii after 24 hours, indicating no toxicity.

Discussion

Polar solvents yield extracts with high phenol and flavonoid contents that enhance antioxidant activity through metal ion chelation and free radical neutralization (32). The aqueous fraction exhibited high total phenol content (123.27 µg GAE/mg), consistent with previous findings on *Ephedra alata* (20,31). The dichloromethane fraction showed a high flavonoid content (35.43 µg QE/mg), aligning with research on *E. alata* (20,31). Phenolic compounds enhance antioxidant properties by scavenging free radicals, while flavonoids protect against oxidative damage by scavenging reactive oxygen species and stimulating the oxidation of other substances (36,37). The extract concentrations correlate with scavenging effects on DPPH radicals, up to a threshold (24). The aqueous fraction displayed moderate free radical inhibition (half inhibitory concentration (IC_{50}) = 25.04 µg/mL) compared to ascorbic acid, while the dichloromethane fraction showed lower activity. A paired t-test between plant fractions and ascorbic acid revealed that ascorbic acid possesses significantly ($P < 0.05$) strong antioxidant activity. The previous studies in *Ephedra alata*, *E. altissima*, *E. aphylla*, *E. foeminea*, *E. gerardiana* and *E. intermedia* exhibited the most potent antioxidant activity (IC_{50} ranged from 1.70 to 262 µg/mL) that support the present research (12,13,20,25,26,31).

Table 2. Half inhibitory concentration (IC_{50}) for 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity by different extracts of *Ephedra gerardiana* stems

Extract's fraction	IC_{50} for DPPH (µg/mL)
Hexane	No activity
Dichloromethane	86.41 ± 6.21 (R^2 = 0.89)
Aqueous	25.04 ± 1.57 (R^2 = 0.91)
Ascorbic acid (control)	3.52 ± 7.57* (R^2 = 0.97)

Values are means ± standard error of three replicates; Value with an asterisk (*) indicates a significant difference at $P \leq 0.05$ compared between the fractions and control.

Table 3. Half inhibitory concentration (IC_{50}) for α-glucosidase inhibition by different extracts of *Ephedra gerardiana* stems

Extract's fraction	IC_{50} for α-glucosidase (µg/mL)
Hexane	Not evaluated
Dichloromethane	No activity
Aqueous	4.33 ± 0.11* (R^2 = 0.97)
Acarbose (control)	220 ± 15.2 (R^2 = 0.98)

Values are means ± standard error of three replicates; Value with an asterisk (*) indicates a significant difference at $P \leq 0.05$ compared between the fractions and control.

With an IC_{50} value of 4.33 µg/mL, *E. gerardiana* aqueous fraction demonstrated stronger α-glucosidase inhibitory activity than acarbose, highlighting its potential to treat postprandial hyperglycemia. This result was supported by the previous result of Jaradat et al (31), where the methanol fraction of *E. alata* had a stronger level of α-glucosidase inhibition (IC_{50} value of 46.16 ± 0.63 µg/mL) when compared to acarbose. A similar outcome was demonstrated by Lee et al. (27) in *E. pachyclada*. Significant antioxidant activity scavenges free radicals, controls oxidative damage, and lowers oxidative stress associated with diabetes (37,38). Diabetes-induced oxidative stress disrupts enzymatic systems, inducing lipid peroxidation and depleting antioxidants (36). Additionally, the aqueous fraction showed no brine shrimp nauplii mortality, serving as a guide for non-toxic effects (35).

Conclusion

The aqueous fraction exhibited both DPPH and α-glucosidase inhibition, with IC_{50} values of 25.04 and 4.33 µg/mL respectively probably due to the presence of high phenolic content (123.27 µg GAE/mg). Additionally, it revealed that fraction has no lethality in the brine shrimp test. This suggests that *E. gerardiana* stem may serve as a source of natural antioxidants and contribute to the development of antidiabetic drugs as it has strong α-glucosidase inhibition.

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Authors' contribution

Conceptualization: Mukti Ram Paudel.
Data curation: Kalpana Dulal, Sapana Ojha-Khatri, Hem Raj Paudel.
Formal analysis: Mukti Ram Paudel.
Investigation: Kalpana Dulal, Sapana Ojha-Khatri.
Methodology: Mukti Ram Paudel.
Resources: Mukti Ram Paudel.
Supervision: Mukti Ram Paudel.
Writing–original draft: Kalpana Dulal.
Writing–review & editing: Kalpana Dulal, Sapana Ojha-Khatri, Hem Raj Paudel, Mukti Ram Paudel.

Conflict of interests

The authors declare that there is no conflict of interest.

Ethical considerations

This study does not involve the use of animal and human models. The approval for plant materials collection from Sagarmatha National Park was officially permitted by the Government of Nepal.

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