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Qualitative and Quantitative Phytochemical Screening and Free Radical Scavenging Activity of Different Parts of *Rubus ellipticus* Sm.

Ananda LAMICHANE[1]*, Susmita KHATRI[2], Mamata DHUNGANA[1], Bijaya TRIPATHI[1], Namrata BHATTRAI[1], Rishiram BARAL[2], Nirmala JAMARKATTEL[1]

[1] School of Health and Allied Science, Pokhara University P.O. Box 427, Pokhara-30, Kaski, Nepal; Department of Pharmacy, Kyungpook National University Daegu, South Korea
[2] School of Health and Allied Science, Pokhara University P.O. Box 427, Pokhara-30, Kaski, Nepal; Department of Business Development, Kaplan Business School, 28 Bridge Street, Sydney, Australia

*Corresponding author: aagyan2018@knu.ac.kr , ORCID ID: 0000-0003-1053-202X

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Abstract

Free radicals are molecules with reactive unpaired electrons which are produced during cell metabolism and create the oxidative stress inside tissue resulting in tissue damage. The aim of the present study was qualitative and quantitative phytochemical screening, determination of total phenolic and total flavonoid content, and free radical scavenging activity of different parts of *Rubus ellipticus* Sm. plant. Root, stem, and leaves of *Rubus ellipticus* Sm. were collected from Annapurna Rural Municipality, Kaski, Western Nepal. Ethyl acetate and ethanol extracts of the plant parts were obtained by subsequent maceration process. The phytochemical screening of most of the extract showed the presence of phenols, carbohydrates, flavonoids, and glycosides. The ethanolic extract of stem showed the higher phenolic content with the value of 343.75 ± 2.21 µg GAE/mg. Ethanolic extract of stem had the highest amount of flavonoid content (1563.17 ± 10.79 µg QE/mg of extract), whereas all the ethyl acetate extracts of root, leaves and stem showed comparable flavonoid content. Ethanolic extracts of leaves showed potent DPPH free radical scavenging activity with IC50 value of 5.03µg/ml while ethyl acetate extract of stem showed the maximum free radical scavenging properties. The result depicted that the ethanolic extract of *Rubus ellipticus* Sm. showed the potent antioxidant activity by scavenging free radicals.

Keywords: Antioxidant, Ethnomedicine, Free Radical Scavenging, Phytochemicals, *Rubus ellipticus* Sm.

1. Introduction

Free radicals are molecules with one or more unpaired electrons (superoxide, hydroxyl, peroxyl) that are produced during cell metabolism are very reactive (Halliwell. 1995). Among different free radicals generated inside the cell during metabolic process, Reactive Oxygen species (ROS) are most profound oxygen free radicals (Deweirdt et al., 2017; Valko et al., 2007). Because free radicals have a strong oxidizing functional group (Baliyan et al., 2022), they can create the oxidative stress inside tissue resulting in tissue damage (Erdemoglu et al., 2006). ROS are mainly generated into the mitochondria, endoplasmic reticulum,

cytosol and plasma membrane of the cell (Polidori & Mecocci. 2022; Sies & Cadenas. 1985). Meanwhile there are some exogenous factors inducing ROS production such as; exposure to radiations, xenobiotic, tobacco etc. (Brown & Borutaite. 2012; Deweirdt et al., 2017). These ROS can cause oxidative damage of lipids, proteins, RNA, DNA, and many small molecules in cells (Halliwell & Gutteridge. 2015). Although the human body produces natural antioxidants to combat those free radicals, but oxidative stress and aging upsurge the production of free radicals in a way necessitating the use of exogenous antioxidants (Indradi et al., 2017).

Antioxidants are the compounds the protect the cells from ROS by donating electrons (Choudhary. 2015; Surabhi & Leelavathi. 2010), prevent the formation of free radical, scavenge, suppress or forms chelates with free radical, repair and eliminate damaged molecules (Cesquini et al., 2003; Gutteridge. 1994; Maxwell. 1995; Raj et al.). Antioxidants produced by the body are crucial for the preservation of healthy cellular processes. The three most effective enzymatic antioxidants are superoxide dismutase, catalase, and glutathione peroxidase. Non-enzymatic antioxidants encompass vitamins E and C, thiol, melatonin, carotenoids, natural flavonoids, and other substances. The standard antioxidant compounds derived from plant sources include vitamin C, vitamin E, carotene, quercetin, and tocopherol. Various plant-derived antioxidants are effective free radical scavengers that are utilized as a nutraceuticals in combination to treat a variety of diseases (Indradi et al., 2017; Raj et al.)

Oxidative stress is caused by an imbalance between antioxidants and reactive oxygen species, which leads to cellular damage, cancer, aging, ischemic injury, inflammation, and neurodegenerative diseases (Erdemoglu et al., 2006; Surabhi & Leelavathi. 2010). Due to the low toxicity and side effect, most of the plants has been using as a natural antioxidant to cure different diseases condition (Baral et al., 2021).

The revival of interest in plant derived drugs is mainly due to the current widespread belief that “green medicine” is safe and more dependable than the synthetic drugs, many of which have adverse side effects (Jigna et al., 2005). Plants are used medicinally in various countries and are the source of numerous potent and powerful drugs (Srivastava et al., 1996). A wide variety of medicinal plant parts such as root, stem, flower, fruit, and some other parts are being used as raw drugs having varied medicinal properties. Some community and folk healers collect a small quantity of plants for local use, while others collect a large amount and supply to the herbal industries (Atanassova et al., 2011; Sharma and Kumar. 2011; Uniyal et al., 2006). Plants produce diverse types of bioactive molecules making them a rich source of different types of medicines. This revival of worldwide interest in medicinal plants reflects recognition of the validity of many traditional claims regarding the value of natural products in health care (Bagewadi et al., 2014). The most important bioactive component of the plants are alkaloids, tannins, flavonoids, and phenolic compound. About 85% of the rural population of Nepal are said to use herbal remedies which is mainly due to the indigenous beliefs and lack of alternatives in rural areas. Acquaintance with different ethnic groups contributes to the development and research on natural products which as a result increases the knowledge about the close relationship between the chemical structure of a compound and its biological properties (Atanassova et al., 2011; Kunwar & Bussmann. 2008).

Natural species of the Rubus genus are not only a source of food, but they are also used as medicine (Sharma & Kumar. 2011). Rubus species are said to be the world's best-known infusion herbarium (Rojas-Vera et al., 2002). According to ethnomedicine, the leaves and

fruit of *R. ellipticus* are used to treat bronchitis, nausea, ulcer, antimicrobial, diabetes, and as a carminative and tonic (Subba et al., 2019; Vadivelan et al., 2009). The fruit extract of *R. ellipticus* has been shown not to be cytotoxic to normal cells, but rather to have a stimulatory effect on their proliferation, with cervical cancerous cells being particularly sensitive (Saini et al., 2014).

R. ellipticus root paste has been reported to be used as a poultice for bone fracture, severe headache , colic pain (Castleman. 1991; Patel et al., 2004). The root bark is also used in diarrhea, dysentery, as abortifacient, emmenagogue and in fractured bones (Kirtikar et al., 2001). It has been reported that its shoot was chewed to relieve stomach upset and that a root decoction was given to warm the stomach (Bhakuni et al., 1987; Yadav et al., 2011). In traditional Tibetan medicine, the inner bark of the *R. ellipticus* plant is prized for its therapeutic properties, which include uses as an anti-diuretic and renal tonic (Pfoze et al., 2012) . The juice of *R. ellipticus*, which has an appealing color and flavor, can be maintained in its natural form and is also useful for preparing squash and edible jam (Vadivelan et al., 2009).

This study was conducted to discover the scientific evidence of *R. ellipticus* using ethnomedicinal uses as a guide. The primary goal of this study is to perform and compare the phytochemical screening, total phenolic and total flavonoid content, and free radical scavenging activity of different parts of *R. ellipticus* Sm.

Table 1. List of the parts of plant with their voucher number

Scientific Name	Family	Local Name	Parts Used	Crude Drugs Voucher Number	Sample Number
Rubus ellipticus Sm.	Rosaceae	Ainselu (Himalayan Raspberry)	Root	PUCD-2020-CR16	CR16
			Stem	PUCD-2020-CR17	CR17
			Leaves	PUCD-2020-CR18	CR18

2. Material and Methods

2.1. Chemicals and Reagents

1,1 Diphenyl-2 Picryl Hydrazyl Radical (DPPH) was purchased from Tokyo Chemical Industry Co. Ltd., Japan. Benedict's reagent, sodium hydroxide, sodium nitrite, sodium carbonate, ethanol, lead acetate, ammonium hydroxide, sodium hydroxide pellets, mercuric chloride, ferric chloride, and hydrochloric acid were supplied from Thermo Fisher Scientific, Pvt. Ltd. India. L-ascorbic acid was purchased from Himedia Laboratory, India. Qalinges Fine Chemicals supplied the sulphuric acid while Ethyl acetate, copper sulphate pentahydrate pure, sodium anhydrous pure, and 1-napthol were purchased from Merck Specialties Pvt. Ltd., Germany.

2.2. Collection and Identification of Plant Material

The experimental plant parts (listed in Hata! Başvuru kaynağı bulunamadı.) were collected (July 2019) from the Annapurna Rural Municipality in Kaski, Western Nepal, at an elevation of 4528 feet above sea level. Plant parts were collected and identified at the National Herbarium Kathmandu, (voucher specimen no. PUH-2022-08) while crude samples were stored at the Pharmacognosy Laboratory of Pokhara University's School of Health and Allied Sciences. The collected parts of the plant were chopped properly and subjected to shed drying. Hot air oven (40° C) was used to remove the moisture in the sample which was regularly monitored by weight variation

test at various time interval. After complete drying, it was finely powdered using a grinder.

2.3. Preparation of Plant Extract

The crude drug was extracted using successive maceration, as described by Pandey and Tripathi (2014) with minor modifications. Ethyl acetate and ethanol were selected as an extracting solvent was based on their polarity and availability of the solvent in the laboratory. 50-100 gm of the crude extract were macerated for 48 hours with enough (1:5 w/v) ethyl acetate to extract the plant sample. Following 48 hours, the filtrate was collected and concentrated using vacuum evaporator. The residue was then again macerated for 48 hours with enough ethanol (1:5 w/v ratio), and the final filtrate was collected and concentrated using vacuum evaporator again.

2.4. Phytochemical Screening

Phytochemical screening was measured according to the previous report explained by Yadav and Agarwala (2011) and Okoduwa, Umar et al. (2016) for qualitative determination of phytochemical compounds with slight modification.

2.5. Total Phenolic Content

The total phenolic content of the extract was determined using the Folin Ciocalteu Method, as described by Fattahi et al., (2014) and Kaur and Kapoor (2002) with minor modifications. 1mL of crude extract (1 mg/mL) was combined with 5 mL of distilled water and 1mL of the Folin-Ciocalteu reagent. After 5 minutes, 1mL of distilled water and 1mL of 10% (W/V) sodium carbonate were mixed and thoroughly shaken. Following 60 minutes interval, the absorbance at 725 nm was measured. Gallic acid is used as a positive control for phenolic compound as a standard. Gallic acid at concentrations ranging (15.63 mg/mL, 31.25 mg/mL, 62.5 mg/mL, 125 mg/mL, 250 mg/mL, and 500 mg/mL) were prepared in ethanol. The calibration curve was plotted using gallic acid as the standard. The calibration curve was then used to calculate the total phenolic content of *R. ellipticus* (Different parts), and the results were presented as mg of gallic acid equivalent per gram dry extract weight.

2.6. Determination of Total Flavonoids Content

The flavonoid content in the extract was determined using the aluminum chloride colorimetric method given by Chang et al., (2002) and Chun et al., (2003) with slightly modification. In ethanol, different concentrations of quercetin (15.63 mg/mL, 31.35 mg/mL, 62.6 mg/mL, 125 mg/mL, 250 mg/mL, and 500 mg/mL) were prepared as a standard. 1 mL of each extract solution (1 mg/mL) was combined with 4 mL of distilled water and 0.3 mL of 5% sodium nitrite. After 5 minutes, 1.3mL of 20% aluminum chloride was added and left for another 6 minutes. The absorbance at 510 nm was immediately measured using a UV spectrophotometer after addition of 2 mL of 1 M sodium hydroxide. Total flavonoid content was determined with the help of calibration curve (Figure 3) and results were expressed as mg quercetin equivalent per gram dry extract weight as shown in Table 5.

2.7. DPPH free radical scavenging method

The free radical scavenging activity of different parts of the R. ellipticus extract was determined using the diphenyl-picrylhydrazyl (DPPH) assay, as described by Akter et al., (2010) and Jabbari and Jabbari (2016). The stock solution (1 mg/mL) was diluted with methanol to a dilution series (50 g - 1000 g/ml). An aliquot of each dilution (2 mL) was mixed with a methanolic solution of DPPH (0.06 nM) and

shaken at room temperature for 30 minutes. Followingly, a control containing a methanolic solution of DPPH (2 mL, 0.06 mM) and ethanol (2 mL) was run. The absorbance was measured at 512 nm against a blank of methanol. Ascorbic acid was used as a standard of comparison. Equation 1 was used to calculate the percentage of free radical scavenging.

DPPH radical scavenging activity (%) = (Ao-A1)/A0*100 ……Equation 1

[Where Ao = Control Absorbance (Ascorbic acid), A1 = Absorbance when a test sample is present].

3. Results and Discussion

3.1. Yield Value Determination

The extraction yield value of *R. ellipticus* stem, root, and leaves in ethyl acetate and ethanol was calculated and listed in the Table 2.

Table 2. Yield value of diffrent plant parts in different sovents

Plant	Common name	Parts	% Yield (Ethyl Acetate extract)	% Yield (Ethanol extract)
Rubus ellipticus Sm.	Aiselu	Leaves	1.66	6.95
		Root	1.68	10.78
		Stem	0.86	3.83

Table 3. Phytochemical screening of ethyl acetate and ethanolic extract

Compounds	Test	Parts of Plants					
		Leaves		Root		Stem	
		EA	Ethanol	EA	Ethanol	EA	Ethanol
Alkaloids	Mayer's Test	-	-	+	-	+	-
Carbohydrate	Molish Test	+	+	+	+	-	-
	Ferric Chloride test	+	+	+	+	+	+
Phenol	Lead Acetate Test	+	+	-	-	+	+
Flavonoid	Alkaline Test	+	-	-	-	-	-
Glycoside	Legal's Test	+	+	+	+	+	-

3.2. Phytochemical screening

The phytochemical screening of different parts extracts of *R. ellipticus* in different solvents showed the presence of alkaloid, carbohydrate, phenol, flavonoid and glycoside which is mentioned in the Table 3.

Ethyl acetate extract of *R. ellipticus* leaves contained phenol, glycosides, and flavonoids, while the root and steam contained carbohydrate and alkaloids as well. In the case of ethanolic extract, leaves contained carbohydrate, phenol, and glycoside, whereas root extract was negative in the lead acetate test.

Meanwhile, the stem extract showed positive phytoconstituent result with phenol, flavonoid, and alkaloid.

3.3. Total Phenolic Content

The total phenolic content of all extracts was determined using the FC method with gallic acid as a standard. As shown in Table 1, all

results were expressed as µg gallic acid equivalent per mg of extract. Among the extracts, ethanolic extract of *R. ellipticus* (Stem) had the highest phenolic content (342.75 ± 2.21 µg GAE/mg of extract), while ethyl acetate extract had the lowest phenolic content (40.92 ± 4.25 µg GAE/mg of extract). **Hata! Başvuru kaynağı bulunamadı.** depicts the calibration curve for gallic acid. All determinations were made in triplicate, and the results were expressed as mean ± SD as shown in Table 1 and Figure 1.

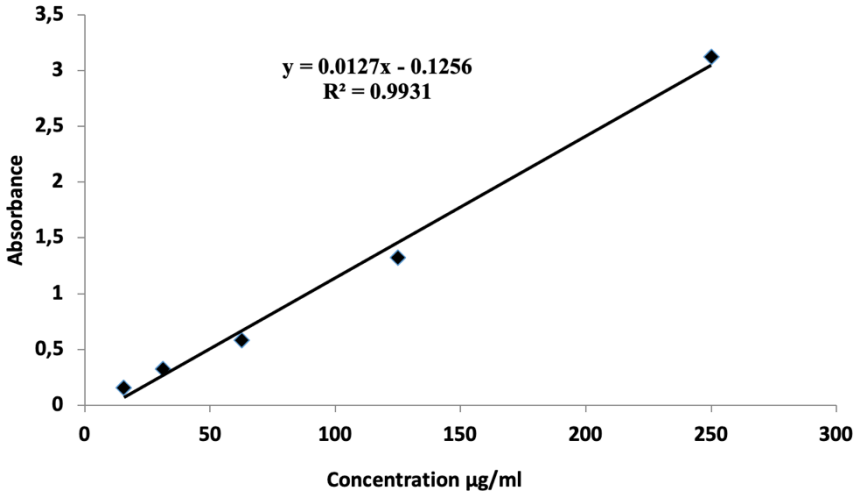

Figure 1. Standard gallic acid calibration curve for total phenolic calculation

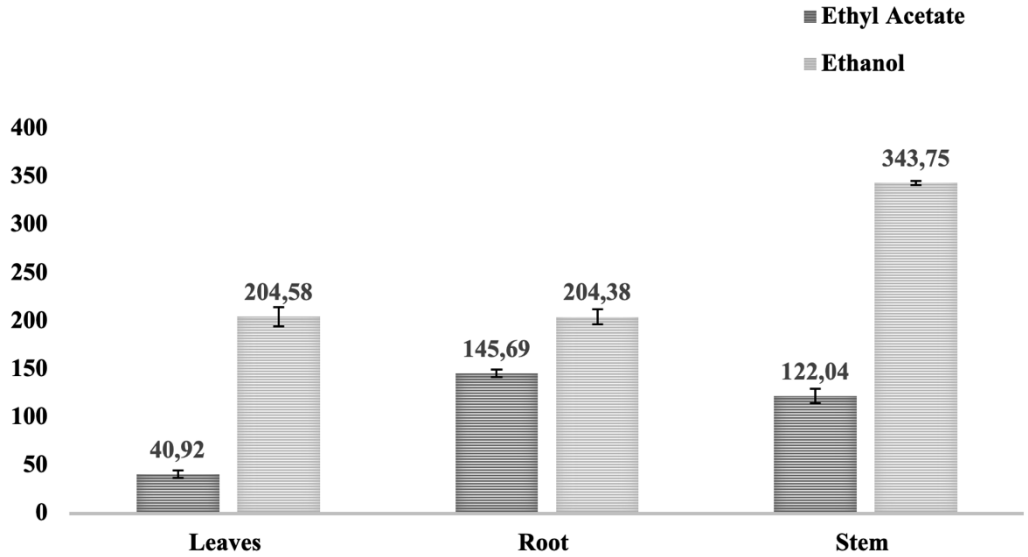

Figure 1. Graphical presentation of total phenolic content expresses as mg GAE/mg of extract

Table 1. Total phenolic content expresses as µg GAE/mg of extract

S.N.	Parts of *Rubus ellipticus* Sm.	Ethyl Acetate (µg GAE/mg of extract)	Ethanol (µg GAE/mg of extract)
1	Leaves	40.92 ± 4.25	204.58 ± 9.82
2	Root	145.69 ± 4.1	204.38 ± 7.95

3	Stem	122.04 ± 7.34	343.75 ± 2.21

3.4. Total Flavonoid Content

The flavonoids were quantified using the Aluminum Chloride colorimetric method, with quercetin as the reference standard. The results were expressed as quercetin equivalents per milligram of extract. Among the experimental extracts, the ethanolic extract of stem had the highest amount of flavonoid content (1563.17±10.79 µg QE/mg of extract) compared to the others (Root and Leaves), whereas all the ethyl acetate extracts, i.e., leaves, root, and steam, had almost comparable amounts of flavonoid, as depicted in Hata! Başvuru kaynağı bulunamadı..

Table 5. Total flavonoid content of expressed as µg QE/mg of extract

Solvent	Leaves	Root	Stem
Ethyl Acetate (µg QE/mg of extract)	68.06 ± 36.39	66.20 ± 15.34	84.84 ± 16.80
Ethanol (µg QE/mg of extract)	529 ± 27.45**	648 ± 39.56**	1563 ± 10.79**

*Note: ** represents that the original concentration of the extract falls out of the range so they were reduced to its half and final value was calculated equivalent to the original concentration.*

Table 6. Percentage scavenging activity of extract and ascorbic acid

S.N.	Parts of *Rubus ellipticus*	Solvents	% DPPH scavenging activity				
			0.1 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	IC_{50}
1	Leaves	Ethyl Acetate	1.97±0.15	0.71±3.3	18.90±2.40	81.01±1.29	58.61
		Ethanol	7.84±4.5	16.87±5.92	91.60±0.75	92.33±0.29	5.03
2	Root	Ethyl acetate	1.50±0.47	2.13±2.54	56.09±1.13	94.30±0.57	36.72
		Ethanol	2.32±4.59	15.50±3.08	87.90±2.01	88.61±1.52	5.47
3	Stem	Ethyl acetate	9.01±1.30	39.66±16.20	53.79±17.34	90.34±1.33	34.56
		Ethanol	7.33±1.95	18.29±2.05	90.29±1.13	92.19±1.19	5.08
4	Ascorbic Acid		13.26±0.5	44.28±0.55	95.37±0.21	96.47±0.21	3.562

3.5. Antioxidant Activity Assay

To assess antioxidant activity, the DPPH free radical scavenging activities of various parts *of R. ellipticus* were measured and shown in Hata! Başvuru kaynağı bulunamadı.. The leaves ethanolic extract demonstrated the most potent free radical scavenging activity, with an IC_{50} of 5.03 g, which is comparable to

the IC_{50} of standard ascorbic acid, as shown in

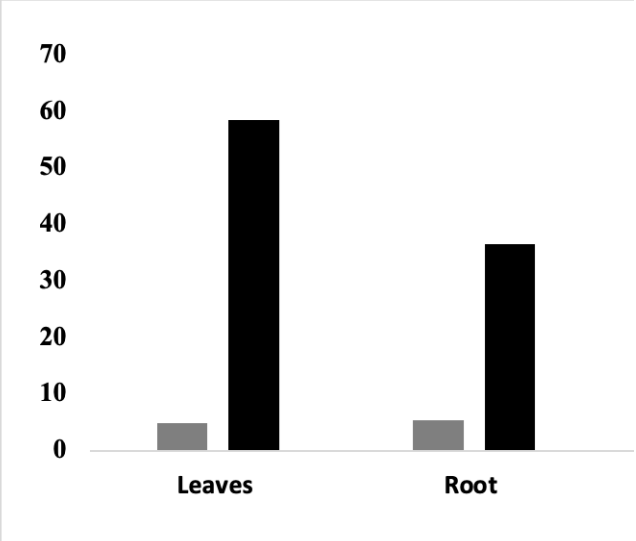

Figure **2**.

4. Discussion

Plants contain a wide range of natural compounds with different molecular families which show various medicinal properties. Plants are the important source of potentially useful structures for the development of new chemotherapeutics agents (Mukherjee et al., 2001).

In this study, we collected the ethnomedicinally used *Rubus ellipticus* Sm. and compared phytochemicals content, free radical scavenging effect, total phenolic, and total flavonoid content of different parts (leaves, stem, root).

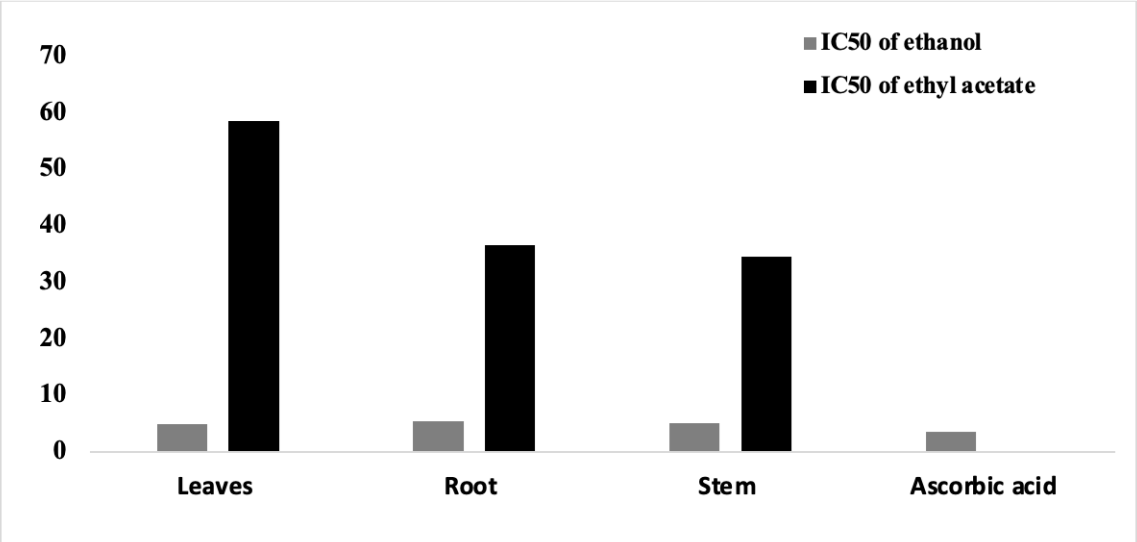

Figure 2. Graphical representation of IC_{50} values of all the extracts along with the standard ascorbic acid

The phenolic compounds are one of the largest and most prevalent groups of plants metabolites. They have therapeutic benefits in part because of their antioxidant capabilities, which include metal chelation, scavenging and suppression of reactive oxygen species, and scavenging of electrophiles. Phenolic compounds have been linked to antioxidative effects on living things because they scavenge singlet oxygen and free radicals (Barros et al., 2007).

Among all the extract of *R. ellipticus*, ethanolic root extract have maximum yield value of 10.78% followed by the ethanolic extract of the leaves and stem, whereas all the three extract of ethyl acetate have a minimum yield value compared to ethanolic extract as depicted in Table 2 which indicates that the plants parts contain more polar compound so that they were extracted in polar ethanol (Majidaee et al., 2020; Raman et al., 2005). The order of different parts of *R. ellipticus* based on the quantity of yielded extraction was root>leaves>stem.

In the qualitative phytochemical study, extract of *R. ellipticus* leaves, stem and root showed the presence of alkaloids, phenols and flavonoids which is even supported by the previously reported study (Sharma & Kumar. 2011; Subba et al., 2019) . In our study, during qualitative phytochemical screening to determine the phenolic compounds, all the extract shows the positive ferric chloride test while only the root extracts show negative lead acetate test, that may be due to the absence of sulfur containing amino acids (Sulfhydryl/thiol group) on the root of *R. ellipticus* (Anup. 2020/04/16).

According to Table 1, the ethanolic and ethyl acetate extract of the stem had the highest amount of phenolic content, followed by the root and leaves, which is supported by Öztürk, M., et al (2004), (Öztürk et al., 2007). According to the previous reported study of Saini et al. 2014, the extraction process of fruits of *R. ellipticus*, showed that the highest amount of phenols were content in an Acid Acetone (PH-2) extract compare to that of other less- polar solvent which is parallel to the results of our study as all ethanolic extract shows higher phenol content compared to ethyl acetate extract. Ethanolic extracts of all *R. ellipticus* samples contained more flavonoids than ethyl acetate extracts as per Hata! Başvuru kaynağı bulunamadı..

The stem of *R. ellipticus* has the highest flavonoid content in ethanolic extract and the highest TFC value in ethyl acetate extract which are comparable to each other as shown in Figure 1. Thus, we can conclude that the flavonoid concentrations are highest in the harder portion of *R. ellipticus*. These above findings clearly showed that ethanol recovered more total phenolics and flavonoids than ethyl acetate extracts, indicating that ethanol is a better solvent solution for the most effective extraction of polyphenols from *R. ellipticus*.

Based on the free radical scavenging activity, all the ethanolic extract of the plant showed comparable radical scavenging properties to each other and showed the IC_{50} value almost similar to the standard ascorbic acid. Also, among the ethyl acetate extract, stem shows the maximum inhibition of the free radical followed by the leaves and root. According to Subba et al., (2019) half-maximal inhibitory concentration value of methanol extract of leaves of *R. ellipticus* was 31 ± 0.2641 mg/mL depicting more potency as compared to our study.

In our study, the potent IC_{50} value of ethanolic extract compared to ethyl acetate extract could be attributed to the presence of more phenolic and flavonoid compounds in ethanolic extract, which is mentioned in previous study as well (Duh et al., 1999; Öztürk et al., 2007; Raman et al., 2005; Saxena et al., 2013). Mostly DPPH assay scavenge neutral and cation free radicals which are extracted in higher proportion in a polar solvent (Saini et al., 2014). According to Badhani et al., (2015) and Karuppusamy et al., (2011), there are various types of bioactive polyphenolic and flavonoid components found in different parts of *R. ellipticus* like Anthocyanin, Ascorbic acid, Chlorogenic acid, Gallic acid, Catechin etc. which are known for their antioxidant properties (Badhani et al., (2015); Karuppusamy et al., (2011); Saini et al., (2014); Schulz et al., (2019); Shikha & Kashyap. (2020)). May be because of the presence of such bioactive components, most of the ethanolic extracts showed the potent free radical scavenging effect.

5. Conclusion

The result revealed the presence of medicinally important bioactive constituents in the different parts of *R. ellipticus* with the potency order of leaves>stem>roots. Eventually, this study might also set a milestone to support and give the

scientifically evidence of using *R. ellipticus* for free radical scavenging properties. Moreover, isolation of the active hit and lead molecules, further evidence-based determination other pharmacological properties could benefit the mankind and assist in the discovery of various novel compounds.

Authors' contributions

Ananda Lamichhane, Susmita Khatri, Mamata Dhungana, Bijaya Tripathi and Namrata Bhattrai collected the plant, prepared the samples, performed all the experiments, and analyzed the data. Ananda Lamichhane wrote the manuscript. Rishiram Baral, and Dr. Nirmala Jamarkattel reviewed the manuscript. The authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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