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EVALUATION OF ANTIOXIDANTS AND ANTIMICROBIAL PROPERTIES OF INDIGENOUS PLANTS: *Elaeocarpus sphaericus* AND *Ficus religiosa*

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Abstract

Elaeocarpus sphaericus (Rudraksha) and *Ficus religiosa* (Peepal) are the two different indigenous and religious plants found in Nepal. These plants are rich in antimicrobial as well as anti-inflammatory properties. The present study was done to determine the antioxidants, anti-microbial properties, as well as to analyze the various phytochemicals found in the methanolic extracts of leaves of the sampled plants. The Antioxidants levels were determined by the DPPH Scavenging Assay. The methanolic extracts of the plants showed antioxidant properties i.e., 98.01 and 122.3 µg/ml for Rudraksha and Peepal, respectively. Likewise, the Antibiotic Susceptibility test was performed by Well-Diffusion assay in Mueller Hinton agar (MHA) plates. The zone of inhibition against the Gram-negative (*Escherichia coli, Pseudomonas aeruginosa),* and Gram-positive (*Staphylococcus aureus,* and *Bacillus cereus)* bacterial isolates were observed, supporting the antimicrobial activity of the plant. Additionally, various qualitative tests were performed for determining the presence/absence of the phytochemicals. Both Peepal and Rudraksha extracts gave positive tests for Flavonoids, Terpenoids, Cyclic glycosides, volatile oils, tannins, flavonoids, phenols, anthraquinone, glycosides, alkaloids, steroids, and reducing sugars, and phenols. Likewise, Saponins were found to be positive only in Peepal extracts with negative result for Phlabotannins and proteins. Thus, this research will help for utilizing the two religiously important plants i.e. Rudraksha and Peepal, for further researches in the medical field and preparation of various ayurvedic medicines.

Keywords: Plant extract, DPPH, Methanol, MHA

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Introduction

Nepal has geographical variation i.e., the Himalayan, Hilly, Plateaus, lowlands and terai; thus, plethora of vegetation flourish in different geological regions. Across the country, various flora with high potentiality in diverse medical and research purposes could be found (Ncube *et al*., 2008; Hardainiyan *et al*., 2015). Of the diverse flora, 14-28% are widely used in the medicines alongside, 74% of the pharmacologically active plant derived components are widely used in various scientific research, ayurvedic medicines and in skin products (Gibbons, 2003).

Elaeocarpus sphaericus (Rudraksha) is symbolically meant to be the tear rolled from the eyes of Lord Shiva (Tewari *et al*., 2013). The plant belong to the family Elaeocarpeceae and is found usually in the Himalayan region, and well known as "Rudraksha" all over Nepal and India. They are evergreen and can be found in the tropical and sub-tropical regions around 2000 meters altitude from the sea level (ranging from 2000 to 3500 m) (Tripathy *et al*., 2016).

In Ayurveda, wearing the bead of rudraksha has a positive effect on health and nerves (Pandey *et al*., 2014). It has its popularity due to its utilization for treatment of various diseases since ancient times (Joshi and Jain, 2014; Hakiman and Maziah, 2014). For instance, the leaves help in treating fever, diabetes, heart problems, headaches, mental disorders, fever, chickenpox as well as other ailments. Additionally, anti-inflammatory as well as antimicrobial properties has also been reported alongside, enhancement and boosting up memory power (Singh *et al*., 2011). Due to its use in treatment of various condition and diverse medicinal properties, Rudraksha is also known as the king of herbal medication. It is used for the release and treatment of stresses as well as feelings of depression, anxiety, along with epilepsy, asthma, liver problems, nervous problems and many more. The various phytochemicals and nutrition in those plants like, rudrakine, grandisines, quercitin, triterpines, etc. has ethnomedical qualities in them and possess sedative as well as other essential properties (Tripathi *et al*., 2015).

Ficus religiosa (Peepal) is another plant that is religiously considered as an incarnation of "Lord Vishnu". It is also referred as sacred figs or Bodhi trees and can be found in Himalayan foothills and various Southeast Asian countries. These are evergreen trees possessing broad leaves. Peepal has different chemical compounds that are beneficial for the treatment of various ailments like asthma, inflammation, epilepsy, stomach ulcers, etc. (Gautam *et al*., 2014). The Peepal leaves can be used as ointments and are diuretics. They help in reducing indigestion, nausea and help in maintaining skin health. These leaves also possess astringent and help to work as a medication for people's health.

The leaves of Peepal and Rudraksha are rich in antioxidants. Moreover, the leaves has been found to contain anticarcinogenic, antimutagenic, antimicrobial, analgesic, and anti-inflammatory properties. They are rich in various constituents like campesterol, stigmasterol, α-amyrin, lupeol, tannic acid, amino acids, hexacosanol, n-octacosane, and quercetin. People nowadays are attracted to Ayurveda due to the side effects from allopathic/

modern medicine, and increase in antibiotic resistance day- by- day (Sakat *et al*., 2009; Weli *et al*., 2018). As the plants are rich in various phytochemicals and other compounds, the medications derived from plants have become one of the interesting fields for study. They are sources of various traditional drugs, medicine, nutraceuticals, food supplements, etc. Therefore, this research focuses on understanding the antioxidants, antimicrobial, and phytochemicals in the leaves of Peepal and Rudraksha. The results of this study could contribute in the field of medicine.

Materials and methods

The two different religiously important plants *E. sphaericus* (Rudraksha) and *F. religiosa* (Peepal) were taken as a sample for this research study. The leaves of *E. sphaericus* and *F. religiosa* were collected from Kathmandu, Nepal in July, 2018.

Bacterial Cultures: For the Antibiotic Susceptibility tests, four different bacterial cultures were used i.e. Gram-positive: (*Staphylococcus aureus* ATCC 25923*), Bacillus cereus* 14579*)* and Gram-negative: *(Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* 15442*)* which were provided by the Department of Plant Resources, Thapathali, Kathmandu.

Preparation of Plant Extract

The healthy and matured leaves of Peepal and Rudraksha were collected and washed in distilled water. They were then air-dried and further dried in the hot air oven at 50°C and finally crushed into dry powder using a mixer. The 20g of leaf powder was subjected to extraction, using hydro- methanolic solution (100% methanol in water) in the ratio of 1:4 and placed on a rotating incubator at 28°C at 150 rpm for 3 days. Furthermore, filtration of the extract was done using a muslin cloth. The filtrate was then evaporated at room temperature to reduce the volume. The concentrated extracts were dissolved in dimethylsulfoxide (DMSO) for a hydro-methanolic extract. The extracts were weighed and stored in the fridge (Mostafa *et al*., 2018; Weli *et al*., 2018). The physical characteristics of the extracts i.e. their color, odour, consistency, sense of touch, as well as yield were performed. The yield % was carried out by the formula:

$$\text{Extract yield \%} = R/S \times 100\% \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{ equation (i)}$$

Where, R= weight of extract and S= weight of plant raw sample (Mostafa *et al*., 2018)

Antioxidant Assays (DPPH scavenging assay)

The stock solution of 1 mg/ml was prepared by dissolving 1.5mg plant extract (Peepal and Rudraksha) and ascorbic acid (as standard) in 1.5 ml methanol in an Eppendorf's tube. Different concentrations of plant extracts and ascorbic acid were prepared. 0.3 mM DPPH was prepared using methanol as a solvent. 300 µl of a sample (a plant extract and ascorbic acid of different concentrations) was mixed with an additional 300 µl of DPPH reagent, mixed, and left for incubation for 30 minutes. Spectrophotometric analysis was done for each sample

using methanol as a blank. The sample absorbance was taken and was plotted in the graph. Similarly, 600 µl of DPPH absorbance was also taken and noted as a control (Sharma *et al*., 2015; Mostafa *et al*., 2018).

$$\text{Percentage scavenging} = \frac{Ao - A1}{Ao} \times 100\ \% \quad \ldots\ldots\ldots\ldots \text{equation (ii)}$$

Where, Ao = Absorbance of DPPH solution,

A1 = Absorbance of DPPH along with different concentrations of extract (Mostafa *et al*., 201)

The IC50 value was calculated and expressed as µg/ml. IC50 value is defined as the concentration of the sample that is required for scavenging 50% of DPPH free radical. The lower values of IC50 indicates the higher amount of antioxidants as it shows the required antioxidant for free radical reduction by 50%of initial concentration (Koirala and Shrestha, 2020). It can be calculated by plotting the graph of the radical scavenging activity against their concentration and is further determined (Bhandari *et al*., 2019).

Antimicrobial susceptibility assay

An Antimicrobial susceptibility assay is done to determine the antimicrobial activity of the extracts against the known pathogenic bacteria. The bacterial cultures; Gram-positive: (*Staphylococcus aureus, Bacillus cereus)* and Gram-negative: *(Escherichia coli, Pseudomonas aeruginosa)* were used. The antibacterial potential was analyzed by the agar well diffusion method (Lakhey *et al*., 2018; Koirala *et al*., 2021). The turbidity of cell suspension was made equivalent to 0.5McFarland (containing a microbial load of 1.5×10^8 CFU/ml). Methanol was used as a negative control. The suspension was spread on an MHA (Mueller Hinton Agar) plate and let stand for around 15 minutes for absorption. Further, 4 different wells were prepared using a sterilized cork borer. Various concentrations i.e. 12.5, 25, 50, 100, 200 and 400 µg in methanol. The sample prepared was placed in different wells followed by 24 hours incubation. Microbial growth inhibition is determined by measuring the diameter of the zone of inhibition (ZOI) in mm. The results were further compared with the Standard Zone Size Interpretation Chart for Antibiotic Susceptibility Test (AST). (Prabhavathi *et al*., 2016; Lakhey *et al*., 2018; Eloff, 2019; Koirala *et al*., 2021).

Phytochemicals screening

Preliminary phytochemical screening was performed to detect the presence/absence of various Phytoconstituents like glycosides, terpenoids, phenolics, flavonoids, and saponins, and tannins following standard methods.

Terpenoids

The sample was taken and dissolved in 0.5 ml Chloroform and 2-3 drops of Conc. Sulfuric acid was added. The mixture was then heated for 2 minutes and observance of grayish reddish-brown color on the interface indicated a positive test for terpenoids (Prabhavathi *et al*., 2016).

Cyclic glycosides

1 ml sample was mixed with 0.5 ml acetic acid. Further, 2-3 drops of Ferric Chloride was added to the solution. The brown ring at the interface determined the positive test for cyclic glycosides (Prabhavathi *et al*., 2016).

Saponins

1 ml distilled water was shaken with 1 ml extract and the foams were observed and persist for around 10 minutes, which indicated positive results for Saponins (Prabhavathi *et al*., 2016).

Tannins

To 2 ml of each extract, 10 % of alcoholic ferric chloride was added. The formation of brownish blue or black color indicated the presence of tannins (Prabhavathi *et al*., 2016).

Flavonoids

1 ml test sample was tested with 1 ml of Sodium Hydroxide. A yellowish color was observed. Further, 5-6 drops of Conc. HCl. Was added to the tube. The yellow color disappeared which denotes a positive test for Flavonoids (Prabhavathi *et al*., 2016).

Phenols

To 2 ml of each extract, 2 ml of 5% aqueous ferric chloride was added. The blue color observed indicated the presence of phenols in the sample extract (Prabhavathi *et al*., 2016).

Phlobatannin

The extract was taken in a test tube and Conc. HCl was added and boiled for 1 minute. The formation of red precipitate indicated the presence of phlobatannins (Prabhavathi *et al*., 2016; Pakuwal and Manandhar, 2021).

Protein

The extract was taken in a test tube was taken and NaOH solution was added, and 1-2 drops of $CuSO_4$ solution was added. The change of color to violet indicated the presence of proteins. (Lakhey *et al*., 2018).

Anthraquinone

The sample was taken and 20 ml benzene was added to it and mixed on a magnetic stirrer for 4 hours. The filtrate (10 ml) was mixed with 0.5 ml ammonia solution (10%) and mixed properly (Bhattacharya and Roy, 2018; Pakuwal and Manandhar, 2020). Observance of pink precipitate indicated presence of Anthraquinone.

Cyclic glycosides

In 1 ml of sample, 2 ml of glacial acetic acid was added which contains 2% $FeCl_3$ solution. To the mixture, 1 ml concentrated H_2SO_4 was added slowly from the side of the wall of the test tube. The formation of a brown ring indicated the presence of glycoside (Bhattacharya and Roy, 2018).

Alkaloids

In 2 ml sample, 2-3 drops of potassium mercuric iodide was added and mixed properly. A white-yellowish or creamy colored precipitate (Bhattacharya and Roy, 2018) indicated presence of Alkaloids.

Steroids

1 ml of extract was dissolved in 10 ml of chloroform and an equal volume of Conc. H_2SO_4 was added by the sides in the test tube. The upper layer shows green with yellow fluorescence indicated a positive test for Steroids (Bhattacharya and Roy, 2018).

Reducing sugars

In 0.5 ml extract solution, 1 mL water acidified with dilute HCl was added and mixed. It was further neutralized with addition of alkali and heated with 0.5 mL Fehling solution (A + B). A reddish brick precipitate indicates the presence of reducing compounds (Bhandari *et al*., 2019).

Volatile oils

The extract was kept on filter paper through a capillary tube and visualized. Transparent filter paper with no yellow color indicated the presence of volatile oils (Bhandari *et al*., 2019).

Data analysis

The tests were performed on triplicates (n=3) and the results were reported as mean± standard deviation (S.D.). One-way ANOVA (Microsoft Excel 2013) was performed and the results were found to be statistically significant ($p < 0.05$).

Results and discussion

Characteristics of extract

The physical characteristics of the methanolic extracts were done and presented in the graphs. Peepal extracts were of reddish brown color with semi – solid consistency and sticky. Likewise, Rudraksha extract was found to be brownish in color with the semi- solid and sticky nature. The % yield was found to be higher for Rudrakhsa i.e. 25 %.

Table 1: Physical characteristics of extracts

Sample	Colour	Odor	Consistency	Sense of touch	Amount of extract (in grams)	% yield
Peepal	Reddish-brown	Characteristic	Semi-solid	Sticky	15	20
Rudraksha	Brownish	Characteristic	Semi-solid	Sticky	20	25

DPPH scavenging assay

DPPH scavenging assay was performed using ascorbic acid as standard. The following results were obtained. % inhibition of the DPPH by ascorbic acid (standard solution), *Elaeocarpus sphaericus* (Rudraksha) was found to have comparatively more antioxidant compared to *Ficus religiosa* (Peepal).

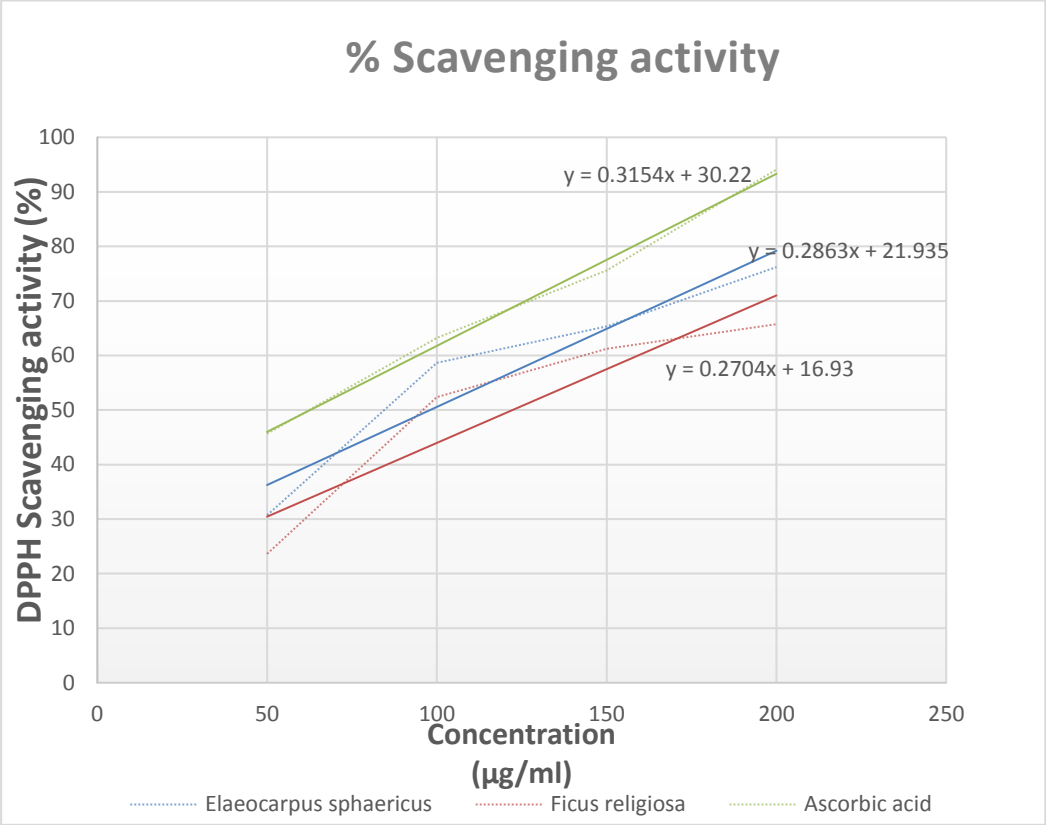

Figure 1: Percentage inhibition of DPPH by *Elaeocarpus sphaericus* and *Ficus religiosa* extracts along with ascorbic acid which was used as a standard.

Table 2: DPPH scavenging activity of *Elaeocarpus sphaericus* and *Ficus religiosa* extracts.

S.N.	Name of the plant	Concentration (µg/ml)	DPPH scavenging %	IC50 values (µg/ml)
1.	*Elaeocarpus sphaericus*	50	30.71	98.01
		100	58.66	
		150	65.33	
		200	76.21	
2.	*Ficus religiosa*	50	23.62	122.3
		100	52.36	
		150	61.21	
		200	65.74	
3.	Ascorbic acid	50	45.66	62.71
		100	63.21	
		150	75.63	
		200	94.09	

Antibiotic susceptibility test

For performing the antimicrobial susceptibility test, gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and gram-negative bacteria (i.e.: *Escherichia coli* and *Pseudomonas aeruginosa)* were used. Following the incubation, zone of inhibitions was observed. The measurements of the zone of inhibition is presented in table 3.

Table 3: Antibiotic susceptibility test

Name of organisms		Peepal *(Ficus religiosa)*				Rudraksha *(Elaeocarpus sphaericus)*				Chloramphenicol (CPL) 30 µg Disk
		50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml	50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml	
Escherichia coli	**Gram negative**	2.4±0.04	4.0±0.08	5.1±0.28	7.0±0.20	2.7±0.0	4.3±0.16	5.5±0.0	7.2±0.08	20 mg/ml
Pseudomonas aeruginosa		1.6±0.08	3±0.08	3.7±0.00	4.5±0.04	2.3±0.16	3.9±0.16	4.6±0.08	5.7±0.04	15 mg/ml
Staphylococcus aureus	**Gram positive**	2.4±0.0	3.6±0.08	4.8±0.04	5.5±0.0	3.2±0.21	3.6±0.12	4.8±0.04	5.8±0.0	18 mg/ml
Bacillus cereus		2.5±0.09	4.2±0.21	5.1±0.1	65.9±0.12	3.0±0.16	4.0±0.09	5.2±0.16	6.4±0.1	22.5 mg/ml

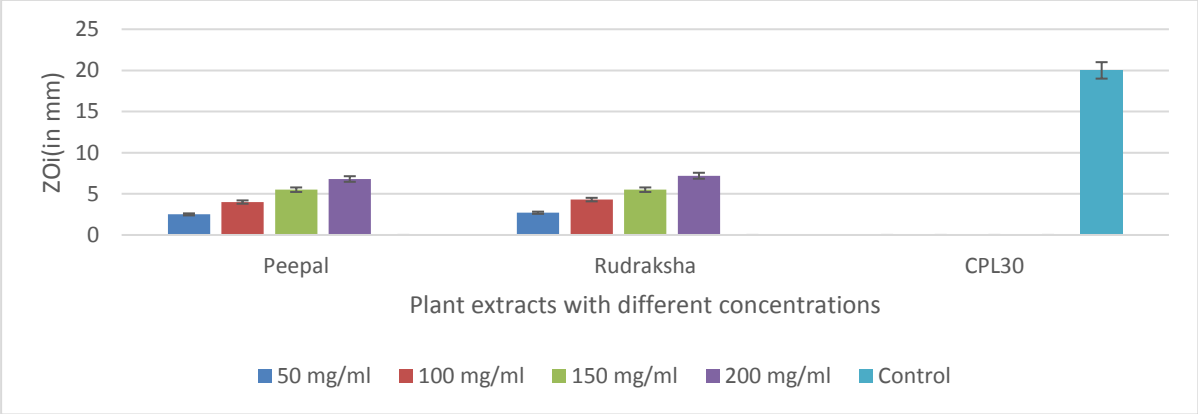

Figure 2: Antimicrobial assay against *Escherichia coli*

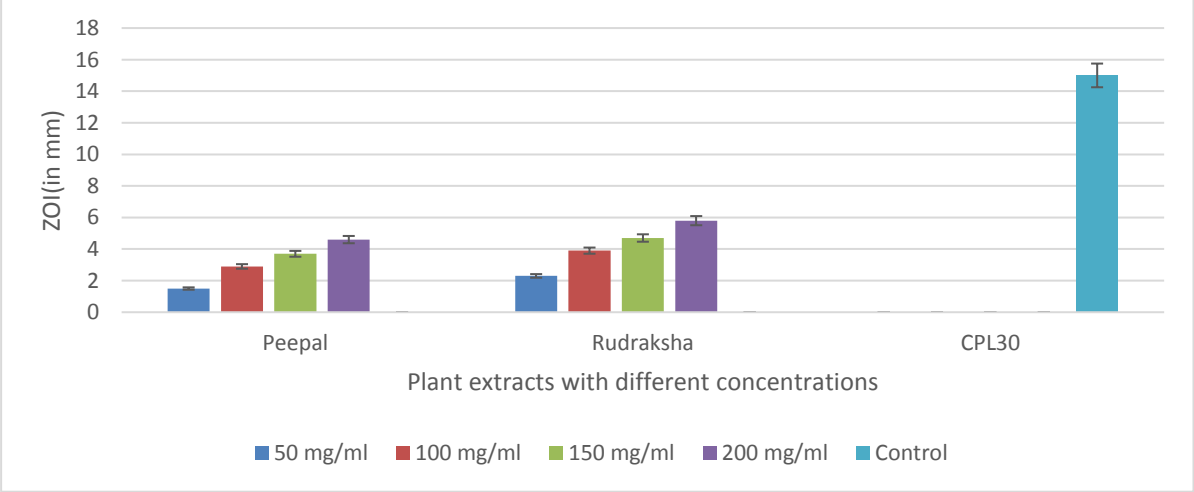

Figure 3: Antimicrobial assay against *Pseudomonas aeruginosa*

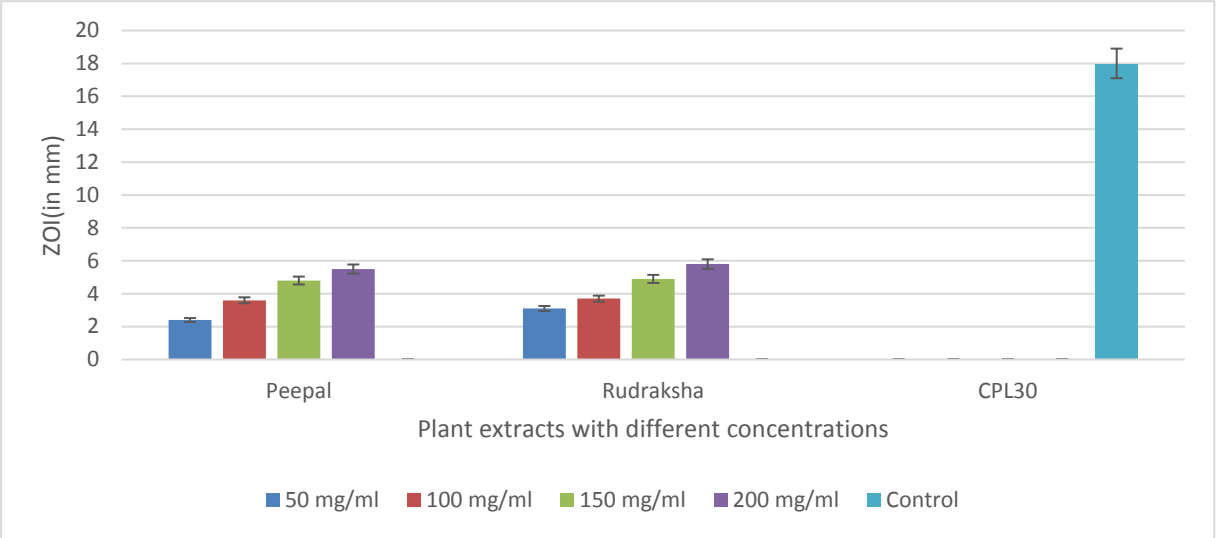

Figure 4: Antimicrobial assay against *Staphylococcus aureus*

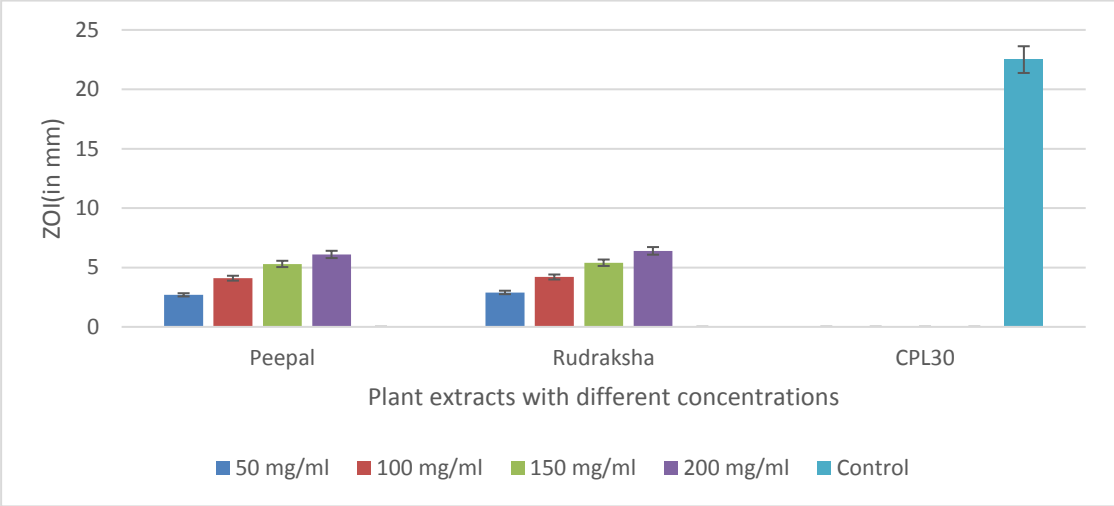

Figure 5: Antimicrobial assay against *Bacillus cereus*

Phytochemicals in extracts

Both Peepal and Rudraksha extracts were found to be positive in Flavonoids, Terpenoids, Cyclic glycosides, tannins, volatile oils, tannins, flavonoids, phenols, anthraquinone, glycosides, alkaloids, steroids, and reducing sugars. Saponins were found to be positive only in Peepal extracts. Likewise, Phlabotannin and Proteins were found to be negative for Peepal extracts. The results are presented in table no. 4.

Table 4: Qualitative tests for phytochemicals

Phytochemicals	**Methanolic plant extracts**	
	Peepal	**Rudraksha**
Terpenoids	+	+
Saponins	+	-
Volatile oils	+	+
Cyclic glycosides	+	+

Tannins	+	+
Flavonoids	+	+
Phenols	+	+
Phlabotannin	-	+
Proteins	-	+
Anthraquinone	+	+
Glycosides	+	+
Alkaloids	+	+
Steroids	+	+
Reducing sugars	+	+

(+ denote presence and – denote absence)

Figure 6: *Ficus religiosa* (Peepal)

Figure 7: *Elaeocarpus sphaericus* (Rudraksha)

*Elaeocarpus sphaericus (*Rudraksha) and *Ficus religiosa* (peepal) can be found widely in South Asian countries. They are enriched with various phytochemicals and can be used as a medicine in various ailments (Weli *et al*., 2018). In this present study, the leaves extracts of these two plants were used for determination of Antioxidant, Antibacterial properties along with phytochemicals. Table 1 describes the physical characteristics of the methanolic extracts of *Elaeocarpus sphaericus (*Rudraksha) and *Ficus religiosa* (peepal). Peepal extract was reddish-brown, sticky, semi-solid with a 20% yield (15 grams of extract) of total dry weight. Likewise, Rudraksha gave brownish colored, sticky, semi- with 25% yield (20 grams of extract) of total dry weight.

Table 2 determines the level of antioxidants in the plant extracts. Antioxidant activity was determined by the DPPH scavenging assay. Two different samples were used i.e. Rudraksha and Peepal. They gave the lowest absorbance at the concentration of 200 µg/ml which shows the greatest antioxidant activity. The DPPH Scavenging % was highest at 200 µg /ml i.e. 76.21% and 65.74 % in *Elaeocarpus sphaericus* and *Ficus religiosa* respectively. Ascorbic acid was used as a standard and DPPH as a control. The antioxidant activity of

the plant extracts was lesser than that of the standard (ascorbic acid). DPPH is a free radical which is reduced by antioxidants. The reduction of DPPH by the sample determines the antioxidants present in the sample, and on reduction, DPPH color is decolorized. The discoloration of the DPPH is measured by the spectrophotometric analysis at 517nm. The radical scavenging assay of a sample is inversely proportional to the absorbance (Kumar *et al*., 2008; Bhandari *et al*., 2019)**.** Antioxidants help to protect against diseases like cancer, dementia, Alzheimer's disease as well as arthritis. They can also be used in food, anti-aging cosmetics, degradation of rubber as well as gasoline prevention (Hassan *et al*., 2020). Therefore, the research indicates that the extracts of the plant leave help in reducing the radical series reaction and are proved to be better sources of antioxidants (Reddy and Vadde, 2021).

As per the research performed by Bhatt and Dahal (2019), various phytochemicals tests like alkaloids, carbohydrates, saponins, terpenoids, etc. along with antimicrobial tests were performed. The extracts showed activity against various bacteria i.e. *E. coli*, *S.* Typhi, and *S. aureus*, and even showed maximum inhibition of 20 mm. Likewise, the seeds showed radical scavenging activity of 28.09 µg/ml. The zone of inhibition was greater compared to this research along with the antioxidants level. This might be because of differences in the protocols and extraction methods of the samples. As per another study performed by Marisetti and Kollie (2016), the IC50 value was found to be around 72 to 78 µg/ml which is lower than the obtained value of this research. These changes can be because of the difference in the geographical locations, as well as the different procedures and methods, climate changes, and the season of sample collection.

The antimicrobial test was performed at 4 different concentrations on MHA i.e. 50, 100, 150, 200 mg/ml respectively. The zone of inhibition was observed after the incubation of MHA plates. According to Gautam *et al*. (2014), Peepal showed antimicrobial activity in the strains of *E. coli* with a 24 mm zone of inhibition along with high antioxidant activity but in this research performed, there was less antioxidant capacity shown by *F. religiosa*. Likewise, in the research conducted by Prakash *et al.* (2017), the leaves of *F. religiosa* showed antimicrobial activity against *E. coli* and showed a zone of inhibition of 10 mm and 12 mm respectively. The zone of inhibition was comparatively less in this research performed. Additionally, the methanolic extracts showed antimicrobial activity against two different microorganisms i.e. *P. aeruginosa* and *S.* Typhi. Likewise, extracts of *Sphaericus ganitrus* Roxb. showed activity against *S. aureus, Bacillus cereus,* and *Pseudomonas* (Khan *et al*., 2004; Sakat *et al*., 2009; Hemaiswarya *et al*., 2009; Kumar *et al*., 2011). In a study performed by Chavan et al. (2019), the Peepal leaves extracts were subjected to screening and were found to be possessing antimicrobial properties against the bacteria i.e. *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*,

and *Staphylococcus aureus*. They also showed the presence of phytoconstituents like glycosides, terpenoids, alkaloids, tannins etc.

The results observed in table 4 determine the phytochemicals. Both Peepal and Rudraksha extracts were found to be positive in Flavonoids, Terpenoids, Cyclic glycosides, tannins, volatile oils, tannins, flavonoids, phenols, anthraquinone, glycosides, alkaloids, steroids, and reducing sugars. phenols except for Saponins. Saponins were found to be positive only in Peepal extracts (Kumar *et al*., 2012). Tannins test, Cyclic glycosides, Flavonoids, and Terpenoids were positive for Peepal extract of a leaf using Methanol and the results observed were also positive as compared to the research. It was negative for Phlabotannins, and Proteins. The phytochemicals tests were also positive for Rudraksha extract except for Saponins which was similar to another research conducted (Singh *et al*, 2011). These phytochemicals are the bioactive compounds that are non –nutritive as well as have the ability of protection against any type of diseased conditions and can be used as lead sources for the discoveries of drugs as well. They have fewer side effects as compared to other synthetic drugs. These phytochemicals have proven health benefits. They help in the protection of the body against cancer cells. Saponins help for decreasing the level of lipid in blood, lowering the risk of cancer as well as help in lowering the blood glucose responses. The other phenolic compounds help for the preservation and protection of plants as well as help in aiding the immune system, protection of cells against damage, reduction of inflammations as well as regulation of hormones. They also help in preserving plants from the outer stresses and rodents (Shi *et al*., 2004).

Conclusion

The two samples Rudraksha (*E. sphaericus*) and Peepal (*F. religiosa*) showed antioxidants value 98.01 and 122.3 µg/ml respectively. Likewise, they showed zone of inhibition against all the four organisms that were used in the study i.e. gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa)* and are rich in phytochemicals Thus, both the plants are useful antioxidant and antibiotic sources along with phytochemicals and can be used in Ayurveda medications.

Conflict of interest statement

The authors have no conflict of interest regarding this study.

Author's contribution statement

Barsha Koirala designed the study and performed all the researches regarding the plant extracts, their phytochemical detection, antioxidants, and antimicrobial tests as well as analysis and interpretation of data.

Likewise, Evance Pakuwal and Hookman Jimi Rai helped to perform work in the lab and assist with the data analysis. Angela Shrestha supervised, checked, reviewed as well as revised the research and final manuscript. All the authors have read and approved this manuscript.

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