

Full Length Research Paper

Assessment of phytochemical content, antioxidant and antibacterial activities of three medicinal plants of Nepal

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Three indigenous medicinal plants, *Hibiscus rosa-sinensis* L., *Phlogacanthus thyriformis* Mabb. and *Lygodium japonicum* (Thunb.) Sw., have been investigated for their phytochemical constituents, antimicrobial and antioxidant activities. All the three plants tested were positive for polyphenols, terpenoids, glycosides, saponins, flavonoids and reducing sugar. The ethanol extract of these medicinal plants were subjected to evaluate their antibacterial properties against four gram negative (that is, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*) and two gram positive bacteria (that is, *Staphylococcus aureus*, *Bacillus subtilis*) by agar well-diffusion method. The ethanol extracts of the three plants prevented the growth of both gram-positive and gram-negative bacteria. The zones of inhibitions obtained ranges from 7 ± 0.17 to 18.3 ± 0.26 mm. Among these three plants extracts, *H. rosa-sinensis* was the most efficient against bacterial activity. Antioxidant activity of the extract was tested using scavenging activity of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical method. Based on the result obtained, *L. japonicum* was found to have the highest antioxidant activity ($IC_{50} = 80\pm 1.3$ $\mu\text{g/ml}$) followed by *P. thyriformis* ($IC_{50} = 127\pm 1$ $\mu\text{g/ml}$) and *H. rosa-sinensis* ($IC_{50} = 225\pm 1$ $\mu\text{g/ml}$). The results were compared with antioxidant activity of ascorbic acid ($IC_{50} = 54\pm 0.5$ $\mu\text{g/ml}$). This study thus suggests that these three plants have great pharmacological importance since they have potent biological activities.

Key words: Natural products, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* polyphenols, biological activity, agar well diffusion method.

INTRODUCTION

Nature has an enormous collection of natural products and secondary metabolites formed by living systems,

markedly from plant origin. Till date, thousands of medicines have been discovered from plants and these

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medicines are really effective in treating diseases from simple fever to life threatening diseases such as cancer, coronary heart diseases, diabetes and infectious diseases (Aruoma et al., 1989; Aruoma, 1998; Lai et al., 2010; Raphael et al., 2002; Reuter et al., 2010). Nowadays, oxygen-containing free radicals in biological systems and their indirect roles as causative agents in the variety of chronic disorders are no longer obscured (Jones, 2008). The role of antioxidant is renowned for inhibiting free radical in biological systems and preventing the prevalence of many chronic diseases and their prolong side effects (Sawa et al., 1999). There are many synthetic antioxidants but they have side effects, hence, there is a need for more potent, less toxic antioxidants (Branien, 1975; Ito et al., 1983).

It has been found that plants having polyphenolic compounds, such as flavonoids, possess antioxidant activity (Alho and Leinonen, 1999). Polyphenols have antioxidative properties which is due to their high reactivity as hydrogen donor or electron donor which stabilize and delocalize the unpaired electron. Because of their fewer side effects, natural antioxidants are gaining more attention and are studied extensively nowadays (Tanizawa et al., 1992; Duh, 1998; Ali et al., 2001; Candan et al., 2003). Moreover, the growing prevalence of multidrug resistance microbes due to haphazardly use of conventional antimicrobial drugs has urged the search for potential compounds from plants for therapeutic, medicinal, aromatic and aesthetic uses (Ng PC, 1994; Dean and Burchard, 1996).

Medicinal plants are rich sources of antimicrobial agents. Many infectious diseases have been known to be treated with herbal extracts (Brantner and Grein, 1994; Somchit et al., 2003; Lee et al., 2007; Newman and Cragg, 2007). The screening of plants for their phytochemical constituents is also a crucial initial step in determining the therapeutic potentiality of a medicinal plant. Phytochemicals are natural and non-nutritive bioactive compounds produced by plants that act as defending agents against external stress and pathogenic worry (Tepe et al., 2005). Based on their biosynthetic origin, there are several categories of phytochemicals, for example phenolics, alkaloids, steroids, terpenes and saponins. Phytochemicals could demonstrate different bioactivities such as antimutagenic, anticarcinogenic, antioxidant, antimicrobial, and anti-inflammatory properties (Okarter and Liu, 2010). Thus, the presence of such bioactive components like terpenoids, alkaloids and flavonoids has also raised the degree of usefulness of medicinal plants.

Nepal's geography comprises an exceptionally varied landscape and hence, this wide variation fosters an incredible variety of ecosystems. Though various medicinal plants are practiced as folk medicines since time immemorial, the investigation of those plants are not carried out very often (Baral and Kurmi, 2006; Rajbhandari, 2001). It had been reported that biological

properties of the plant is influenced by genetic, geographical, and seasonal factors as well as the developmental stages of the concerned plant, its parts/tissues (Kaura et al., 1998; Kaushik et al., 2007).

Therefore, taking account of these reports, here we have attempted to analyze phytochemical, antibacterial and antioxidant properties of three ethnomedicinal plants, namely, *H. rosa-sinensis* L., *Phlogacanthus thyriformis* Mabb. and *Lygodium japonicum* (Thunb.) Sw. of Nepal.

MATERIALS AND METHODS

Collection and processing of plants

Three plant species of ethnomedicinal values were selected for the analysis of their phytochemical constituents, antibacterial and antioxidant activities, based on the result of our previously reported research and available literatures (Subba et al., 2016; Ningombam and Singh, 2014; Obi et al., 1998). Taxonomic information, organs used for the analysis and traditional claims are presented in Table 1. The fresh flowers of *P. thyriformis* Mabb. and leaves of *L. japonicum* (Thunb.) Sw. with no apparent physical, insect or microbial damage were collected from Chanuwa VDC, Ward No. 8, Dhankuta, Nepal and were dried in sun shade. *H. rosa-sinensis* L.'s fresh flowers were collected from Purano Naikap, Kalanki, and Kathmandu Nepal and were dried in hot-air oven at 60°C. The plants were identified with the help of available literature (Hara and Williams, 1979; Hara et al., 1978) and authenticated by the taxonomist at Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal. The specimens were deposited in the National Herbarium and Plant Laboratories, Godavari (KATH). About 100 g of dried sample of each plant was ground to powder and exhaustively extracted with 600 ml ethanol using Soxhlet extractor and the extract was concentrated under reduced pressure using a rotary evaporator and then stored in an air tight container for further study.

Chemicals and standards

The chemicals used were Ethanol (Merck, Germany), DPPH and Ascorbic acid (Sigma Aldrich, USA). All other chemicals used were of the highest commercially available grade. For absorption measurement, double beam U-2800 UV-visible spectrometer, HITACHI, Japan, was used.

Phytochemical screening

The preliminary qualitative phytochemical analyses of the ethanolic extracts were carried out using standard procedures to identify the various groups of constituents (Hara and Williams, 1979; Culie, 1982; Alamzeb et al., 2013). Briefly, following procedures were used.

Test for alkaloids

About 3 ml of extract was stirred with 3 ml of 1% HCl on steam bath. 2 × 1 ml of mixture was taken separately and put in two test tubes. Few drops of Dragendorff's reagent were added in one tube and occurrence of orange red precipitate was taken as positive. In the second tube, Mayer's reagent was added and appearance of buff colored precipitate was taken as positive test for the presence of alkaloids.

Table 1. Medicinal plants tested for phytochemical content, antioxidant and antibacterial activities (Ningombam and Singh, 2014).

Species and part of the plant used	Family	Traditional claims*
<i>Hibiscus rosa-sinensis</i> L.(flowers)	Malvaceae	The plant is believed to act as anti-diabetic, expectorant, diuretic, cough cold and hair loss.
<i>Lygodium japonicum</i> (Thunb.) Sw. (leaves)	Lygodiaceae	Crushed leaves are used in fresh cuts and wounds. China people use this plant to treat hepatitis and dysentery.
<i>Phlogacanthus thyriformis</i> Mabb.(flowers)	Acanthaceae	The whole plant is believed to act as stimulant, astringent, aphrodisiac, diuretic, anti-dysenteric and antipyretic. Leaf juice is used in cough, asthma, rheumatism and high blood pressure.

Test for flavonoids

Ammonia solution 0.1N (5 ml) was added to the aqueous filtered fraction of each sample followed by the addition of concentrated H₂SO₄. A yellow coloration that disappears on standing indicated the presence of flavonoids.

Test for terpenoids (Salkowski test)

Each extract (0.5 g) was added into a test tube containing 2 ml of chloroform. Concentrated H₂SO₄ (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

Test for polyphenols

Crude extract was mixed with 2 ml of 2% solution of FeCl₃. A blue-green or black coloration indicated the presence of phenols.

Test for quinones

A small amount of extract was treated with concentrated HCl and observed for the formation of yellow color precipitate, indicating the presence of quinones.

Test for glycosides (Molisch's test)

Crude extract was mixed with 2 ml of Molisch's reagent and the mixture was shaken properly. After that, 2 ml of concentrated H₂SO₄ was poured carefully along the side of the test tube. Appearance of a violet ring at the interphase indicated the presence of carbohydrate.

Test for reducing compounds (Fehling's test)

Equal volume of Fehling A and Fehling B reagents were mixed together and 2 ml of the mixture was added to crude extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

Test for saponins

Powered sample (1 g) was boiled in 10 ml of distilled water in a water bath and filtered. 5 ml of the filtrate was mixed with 2 ml of distilled water and shaken vigorously and observed for a stable persistent froth.

Test organisms

The clinical isolates of pathogenic bacteria of gram negative *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella Typhi*, *Proteus mirabilis* and gram positive *Bacillus subtilis* and *Staphylococcus aureus* were obtained from the laboratory of Department of Microbiology, Teku Hospital, Kathmandu. They were then maintained on agar plates and were stored at 4°C in refrigerator.

Antibacterial assay

Inhibition of bacterial growth was tested by using the agar well diffusion method (Dingle et al., 1953). About 100 µl suspension of tested microorganisms was spread on Muller-Hilton Agar (MHA) medium. Wells were made on the agar plates using the sterile cork borer (6 mm in diameter). Ethanol extracts of different concentrations (0.3 and 0.6 mg/well) were loaded into the wells along with

solvent dimethyl sulfoxide (DMSO). A broad spectrum standard antibiotic tetracycline 0.3 mg/well was used as a positive control and DMSO as a negative control. The plates were then incubated for 24 h at 37°C. After incubation, the growth inhibition rings were quantified by measuring the diameter of the zone of inhibition in mm (from the edge of the well). All tests were performed in triplicate.

DPPH radical scavenging activity (RSA) assay

The free radical scavenging activity of samples and standard ascorbic acid solution in ethanol was determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH·) free radical method, proposed by Blois (1958). The plant samples at various concentrations (15 to 250 µg/ml) were added to a 100 µM solution of DPPH in ethanol. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The measurement was performed in triplicates. The antioxidant activity of the samples was expressed as IC₅₀ (inhibitory concentration), which was defined as the concentration (µg/ml) of sample required to inhibit the formation of DPPH radicals by 50%. Ascorbic acid was used as positive control. Free radical scavenging activity was calculated by using the following equation:

$$\text{Free radical scavenging activity (\%)} = (A_0 - A_T) / A_0 \times 100$$

where A₀ = Absorbance of DPPH solution and A_T = Absorbance of test or reference sample after 30 min of incubation. The % scavenging was then plotted against concentrations used and from the graph IC₅₀ was calculated.

Statistic

The results are expressed as mean \pm Standard deviation (SD).

RESULTS AND DISCUSSION

Screening of phytochemicals

As phytochemicals often play an important role in plant defense against prey, microorganism, stress as well as interspecies protections, these plant components have been used as drugs for millennia (Mandal et al., 2010; Rao, 2003). Hence, phytochemicals screening serves as the initial step in predicting the types of potential active compounds from plants. Table 2 shows the results of phytochemical analysis of all plant extracts.

The results showed that the polyphenol compounds were found in all the three plant extracts. Terpenoids and glycosides were observed in *P. thyriformis* and *L. japonicum*. Saponin was presented only in *H. rosa-sinensis*. Our results are in agreement with Bhaskar et al. (2011) who stated that ethnolic fraction of *H. rosa-sinensis* flower had saponins but not with Afify and Hassan (2016). Flavonoids were present in *P. thyriformis* and reducing sugars was found to be present in both *H. rosa-sinensis* and *L. japonicum*. In contrast, alkaloids and quinones were found to be absent in all the plant extracts. The compounds like flavones, phenolic acids and steroidal glycosides had been reported for ethanolic root extract of *L. japonicum* and other genus of family Lygodiaceae (Chen et al., 2010; Song et al., 2001; Zhang et al., 2006). Phytochemical like tannins, flavonoids, saponins, gums, carbohydrates, steroids, alkaloids, reducing sugar, and terpenoids have been reported in the leaves of *P. thyriformis* (Das et al., 2015). Similarly phytochemicals like tannin, phlobatannins, cardiac glycosides, flavonoids, terpenoids, saponins and others are reported to be determined in leaves, stems and roots, and additionally anthocyanins in flowers of *H. rosa-sinensis* (Patel et al., 2012; Gauthaman et al., 2006, Bhaskar et al., 2011).

Phytochemical investigation of ethanolic extract of three different plant extracts revealed the phytochemical constituents that are widely considered to have medicinal and pharmacological effects. The presence of polyphenols and flavonoids, widely known for their antioxidant properties, indicates that these plants are probably endowed with multiple biological effects including anti-inflammatory and anticancer activities. The presence of terpenoids indicates a wide range of biological activities against cancer, malaria, inflammation and variety of infectious disease. The presence of glycosides contributes to the wide range of biological activities like antioxidant, antipyretic, and anti-inflammation. The presence of saponins, acting as antioxidant and anti-inflammatory agents, is useful in lowering cholesterol, but also in bone health and stimulation of

immune system (Lacaille-Dubois and Wagner, 2000; Traore et al., 2000; George et al., 2002). Use of *H. rosa-sinensis* as ingredient of shampoo is well supported by the presence of saponin in phytochemical screening. The observed pharmacological properties of these plants possibly have been attributed to the presence of flavonoids, terpenoids, saponins, and tannins. The results obtained here are in good correlation with previously reported results (Gauthaman et al., 2006).

Antioxidant activity by DPPH assay

The ethanol extracts of *P. thyriformis*, *L. japonicum* and *H. rosa-sinensis* were assessed for free radical scavenging activity. The results obtained shows that the extracts of *L. japonicum* exhibited the highest radical scavenging activity, with inhibitions of DPPH radical starting from 59.006% at concentration of 100 $\mu\text{g/ml}$ to 85.86% at concentration of 500 $\mu\text{g/ml}$, followed by *P. thyriformis* starting from 72.98% at concentration of 200 $\mu\text{g/ml}$ to 81.05% at concentrations of 500 $\mu\text{g/ml}$. *H. rosa-sinensis* showed the lowest radical scavenging activity, with DPPH radical inhibitions starting from 67.39% at concentration of 300 $\mu\text{g/ml}$ to 79.81% at concentration of 500 $\mu\text{g/ml}$ (Figure 1). The anti-oxidant activities of three plants extracts were consistent with the gradual increment of concentration. The IC_{50} was calculated from the graph obtained by plotting the % of scavenging against concentrations used (Figure 1). The IC_{50} values were found to be 80 ± 1.3 $\mu\text{g/ml}$ for *L. japonicum*, 127 ± 1 $\mu\text{g/ml}$ for *P. thyriformis* and 227 ± 1 $\mu\text{g/ml}$ for *H. rosa-sinensis*. However, all extracts were found to be less active than ascorbic acid (AA) with IC_{50} values 54 ± 0.5 $\mu\text{g/ml}$, a standard antioxidant drug (Figure 2). Although the promising free radical scavenging property of *P. thyriformis* has been reported (Upadhyay, 2009), it has not been obtained in this research. However, the strong antioxidant property observed in this experiment for *L. japonicum* is well supported by result of phytochemical analysis, indicating the presence of constituents like flavones and phenolic acids, being reported as strong antioxidants previously (Song et al., 2001). In contrast to the results obtained here, the total antioxidant capacity of *H. rosa-sinensis* extract has been reported nearly two fold higher than that of butylated hydroxytoluene (BHT) (Faten et al., 2012). This may be attributed to the fact that biological properties of the plant is influenced by genetic, geographical, seasonal factors, the developmental stages of the plant, its parts/tissues as well as the type of solvent used in extraction procedures (Kaura et al., 1998; Kaushik et al., 2007).

Antibacterial activity of plant extracts

In recent years, the research on searching phytochemicals possessing antimicrobial properties have been

Table 2. Phytochemical constituents of the analyzed species.

Species	Reducing compounds	Glycosides	Polyphenols	Quinones	Alkaloids	Flavonoid	Terpenoids	Saponin
<i>H. rosa-sinensis</i>	+	-	+	-	-	-	-	+
<i>P. thyriformis</i>	-	+	+	-	-	+	+	-
<i>L. japonicum</i>	+	+	+	-	-	-	+	-

Table 3. Antibacterial activities of ethanol extract of the test plants.

Species	Bacteria	Mean Zone of Inhibition (mm)*			DMSO
		Plant extract		Tetracycline	
		0.6 (mg/well)	0.8 (mg/well)	0.3(mg/well)	
<i>H. rosa-sinensis</i> L.	<i>B. subtilis</i>	NA	NA	12.4 ± 0.36	NA
	<i>S. aureus</i>	13.0 ± 0.32	16.4 ± 0.4	26.5 ± 0.5	NA
	<i>E. coli</i>	16.3 ± 0.2	18.3 ± 0.26	26.3 ± 0.26	NA
	<i>S. typhi</i>	14.2 ± 0.25	16.2 ± 0.25	11.3 ± 0.20	NA
	<i>K. pneumonia</i>	8 ± 0.5	11 ± 0.5	NA	NA
	<i>P. mirabilis</i>	12.5 ± 0.5	14.8 ± 0.7	24 ± 0.5	NA
<i>P. thyriformis</i> Mabb.	<i>B. subtilis</i>	10.1 ± 0.3	12 ± 0.5	NA	NA
	<i>S. aureus</i>	8 ± 0.5	11.2 ± 0.25	14.3 ± 0.3	NA
	<i>E. coli</i>	8 ± 0.5	11.2 ± 0.25	16 ± 0.5	NA
	<i>S. typhi</i>	NA	NA	16 ± 0.5	NA
	<i>K. pneumonia</i>	NA	NA	NA	NA
	<i>P. mirabilis</i>	NA	NA	20 ± 0.5	NA
<i>L. japonicum</i> (Thunb.) Sw.	<i>B. subtilis</i>	7 ± 0.17	9 ± 0.25	11 ± 0.5	NA
	<i>S. aureus</i>	NA	NA	16 ± 0.5	NA
	<i>E. coli</i>	NA	NA	16 ± 0.5	NA
	<i>S. typhi</i>	7 ± 0.35	8 ± 0.35	16 ± 0.5	NA
	<i>K. pneumonia</i>	NA	NA	NA	NA
	<i>P. mirabilis</i>	9.3 ± 0.32	10 ± 0.5	26 ± 0.5	NA

Diameter of the well is 6 mm. Mean zone inhibition are expressed as means ± SD (n = 3) in millimeter (mm). NA: Not active.

in demand due to their potential use in the therapy of various chronic and infectious diseases. In addition, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes (Davies, 1994; Harbottle et al., 2006). The results of antimicrobial screening of the ethanol extracts of three plants *H. rosa-sinensis*, *P. thyriformis* and *L. japonicum* are presented in Table 3. The antimicrobial activities of all the plant extracts against the six bacteria strains examined were assessed by the presence or absence of inhibition zones.

The result of antibacterial sensitivity test reveals that all the extracts have antibacterial activity against both gram positive and gram-negative bacteria. In addition, among the three plant extracts, *H. rosa-sinensis* showed the high antibacterial activity against *E. coli*, *S. typhi*, *S. aureus*, *K.*

pneumoniae and *P. mirabilis* with zone of inhibition ranging from 8 to 18 mm (Figure 3). Promising antimicrobial effect of flower of *H. rosa-sinensis*, higher than that of leaves, have been reported against *S. aureus*, *E.coli*, *B. subtilis*, *S. typhimurium* and *Salmonella* species (Uddin et al., 2010). The plant extracts of *P. thyriformis* showed the antibacterial activity against *B. subtilis*, *S. aureus*, *S. typhi* and *E. coli* with zone of inhibition ranging from 8 to 14 mm (Figure 4). Antimicrobial activity of *P. thyriformis* had also been reported previously (Singh et al., 2010), whereas bacterial strains (*B. subtilis*, *Salmonella Typhi*, *P. mirabilis*) are susceptible to the extract of *L. japonicum* with zone of inhibition ranging from 7 to 10 mm (Figure 5). Moderate antimicrobial activity had previously been reported for *L. japonicum* against *K. pneumoniae*, *S. aureus*, *Bacillus*

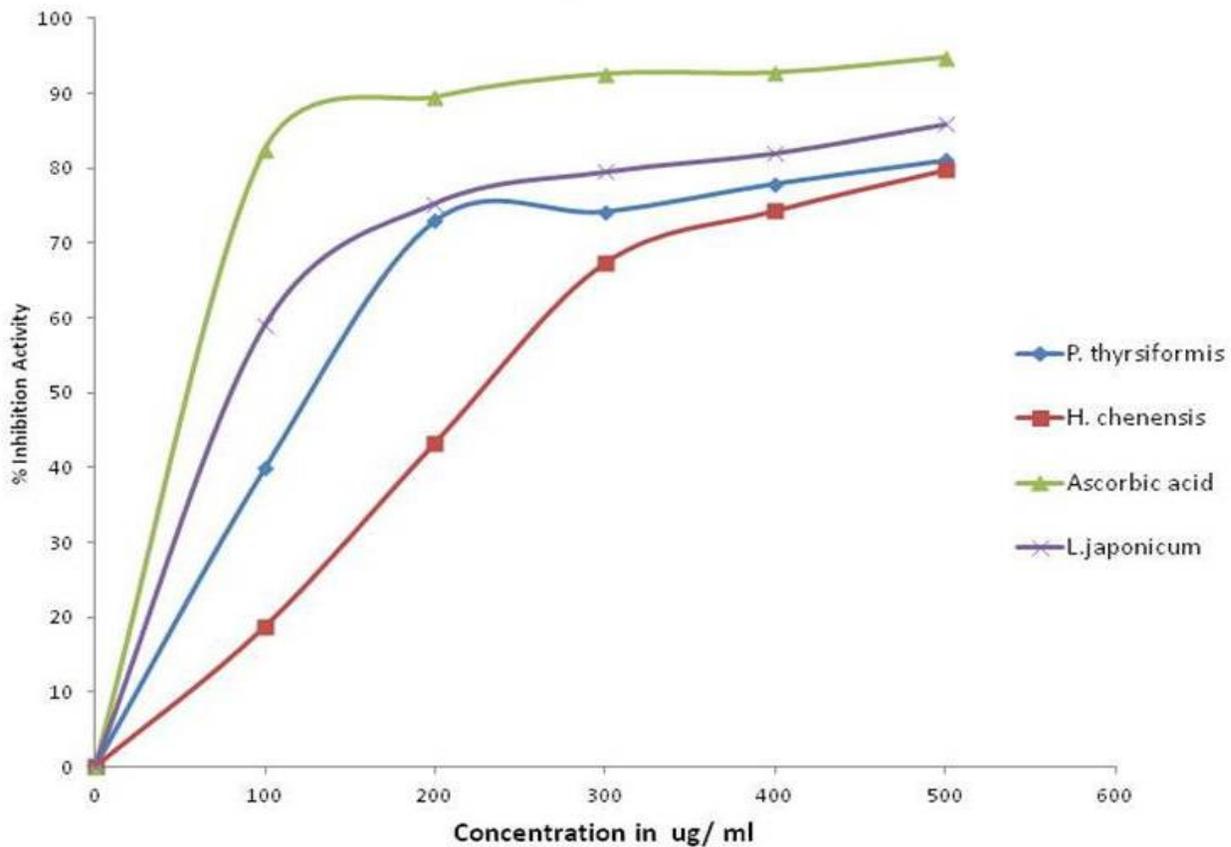


Figure 1. Antioxidant properties of analyzed species with standard ascorbic acid.

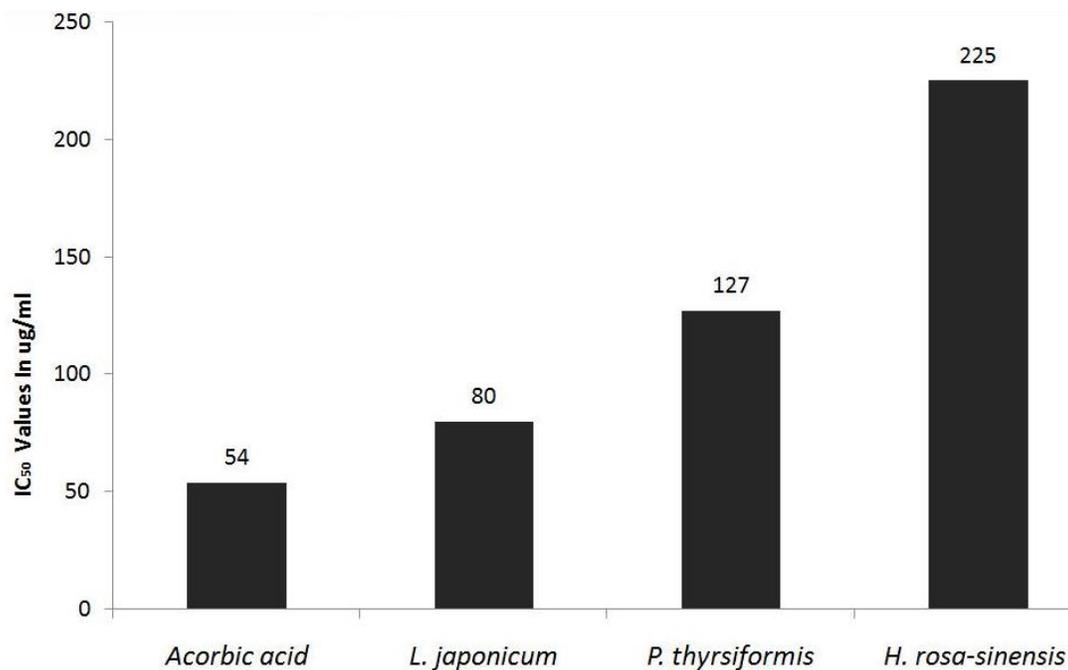


Figure 2. The IC₅₀ values of plant extracts on DPPH (lower values indicates more powerful antioxidant capacity).

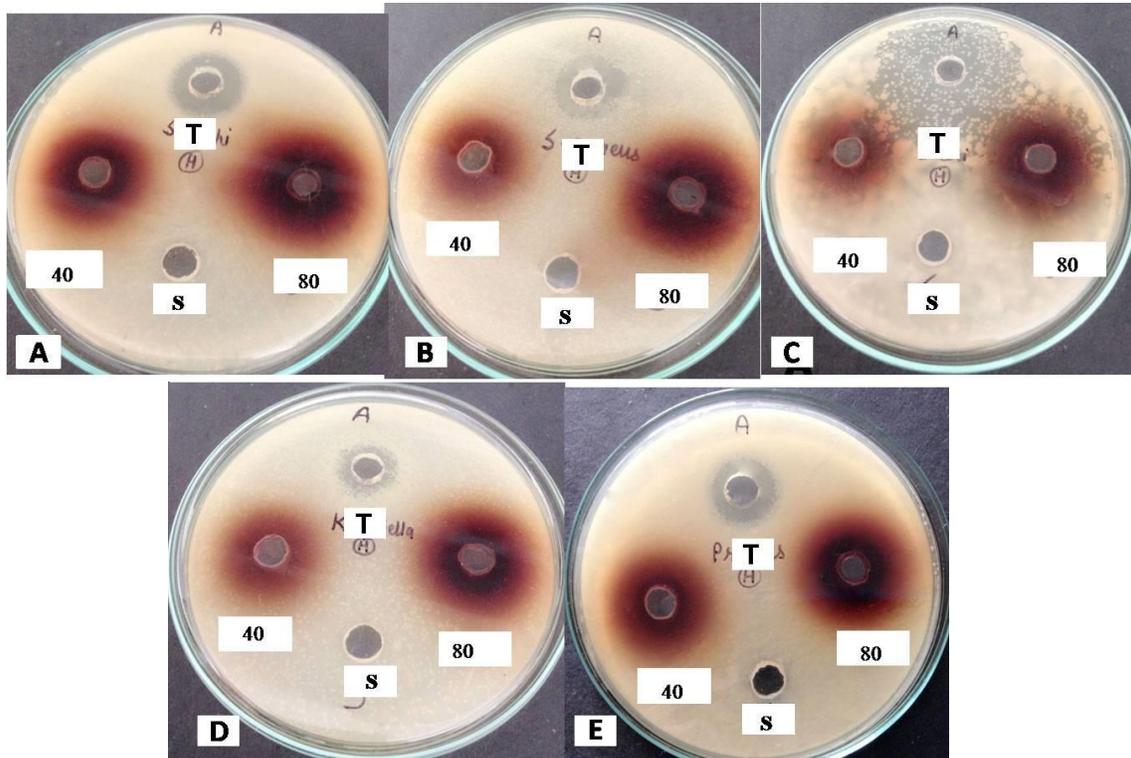


Figure 3. Agar well diffusion test demonstrating inhibition zones by *H. rosa-sinensis* flower extract, where; A: *S. typhi*, B: *S. aureus*, C: *E. coli*, D: *K. pneumoniae*, E: *P. mirabilis*, S: DMSO, T: Tetracycline 0.3 mg/well, and 40, 80: Plant extract at concentration of 0.3 and 0.6 mg/well, respectively.

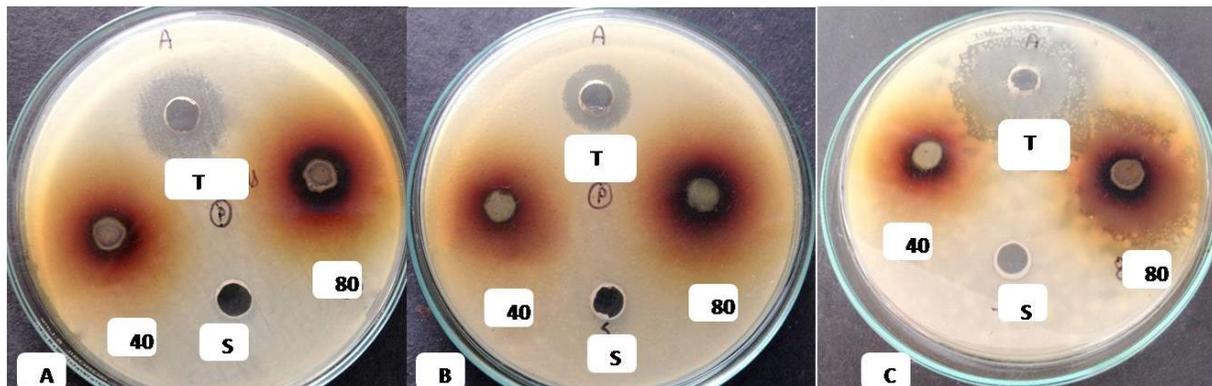


Figure 4. Agar well diffusion test demonstrating inhibition zones by *P. thyriformis* flower extract, where; S: DMSO, T: Tetracycline 0.3 mg/well, 40, 80: Plant extract at concentration of 0.3 and 0.6 mg/well, respectively, A: *B. subtilis*, B: *S. aureus* and C: *E. coli*.

cereus, *Vibrio cholerae* and *Candida albicans* (Vashist and Jindal, 2012).

The study of antibacterial activities in the three plants indicates the potential effect against both gram positive and gram negative bacteria. Among the three plant extracts *H. rosa-sinensis* was the most effective while antibacterial effect of two other extracts were lower. Although, they were effective against different strains,

this research posits that their effectiveness was rather similar to each other.

CONCLUSION

Based on the results of the present study, the ethanolic extract of *L. japonicum* with moderate antimicrobial

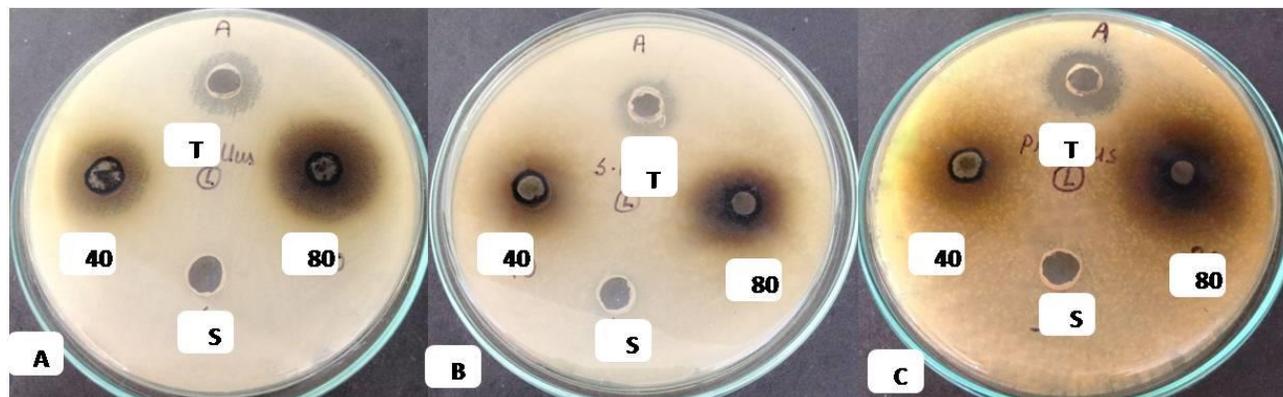


Figure 5. Agar well diffusion test demonstrating inhibition zones by *L. japonicum* leaves extract. Where S: DMSO, A: Tetracycline 0.3 mg/well, 40, 80: Plant extract at concentration of 0.3 and 0.6 mg/well, respectively A: *B. subtilis*, B: *S. typhi*, C: *P. mirabilis*.

activity showed the strongest antioxidant activity. On the other hand, the extract of *H. rosa-sinensis* with the highest antimicrobial activity showed the lowest antioxidant activity. This suggests that compounds responsible for the antimicrobial activity and antioxidant activity in these plants are not same. These results highlight the biological activities of *P. thyriformis*, *L. japonicum*, and *H. rosa-sinensis* extracts and give scientific support to their traditional use in folklore medicine for the treatment of several ailments. In conclusion, our findings showed that *L. japonicum* and *H. rosa-sinensis* have the potential to be explored further to identify the antioxidative and antibacterial compounds in these plants, respectively. It was concluded that further bioassay-guided fractionation approaches will be required on these species to isolate the bioactive compounds responsible for their promising antioxidant and antimicrobial activity.

Conflict of interests

The authors have not declared any conflict of interests.

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