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RESEARCH ARTICLE

Phytochemical Analysis and Biological Activities of Different Extracts of Walnut (*Juglans regia* Linn.) Kernels

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Abstract

The walnut tree (*Juglans regia* Linn.) is a valuable medicinal plant. Almost all parts of this plant have significant nutritional and curative properties. The walnut fruit has been claimed to possess antioxidant, anti-inflammatory, anti-hypertensive, antimicrobial properties and high nutritional value. The present work is focused on the phytochemical screening and study of antioxidant and antimicrobial properties of *Juglans regia* kernels extracted from four different solvents; methanol, ethyl acetate, chloroform, and hexane. The highest yield of extract was obtained with methanol. The phytochemical screening showed the presence of alkaloids, flavonoids, tannins, phenolic compounds, glycosides, terpenoids, quinones, reducing sugars, and saponins in methanol extract. The total phenolic content was higher in methanolic extract. Methanolic and chloroform extracts possessed more flavonoid content than ethyl acetate and hexane extracts. The DPPH radical scavenging assay showed that the IC_{50} value of methanolic extract was 78.77 µg/mL with TPC 67.65 mg GAE/g and TFC 391.00 mg QE/g. The IC_{50} value obtained for ethyl acetate extract was 135.28 µg/mL with TPC 36.56 mg GAE/g and TFC 212.00 mg QE/g. The IC_{50} value for chloroform extract was 313.72 µg/mL with TPC 13.08 mg GAE/g and TFC 393.73 mg QE/g. The IC_{50} value of hexane extract was 898.99 µg/mL with TPC 6.26 mg GAE/g and TFC 77.82 mg QE/g. Only methanolic extract showed the antimicrobial activity against *Proteus vulgaris, Pseudomonas aeruginosa,* and *Staphylococcus aureus.*

Keywords: *J. regia*; Antioxidant; Phenolic content; Flavonoid content; Antimicrobial

Introduction

Plants are valuable source of secondary metabolites and possess wide range of pharmacological activities like antimicrobial, antioxidant and free radical scavenging activity [1, 2, 3]. Extensive studies and research have been carried out on bioactive compounds and phytochemical acquired from plant parts, herbs and fruits worldwide [4, 5, 6]. Each medicinal plant species has its own nutritional composition. The nutrients such as carbohydrates, fats, proteins, vitamins and minerals are essential for the physiological function of human body.

Walnut is one of the oldest food sources which is rich in nutrients. The walnut tree is a plant introduced from Persia. It was considered as “the food of gods” and the Greeks called the walnut “kara”, that means “head”, for its similarity to the human head and its content, the kernel, to the brain [7]. The edible part of walnut fruit (seed or kernel) is consumed fresh or toasted. In Asia and Europe, walnut seeds are also used as antimicrobial, anthelmintic, and

antidiarrheal treatment in traditional medicines [8]. The walnut seed or kernel represents 30–40% of the nut weight, depending mainly on the variety. The seed of this tree nut shows high levels of oil (52–70%) which contains polyunsaturated fatty acids (73%), mono-unsaturated fatty acids (18%), saturated fatty acids (9%), high antioxidant activity and presence of omega-6 and omega-3 makes the walnut healthy for human diet [9]. In addition to oil, walnuts provide considerable amounts of proteins, carbohydrates, vitamins, fiber and minerals and also contains high amounts of various phenolic compounds [7, 10]. The kernel is popular and valued for nutrition whereas the by-products (leaves, green husks and membrane septum) are also considered as the source of healthcare and have been widely used in traditional medicine [11, 12].

In Nepal, walnut is commonly called "Okhar". It has a different name in different countries such as: "akhrot" in India, "he tao" in China, "Joz" in Arab, "Nogal comun" in Spain and "Noyer commun" in France [12, 13, 14, 15]. Common walnut is a large, wind pollinated, monoecious, dichogamous, perennial tree cultivated for its high-quality wood and nuts. *J. regia* have been observed to grow at the altitude of 1100 – 3000 m in Nepal. The optimal region for the suitable growth of *J. regia* is the temperate region having an average temperature of around 20°C and with the precipitation exceeding 600 mm. In Nepal, walnut is commercially cultivated in Mustang and Jumla but walnut tree can be found across the temperate region of the whole country [16].

Walnut is a crop of high economical interest as its seed is very important to the food industry. The fresh natural kernels are consumed mainly as whole nuts or used in various confectioneries. Walnut kernels are rich in macronutrients such as carbohydrates, fats and proteins. Walnut consists of high amount of starch, sugar, dietary fiber, polysaturated fat and proteins. In case of calories, the three-fourth of calorie in walnut is provided by fat [17]. It is a perfect source of vitamin E (α-tocopherols and γ-tocopherols) and also contains varieties of vitamin B and vitamin K [18]. Vitamin E is strong fat-soluble antioxidant, so the high amount of vitamin E plays a significant role in the protection of mucus and skin cell membranes against the harmful effects of free radical. Also, walnut kernels serve as a good source of a wide variety of flavonoids, phenolic acids and related polyphenols. Although phenolic compounds have no known nutritional function, they may be important for human health owing to their good antioxidant, antiatherogenic, anti-inflammatory and antimutagenic properties [17, 18]. According to researches, consuming walnut daily protects the body against: heart diseases, some certain cancer types, diabetes type 2, and other health problems [19, 20]. Walnut fruit is similar to heart shape and it is considered as the number one heart health-friendly hard-shell fruit. The reason for this is that walnut contains much more antioxidants than the other hard-shell nuts. The antioxidant substances found in walnut are more effective than vitamin E [20]. Walnut reduces cholesterol, oxidative stresses caused by free radicals, and the inflammations that damage health [21, 22].

Herein, we investigate the phytochemical composition and bioactivities of *Juglans regia* kernels extracted with methanol, ethyl acetate, chloroform, and hexane. This study evaluates the antioxidant and antimicrobial properties of these extracts, aiming to highlight the nutritional and medicinal potential of walnut kernels. Our findings will contribute to understanding the health benefits and

therapeutic applications of walnut extracts.

Materials and Methods

Collection of plant material

The kernel of soft-shelled Nepali walnut (*Juglans regia*), imported from Jumla district, was purchased from the local market of Asan, Kathmandu, Nepal in June, 2019. The kernels were washed, dried in shade, grinded with the help of an electric grinder and stored in a clean plastic bag until the further use.

Preparation of plant extract

The cold percolation extraction method was applied for the extraction of plant material. At first, 500 mL hexane was added to the clean and dry glass jar with 100 g powdered kernel and kept for 7 days at room temperature. Then the mixture was decanted and filtered through muslin cloth. The residue was again soaked in hexane and filtered till the hexane in sample become colorless. Thus, obtained filtrate was concentrated with the help of Rotavapor (IKA RV10), under reduced pressure by maintaining temperature lower than the boiling point of hexane. Then the concentrated filtrate was kept in beaker wrapping with aluminum foil containing small pores to facilitate the evaporation of solvent. The residue was completely air dried and sequentially transferred to other solvents: chloroform, ethyl acetate and methanol respectively and the extraction process was repeated for each solvent. After complete evaporation of solvents, the solid extracts were obtained and was stored in the freezer for further analysis. The percentage yield of the extracts was calculated using the following formula:

$$\% \text{ yield of extract} = \frac{\text{weight of extract in g}}{\text{weight of powdered sample in g}} \times 100\%$$

Phytochemical Analysis

The kernel extracts were subjected to various qualitative tests by following the standard protocol to identify the presence of different classes of secondary metabolites present in the extract [23, 24, 25].

Antioxidant Activity

The antioxidant activity of the extracts was determined by a DPPH assay [26, 27]. The stock solution of 1000 ppm of each extract (hexane, chloroform, ethyl acetate, and methanol) were serially diluted to prepare the test solutions of 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm concentration. 2 mL of test solution from each concentration was added to 2 mL of 0.2 mM DPPH solution and the mixture was shaken vigorously. After keeping in a dark for 30 min, remaining DPPH was determined by UV- Visible spectrophotometer (BioTek, EPOCH 2) at 517 nm against methanol and DPPH as a blank solution. Ascorbic acid was used as a positive control. The mean values were obtained from triplicate experiments. The percentage of free radical scavenging activity was calculated using the following equation:

Radical scavenging (%) = $[(A_o - A_s) / A_o] \times 100\%$

where,

A_o = Absorbance of the control (DPPH solution + methanol)

As = Absorbance of test sample

The IC_{50} value is the concentration of the sample required to scavenge 50 % of DPPH free radical and was calculated using the inhibition curve by plotting extract concentration versus the corresponding scavenging effect.

Antimicrobial Activity Screening

Agar well diffusion method was used for the antimicrobial screening of the crude plant extracts. Different strains of gram-positive bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*Proteus vulgaris and Pseudomonas aeruginosa)* were obtained from Department of Plant Resources, Thapathali,

Kathmandu. The working solution was made by dissolving 0.005 g of each crude extract in 1 mL solvent (10% aqueous DMSO with 5% polysorbate 80). The standard inoculum was swabbed carefully and uniformly all over the sterile Mueller-Hinton Agar (MHA) plate. The inoculated plates were left to dry for a day in laminar airflow. The wells were made in the incubated media plates where 20 µl of the working solution of the plant extracts were loaded using micropipette. DMSO was used as negative control and Neomycin was used as a positive control in the separate well for the antibacterial activity. The plates were then observed for the zone of inhibition (ZOI) produced by the antibacterial activity.

Total Phenolic Content

The total phenolic content in plant extract was analyzed by Folin-Ciocalteu colorimetric method based on the oxidation-reduction reaction. Gallic acid was used as a standard [28]. For this test, 1 mL extract from each concentration (20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm) was mixed with 5 mL 10% Folin-Ciocalteu reagent (FCR) and 4 mL 7% sodium carbonate solution. Then the blue colored solution was shaken well and kept in the water bath at 40°C for 30 minutes. Then the absorbance of the solution was measured at 760 nm wavelength using the spectrophotometer. Similar experiment was carried out for gallic acid in triplicate of each concentration and the mean absorbance values were plotted to get the calibration curve. The total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram of extract in dry weight (mg/g).

Total Flavonoid Content (TFC)

The total flavonoid content of the plant extract was determined by Aluminum Chloride colorimetric assay. Quercetin is used as a standard [28]. For the experiment, 1mL of the quercetin of each concentration (20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm) was poured into a 20 mL test tube and 4 mL distilled water was added to it. Then, at zero-time, 0.3 mL 5% $NaNO_2$ was added to the test tube. After 5 minutes, 0.3 mL 10% $AlCl_3$ and after 6 minutes 2 mL of 1M NaOH were added to the mixture. The total volume of the mixture was made 10 mL by adding 2.4 mL distilled water and was shaken thoroughly. Finally, the absorbance of the mixture was measured at 510 nm wavelength using the spectrophotometer against a blank solution containing all the reagents except quercetin. The average absorbance values obtained from the triplicate of different concentrations of quercetin were used to plot the calibration curve. The solution of 1000 ppm of extract was prepared by dissolving 10 mg extract in 10 mL methanol. Then the absorbance values were measured in triplicate following the same procedure as in quercetin. The total flavonoid content of the extracts was expressed as mg quercetin equivalent (QE) per gram of extract in dry weight (mg/g).

Results and Discussion

Determination of Percentage Yield

The percentage yield of different extracts of *J. regia* is given in **Table 1**. The methanol extract had the highest percentage yield whereas hexane extract had the lowest.

Table 1: Percentage yield of different extracts of walnut kernels

Part of Plant	Methanol extract (%)	Ethyl Acetate extract (%)	Chloroform Extract (%)	Hexane extract (%)
Kernels	14.69	12.96	10.73	7.52

Phytochemical screening

Phytochemical screening of different extracts of kernels of *J. regia* was carried out according to the standard procedure and the results are shown in **Table 2**.

Table 2: Phytochemical screening of different extracts of walnut kernels

S.N.	Class of compounds	Hexane extract	Chloroform extract	Ethyl Acetate extract	Methanol extract
1	Alkaloids	-	-	-	+
2	Terpenoids	+	+	+	+
3	Coumarins	-	-	-	+
4	Flavonoids	+	+	+	++
5	Polyphenols	+	+	+	++
6	Quinones	-	-	-	+
7	Glycosides	-	-	-	+
8	Reducing sugar	-	+	-	+
9	Saponins	-	-	-	+
10	Tannins	-	-	-	+

Where, '+' means present and '-'means absent

Result showed the presence of most of the phytochemicals in the polar extract, methanol. Terpenoids, polyphenols, flavonoids and reducing sugar were present in both polar and non-polar solvents. Several studies support the result obtained in Table 2. The presence of alkaloids, saponins, tannins, glycosides and carbohydrates in the kernel of *J. regia* and other species of walnut has been reported [29, 30]. The strong presence of polyphenols, flavonoids and quinones makes the walnut a good source of a healthy diet [31].

Antioxidant Activity

Antioxidant activity of each extract was measured by using DPPH free radical. This method is based on the reduction of DPPH solution in presence of a hydrogen donating antioxidant [32]. Ascorbic acid was taken as standard. **Table 3** shows the absorbance values, % radical scavenging activity, and IC_{50} values of extracts and ascorbic acid taken at different concentrations. **Fig. 1** represents a plot between sample concentration and % free radical scavenging activity.

Table 3: Antioxidant activity of different extracts of walnut kernels

Concentration (µg/mL)	Absorbance values					% Free radical scavenging activity				
	HE	CE	EE	ME	AA	HE	CE	EE	ME	AA
0	0	0	0	0	0	0	0	0	0	0
20	1.054	1.035	0.991	0.899	0.859	0.37	2.2	6.3	15.01	18.81
40	1.045	1.017	0.867	0.805	0.751	1.28	3.88	18.04	23.89	29.01
60	1.033	0.973	0.831	0.630	0.516	2.36	8.01	21.41	40.45	51.22
80	1.009	0.916	0.724	0.477	0.157	4.59	13.42	31.56	54.93	85.16
100	1.003	0.897	0.683	0.432	0.080	5.12	15.21	35.41	59.16	92.43
IC_{50} value (µg/mL)						898.99	313.72	135.28	78.77	53.99

Indications: HE: Hexane extract, CE: Chloroform extract, EE: Ethyl acetate extract, ME: methanol extract, AA: Ascorbic acid and Absorbance of control (methanol + DPPH solution) = 1.058

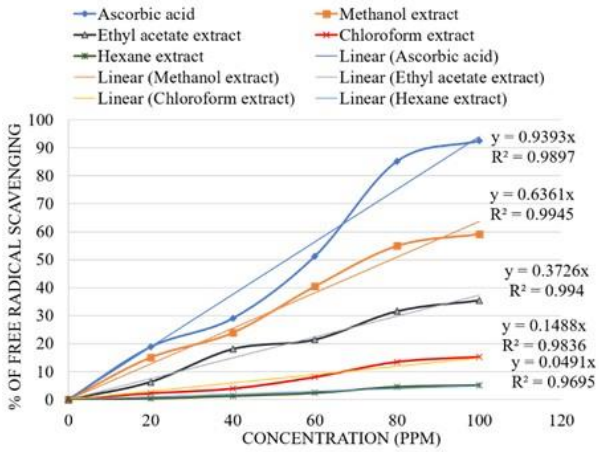

Figure 1: *Comparison of % radical scavenging between ascorbic acid and four walnut extracts*

The IC_{50} value of the methanolic extract of walnut kernel was lower than that of other extracts which implies methanolic extracts have high antioxidant properties. Thus, methanolic extract can act as the potential natural antioxidant. Also, the antioxidant activity of the

extract was found to be increased with increase in polarity of the solvent. Hence, polar solvents can be used to extract the antioxidants from the plant sample.

The antioxidant potential is in an inverse relation with IC_{50} value, which can be calculated from the Linear regression of the % of free radical scavenging (in Y-axis) versus concentration of extract (in X-axis). Lower the value of IC_{50} indicates high antioxidant potential [33]. The percentage scavenging of DPPH radical was found to be increased with increase in the concentration of the extracts from 20 ppm to 100 ppm. The methanolic extract of walnut kernels showed the higher antioxidant activity (IC_{50} value of 78.77µg/mL) than other extracts. The standard ascorbic acid showed the highest antioxidant activity with an IC_{50} value of 53.99 µg/mL. For the same concentration range, ethyl acetate extract showed IC_{50} value of 135.28 µg/mL, chloroform extract with the IC_{50} value 313.72 µg/mL and hexane extract showed the least antioxidant activity with the IC_{50} value of 898.99 µg/mL. In previous study, the IC_{50} values of walnut extracts were determined to be 70 µg/mL in methanol extract [8], 10.3±0.8 µg/mL in methanolic extract and 960±1.4 µg/mL in ethyl acetate extract [7]. These data support the results obtained in **Table 3.**

Determination of Total Phenolic Content (TPC)

The total soluble phenols present in the extracts were evaluated by using Folin-Ciocalteu colorimetric method [28]**.** Gallic acid was used as standard. TPC of different extracts was calculated from the calibration curve of gallic acid by regression equation, followed by the formula C= cV/m and expressed as mg gallic acid equivalents (GAE) per gram of extract in dry weight (mg/g). The calibration curve for the standard gallic acid is shown in **Fig. 2** and TPC of different extracts is given in **Table 4.**

Table 4: Total phenolic content of different extracts of *Juglans regia* kernels

S.N.	Walnut kernel extract	TPC (mg GAE/ g)
1	Methanolic extract	67.65
2	Ethyl acetate extract	36.56
3	Chloroform extract	13.08
4	Hexane extract	6.26

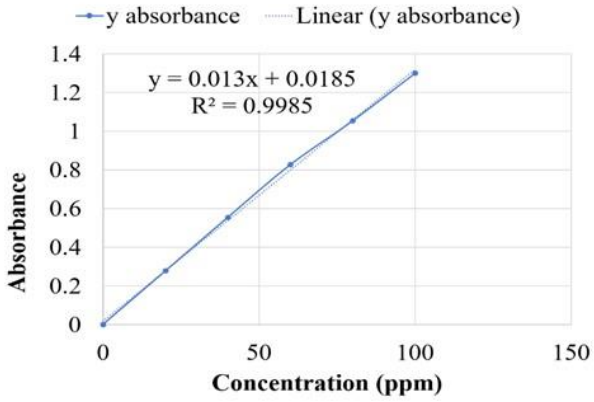

Figure 2: *Calibration curve for standard gallic acid solution.*

From the above result, it is vivid that methanolic extract of kernel contains high phenolic compounds than other extracts. This result is in good agreement with previous studies which showed TPC of 66.55 ± 4.98 mg GAE/g [36] and 50.3 ± 0.7 mg GAE/g [37]. The variation may be due to the difference in the environmental and geographical factors, storage condition, temperature, chemical quality and accuracy of instruments. Phenolic compounds have been known to possess high antioxidant properties due to their free radical scavenging properties. It has been reported that the extract

containing polyphenols possess a good antioxidant activity. Although, the quantitative determination of phenolic compounds in plant extracts may be hampered by their structural complexity, diversity, nature of analytical assay method, selection of standard and presence of interfering substance [35, 36, 37].

Determination of Total Flavonoid Content (TFC)

The aluminum chloride colorimetric assay was used for the estimation of the total flavonoid present in the extract by using quercetin as a standard [28]. The TFC of different extracts was calculated from the calibration curve of quercetin by regression equation, followed by the formula C= cV/m and expressed as mg quercetin equivalent (QE) per gram of extract in dry weight (mg/g). The calibration curve for the standard quercetin is shown as **Fig. 3** and TFC of different extracts is given in **Table 5.**

Table 5: Total flavonoid content of different extracts of *Juglans regia* kernels

S.N.	Walnut kernel extracts	TFC (mg QE/ g)
1	Methanolic extract	391.00
2	Ethyl acetate extract	212.00
3	Chloroform extract	393.73
4	Hexane extract	77.82

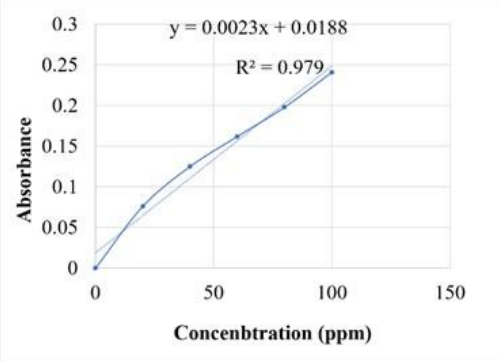

Figure 3: *Calibration curve for standard quercetin solution.*

Chloroform and methanolic extracts are found to contain much higher flavonoid content than in ethyl acetate and the least in hexane extract. The results are in good agreement with that of the previous studies which reported the TFC in walnut kernels between 89.12 to 130.79 mg QE/g [38, 39]. Flavonoid compounds are capable of effectively scavenging the free radicals because of their phenolic hydroxide group and possess antioxidant properties. The antioxidant properties of flavonoids depend upon their structure, particularly hydroxyl position in molecule. The quantitative determination of flavonoid compounds maybe hampered by their structural complexity, diversity, nature of analytical assay method, selection of standard, and presence of interfering substances [35, 36, 37].

Antimicrobial Activity

The antimicrobial screening of four different extracts (methanol, ethyl acetate, chloroform and hexane) of walnut kernel were performed by agar well diffusion method and the result obtained are given in **Table 6** and **Fig.4.**

Table 6: Antimicrobial screening of different extracts of walnut kernels

S.N.	Microorganism	Zone of Inhibition (ZOI) in mm				
		Methanol extract	**Ethyl acetate extract**	**Chloroform extract**	**Hexane extract**	**Positive cor**
	Bacteria					Chloramphe
1	*Proteus vulgaris*	16.81	0	0	0	0
2	*Pseudomonas aeruginosa*	22.84	0	0	0	0
3	*Staphylococcus aureus*	12.45	0	0	0	28.4

Chloramphenicol with concentration of 60 µg/mL was used as positive control in the

antimicrobial activity screening of bacteria.

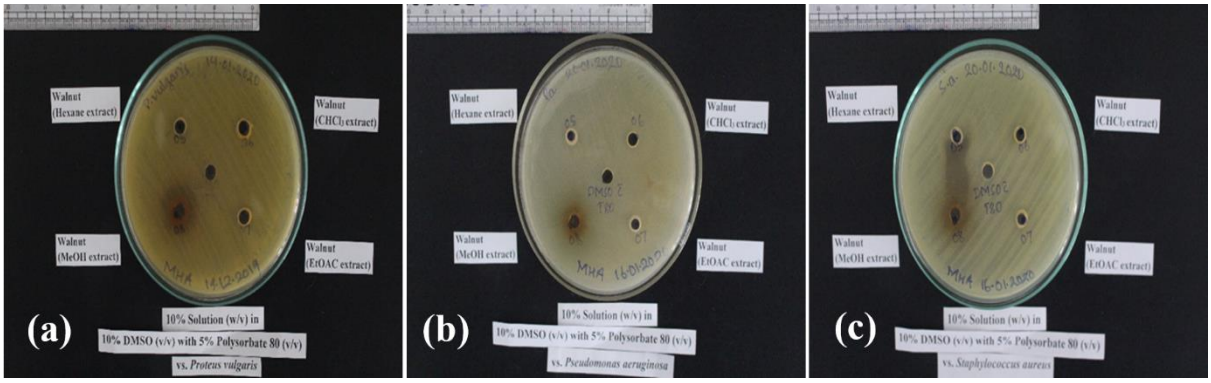

Figure 4*: ZOI of methanol extracts against (a) P. vulgaris, (b) P. aeruginosa and (c) S. aureus*

The result indicates the methanolic extract of walnut kernels possessed 16.81 mm, 22.84 mm and 12.45 mm zone of inhibition for *P. vulgaris, P. aeruginosa* and *S. aureus* respectively. There is significant inhibitory activity against both gram-positive (*S. aureus*) and gram-negative bacteria (*P. vulgaris* and *P. aeruginosa*). According to the observations, the extraction yield increases as solvent polarity increases. This relationship between extraction yield and solvent polarity adds a significant aspect to our knowledge of the extraction process. The detailed phytochemical screening of the extracts offers an extensive knowledge of the chemical composition of the plant material. The finding that the methanol extract contained all the tested phytochemical indicates that phytochemicals from walnut kernel should be extracted using a solvent with high polarity. The relationship of solvent polarity with TPC, TFC and antioxidant activity highlights that an increase in the polarity of the solvent increases phytochemical content and antioxidant potential. The selective antimicrobial activity of methanolic extract against *P. vulgaris*, *P. aeruginosa*, and *S. aureus* is a notable finding. This antimicrobial activity adds to the potential application of plant extracts in the fight against certain pathogens. The total flavonoid content (TFC) in the chloroform and methanolic extracts was measured as 393.73 mg QE/g and 391.00 mg QE/g, respectively. These values surpass those reported in other studies, which indicated TFC levels ranging from 89.12 to 130.79 mg QE/g [38] and 93.07±7.86 mg QE/100g [39]. The methanolic extract of kernel contains high phenolic compounds than other extracts which is 67.65 mg GAE/g. This result exceeds the previous studies which showed TPC of 50.3 ± 0.7 mg GAE/g [37], 58 ±3 mg GAE/g [34], 22 mg GAE/g [40]. The methanolic extract of walnut kernels showed the higher antioxidant activity (IC_{50} value of 78.77µg/mL) than other extracts. In other studies, the IC_{50} values of walnut extract in methanol were determined to be 70 µg/mL [36] and 10.3±0.8 µg/mL [8]. The relationship between bioactive compounds and polarity of solvent can be established from this study. The observed trend indicates that when the solvent from methanol to hexane is changed, the yield of extract, presence of phytochemicals, antioxidant activities, total phenolic and flavonoid contents, and antimicrobial activities all decrease. This is primarily because these solvents have different polarity and selectivity when it comes to extracting different kinds of phytochemicals from plants. Compared to less polar solvents like ethyl acetate, chloroform,

and hexane, methanol, a polar solvent, typically extracts a greater spectrum and higher concentrations of bioactive chemicals. Because phenolic chemicals are known to be effective at scavenging free radicals, the high level of antioxidant activity found in methanol extracts is likely caused by their presence. The presence of bioactive substances with established antibacterial qualities, such as tannins and flavonoids, may be related to the antimicrobial activity seen in methanol extracts.

Conclusions

In conclusion, the study demonstrates that the methanolic extract of Juglans regia kernels is the most effective in terms of extract yield, antioxidant activity, and antimicrobial properties compared to extracts obtained using ethyl acetate, chloroform, and hexane. The methanolic extract exhibited the highest TPC and TFC, as well as the lowest IC50 value in the DPPH radical scavenging assay, indicating strong antioxidant potential. Additionally, it was the only extract showing significant antimicrobial activity against *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. These findings highlight the methanolic extract of *Juglans regia* kernels as a promising source of natural antioxidants and antimicrobial agents

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Author's Contribution Statement

Nabin Das: Writing original draft, Data curation, Investigation, Formal analysis, Visualization. **Pooja Tandukar**: Data curation, Visualization. **Muna Niraula**: Data curation, Formal analysis. **Daman Raj Gautam**: Writing-review and editing, Resources, Project administration, Conceptualization. **Ishwor Pathak**: Writing-review and editing, Supervision, Investigation, Conceptualization.

Conflict of Interest

The authors do not have any conflict of interest throughout this research work.

Data Availability Statement

The data supporting this study's findings are available from the corresponding authors upon reasonable request.

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