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# Determination of Phenolic and Flavonoid Content, Antioxidant and α-Amylase Inhibitory Activity of Leaf and Flower Extracts of Clerodendrum infortunatum and Hibiscus rosa sinensis Growing in Bara Nepal

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## ABSTRACT

In the human body, oxidative stress imposed due to the increase in the concentration of free radicals such as reactive oxygen species (ROS) contributes to the initiation and progression of different diseases. The synthetic antioxidants have low efficacy with side effects, for that plant-derived natural antioxidant can prevent oxidative stress by reducing the risk of having these diseases. In the present study, the methanolic extracts of two traditionally used medicinal plants (leaf and flower) have tested for their antioxidant capacity, α-amylase enzyme inhibitory activity and determination of total phenolic and flavonoid content in the plant extracts. The antioxidant activity was evaluated by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The highest DPPH radical scavenging was shown by *Clerodendrum infortunatum* ($IC_{50}$ = 71.95±0.51µg/mL) whereas leaf and flower of *Hibiscus rosa sinensis* showed moderate antioxidant activity ($IC_{50}$ = 98.74±1.91 and 117.23±7.72 µg/mL) respectively. Among the tested plant extracts, the highest amount of total phenolic and flavonoid content was found in the methanolic extract of *C. infortunatum* with TPC of 87.07±9.22 mg GAE/g and TFC 34.40±2.00 mg QE/g. The antidiabetic activity of plant extracts was evaluated by the α-amylase enzyme inhibition assay. The leaf extract of *C. infortunatum* showed moderate □-*amylase inhibition activity (*$IC_{50}$ = 118±4.80 µg/mL) whereas the standard acarbose has ($IC_{50}$ = 12.96±0.22 µg/mL).

**Keywords:** Oxidative stress, antioxidant, Hibiscus rosa sinensis, Clerodendrum infortunatum, phenols, flavonoids

## Introduction

Varieties of free radicals are generated in the human body due to exposure to different environmental conditions. The reactive oxygen species such as hydroxyl ion, hydrogen peroxide, superoxide, singlet oxygen species and so on are normally generated in the cells during metabolism. These reactive oxygen species may cause severe oxidative damage to proteins, lipids, enzymes and DNA with subsequent tissue injury. The antioxidant compounds play a significant role to scavenge the reactive free radicals by the termination of chain reaction (Saeed et al.2012).

The free radicals generated in our body may cause several diseases such as cancer, neurodegeneration and inflammation. The natural antioxidant found in plants as secondary metabolites (phenolics, flavonoids, tannins and proanthocyanidins) may protect against several diseases (Gulcin, 2012). In this way, medicinal plants could be good sources of such antioxidant compounds that can also be used as food preservatives (Peschel et al. 2006).

It has been reported that most medicinal plants which are rich sources of phenolic and flavonoid compounds are also potent antioxidants and α-amylase enzyme inhibition (Shoib et al. 2015). Phenolic and flavonoid compounds are widespread in medicinal plants where they act as free radical scavengers and □-*amylase enzyme inhibitors (*Shoib et al. 2015). Free radicals are generated in the cells of the human body known to be involved in many acute and chronic disorders such as diabetes, ageing, immunosuppression and neurodegeneration (Harman, 1998). The use of antioxidants contributes to the protection from diseases and reduces morbidity and mortality from degenerative disorders (Gulcin, 2012).

Alpha-amylase is a prominent enzyme found in the pancreatic juice and saliva which breaks down the large insoluble starch molecules into absorbable molecules. On the other hand, mammalian α-glucosidase in the mucosal brush border of the small intestine catalyzes the end step of digestion of starch and disaccharides that are abundant in the human diet. Inhibitors of α-amylase and α-glucosidase delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion (Kazeem et al. 2013). An efficient means of lowering the levels of postprandial hyperglycemia has been offered by α-amylase and α-glucosidase

inhibitors. Several inhibitors of α-glucosidase have been isolated from medicinal plants to serve as an alternative drug with increased potency and lesser adverse effects than existing synthetic drugs (Poovitha and Parani, 2016).

The plant *Hibiscus rosa sinensis* is a perennial shrub have been used as an ethnomedicine for the treatment of various diseases such as hypertension, wound healing, skin diseases, hair diseases, diabetes mellitus and cancer. The plant is reported as a good source of secondary metabolites such as polysaccharides, anthocyanins, flavonoids, polyphenols, pectin and organic acids (Dahia and Kaur, 2009). The plant *Clerodendrum infortunatum* Linn. is commonly known as bhat or hilly glory bower. It is reported that the plant is one of the most well-known natural health remedies in traditional practices and Siddha medicine (Rajurkar, 2010). Fresh leaves of the plants have been used for the treatment of diarrhoea, liver disorder and headache (Duke, 2010). The leaf and root of the plant have been reported to be used as antidandruff, antipyretics, ascaricide, antidiabetic, antimalarial, and skin diseases (Duke, 2010).

Although these two medicinal plants are widely used in traditional medicine, few studies have been conducted on the biological activities of the plants. The phenolic and flavonoid compounds are widespread in the plants where they act as an antioxidant and α-amylase inhibition activity. The main objective of this research was to analyze the phytochemicals present in plant extracts, determine the total phenolic and flavonoid content, evaluate the antioxidant activity and α-amylase inhibition activity of leaf and flower extracts of *Clerodendrum infortunatum* Linn. and *Hibiscus rosa sinensis* growing in Bara district of Nepal.

*Clerodendrum inf ortunatum* Linn

*Hibiscus rosasinensis*

**Figure 1: Photographs of plant sample used in the study.**

## 2. Materials and Methods

### *2.1 Equipment*

The major equipment used in this research were a grinding mill, pipettes, micropipettes, weighing balance (Metler Toledo, ME 204), hot air oven (Yamato Scientific DF412), and rotatory evaporator (Buchi R200), water bath (Buchi R200), spectrophotometer. 96-micro-plate reader (Epoch2, BioTek, Instuments, Inc., USA)

### *2.2 Chemicals*

Most of the chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich, New Delhi, India. Some important Chemicals and reagents used in this study were methanol, acetone, hexane, dimethyl sulphoxide (DMSO), Folin-Ciocalteu's phenol reagent (FCR), gallic acid, quercetin, ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), $NaNO_2$, $AlCl_3$, and NaOH were of Sigma Aldrich. The reagents used during phytochemical analysis were prepared by using solvents of analytical grade and double-distilled water. The working solution of α-amylase and acarbose (Sigma Aldrich) in desired concentrations was prepared by successive dilutions of the corresponding stock solutions throughout the experiment.

### *2.3 Plant materials and preparation of extracts*

The leaves and flowers of *C. infortunatum* Linn. and *H. rosa sinensis* were collected in Bara district of Nepal. The plants were identified at the Central Department of Botany, Tribhuvan University. The leaves and flowers were dried in shade at room temperature, then chopped and ground to a fine powder in a mechanical grinder. The dried leaves and flower powder (100 g) were dipped in 80% methanol (300 mL) for 72 h with frequent shaking. The mixture was decanted and filtered. The filtrates were concentrated by a rotatory evaporator. The extracts were dried and stored at 4 $^{o}$C in storage vials for experimental uses.

### *2.4 Total phenolic content*

The total phenolic content of the extracts was determined by the Folin-Ciocalteu phenol method (Ainsworthi, 2007). Briefly, different concentrations of 10, 20, 30, 40, 50, 60, 70, and 80 µg/mL gallic acid solution as standard were loaded on 96 well pate reader in triplicate by diluting stock solution with distilled water. Then 20 µL of plant sample (500 µg/mL) was loaded on 96 well plates in triplicates. Then to each well 100 µL, Folin-Ciocalteu phenol was separately added followed by 80 µL $Na_2CO_3$. The mixture was kept in dark for 15 minutes and the absorbance was measured at 765 nm. The phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight (mg GAE/g) (Lu et al. 2011).

The total phenolic content in all the samples was calculated by using the formula:

$$C = \frac{cV}{m} \text{ ..... (1)}$$

Where, C = total phenolic content mg GAE/g dry extract, c = concentration pf gallic acid obtained from calibration curve in mg/mL, V= volume of the extract in mL and m = mass of extract in gram.

### *2.5 Total flavonoid content*

The total flavonoid content of the crude extract was determined by the aluminium chloride colorimetric method (Chang et al. 2002). In brief, the solution of different concentrations of quercetin was prepared and loaded in a 96-well plate reader (n=3). Then the 20 µL of plant extract (500 µg/mL) was loaded into each well of the 96-well plate reader maintaining the final volume of 130 µL. To each well 60 µL ethanol, 5 µL $AlCl_3$ and 5 *µL potassium acetate w*ere added separately. The mixture was kept in dark for 30 minutes and the absorbance was measured at 415 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg quercetin equivalent per g dry weight (mg QE/g).

### *2.6 Antioxidant properties*

The antioxidant activity of the plant extracts was evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as described earlier with a slight modification (Villano et al. 2007). In brief, the solution of plant extract and control (ascorbic acid) were mixed with DPPH solution and incubated in dark at room temperature for 1 h. The absorbance of the mixture was measured at 517 nm. The capacity of the plant extract to scavenge DPPH radical was determined from:

$$\text{DPPH scavenging effect (\% inhibition)} = \left(\frac{A_{Control} - A_{Sample}}{A_{Control}}\right) \times 100 \text{ ..... (2)}$$

Where $A_{control}$ = absorbance of control, $A_{sample}$ = absorbance of sample.

### *2.7 Alpha amylase inhibition*

The α-amylase inhibition was performed at 37 $^{o}$C in which 2-chloro-4-nitrophenyl α-D-maltotriose (CNPG3) was used as the substrate (Khadyat et al. 2020). In brief, 50 units of α-amylase was diluted to 3.75 unit by dissolving in 20 mM phosphate buffer (pH 6.95, containing 6.7 mM NaCl). The plant extracts dissolved in 50% DMSO and the solutions were prepared by serial dilution in 50% DMSO where acarbose was used as a positive control. In the 96 well plate reader, to the 80 µL of α-amylase 20 µL of plant extracts was added with a positive and negative control in triplicates (n = 3). The mixture was incubated at 37 $^{o}$C for 15 minutes followed by the addition of 100

µL substrate. The progress of the reaction was measured at 405 nm.

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100 \ldots\ldots\ldots\ldots\ldots (3).$$

The $IC_{50}$ was calculated by using Graph Pad Prism, a computer software.

## 3. Results and Discussion

### *3.1 Phytochemical analysis*

The results of the preliminary phytochemical analysis are shown in Table 1.

**Table 1: Phytochemical analysis of plant extracts.**

| S.N. | Group of compounds | $J_1$ | $J_2$ | $J_3$ |
|---|---|---|---|---|
| 1. | Alkaloids | - | + | + |
| 2. | Flavonoids | + | + | + |
| 4. | Coumarins | + | - | - |
| 5. | Glycosides | + | + | + |
| 6. | Polyphenol | + | + | + |
| 7. | Saponin | + | + | + |
| 8. | Tannins | - | + | - |
| 9. | Terpenes | + | + | + |
| 10. | Quinones | + | + | + |

+ = presence and - = absence

$J_1$ = *Clerodendrum infortunatum* leaf, $J_2$ = *Hibiscus rosa sinensis* leaf and $J_3$ = *Hibiscus rosa sinensis* flower.

The results showed that plant extracts are rich sources of plant secondary metabolites such as alkaloids, flavonoids, coumarins, glycosides, polyphenols, saponins, tannins, terpenes and quinones and the results seem to be more similar to the previously reported results (Hazarika and Saha, 2017, Tiwari et al. 2015). The few variations in the results may due to the different environmental conditions, methods and seasons of sample collection, and extraction procedures and also due to lab setup and chemical grades.

### *3.2. Phenolic and flavonoid content*

The total phenolic content of the methanolic leaf and flower extracts, calculated from the calibration curve ($R^2$ = 0.9746) was 87.07±9.22 to 54.75±1.93 mg GAE/g and the total flavonoid content ($R^2$ = 0.9853) was 34.40±2.00 to 14.11±2.08 mg QE/g (Table 2). The phenolic compounds present in the plant extracts have redox properties, which allow them to act as antioxidants (Soobrattee et al. 2005). Flavonoids including flavones, flavonols, and condensed tannins, are plant secondary metabolites and their antioxidant activity depends upon the presence of free OH groups. Flavonoids have antioxidant activity both *in vitro* and *in vivo* experiments (Geetha et al. 2003, Shimoi et al. 1996).

**Table 2: Total phenolic and flavonoid content of the plant extracts.**

| Plant extracts | Total phenolic content (mg GAE/g) | Total flavonoid content (mg QE/g) |
|---|---|---|
| *Clerodendrum infortunatum* leaf | 87.076±9.229 | 34.40±2.00 |
| *Hibiscus rosa sinensis* leaf | 68.034±2.406 | 24.98±1.69 |
| *Hibiscus rosa sinensis* flower | 54.752±1.938 | 14.11±2.08 |

Phenolic compounds have been known to possess high antioxidant properties due to their free radical scavenging properties. It has been reported that extract containing a large amount of polyphenol content possesses a greater antioxidant activity. The total flavonoid content in *Clerodendrum infortunatum* leaf was found lower than that of the previously reported value (64.56 mg QE/g) (Ghosh et al. 2014).

### *3.3 Antioxidant activity*

The results of DPPH radical scavenging against the different concentrations of plant extracts are shown in Figure 2. The antioxidant potential of plant extracts is expressed in $IC_{50}$ and the plant extracts having lower $IC_{50}$ show the greater antioxidant potential. The results of antioxidant activity in $IC_{50}$ are shown in Table 4. The plant extract of *Clerodendrum infortunatum* showed moderate antioxidant activity ($IC_{50}$ = 71.95±0.65 µg/mL) whereas the plant extracts of *Hibiscus rosa sinensis* leaf and *Hibiscus rosa sinensis* flower showed poor antioxidant activity. The moderate antioxidant activity of *Clerodendrum infortunatum* is due to the high content of total phenolics and flavonoids.

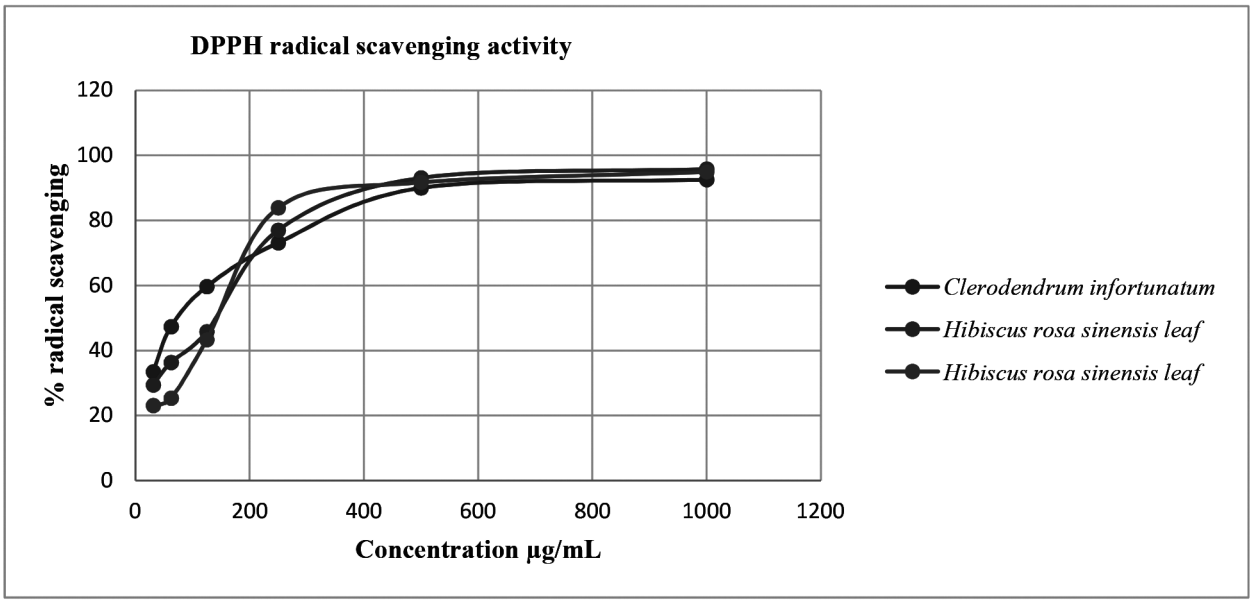


**Figure 2: DPPH radical scavenging against the different concentration of plant extracts.**

The antioxidant potential of plant extracts expressed in $IC_{50}$ is shown in Table 3.

**Table 3: DPPH radical scavenging activity ($IC_{50}$) of plant extracts.**

| Plant extracts | $IC_{50}$ (µg/mL) |
|---|---|
| Quercetin | 3.49±0.15 |
| *Clerodendrum infortunatum* | 71.95±0.65 |
| *Hibiscus rosa sinensis* leaf | 98.74±1.91 |
| *Hibiscus rosa sinensis* flower | 117.23±7.72 |

The leaf extract of *Clerodendrum infortunatum* behaves as moderate free radicals that may include therapeutic use against oxidative stress.

### *3.4 □-Amylase inhibitory activity*

The plant extracts reduce postprandial hyperglycemia by suppressing the hydrolysis of polysaccharides and are found useful to control diabetes mellitus (Layer et al. 1986, Tundis et al. 2010). Many herbal extracts have been reported for antidiabetic activities and are used in Ayurveda for the control of diabetes. Due to the lack of sustained scientific evidence, these medicinal plants are not gaining much importance.

**Table 4: α-amylase enzyme inhibitory activity ($IC_{50}$ ) of plant extract and standard acarbose**

| Plant extract | $IC_{50}$ (µg/mL) |
|---|---|
| *Clerodendrum infortunatum* | 118.2±4.80 |
| Acarbose | 12.96±0.22 |

In this present study, three medicinal plant samples growing in Bara district of Nepal were analyzed for their α-amylase enzyme inhibitory properties and the results ($IC_{50}$) are shown in Table 4. The extract of *Clerodendrum infortunatum* showed moderate α-amylase inhibition activity of $IC_{50}$ = 118.2±4.80 µg/mL as compared to that of the standard acarbose $IC_{50}$ = 12.96±0.22 *µg/*mL.

## 4. Conclusions

In this study, three medicinal plant extracts revealed their phytochemical analysis, determination of total phenolic and flavonoid content, and antioxidant and α-amylase inhibitory activity. Among the studied plant extracts, the leaf extract of *Clerodendrum infortunatum* was found rich in secondary metabolites. The plant extract of *Clerodendrum infortunatum* has the best antioxidant activity that plays a significant role to reduce the oxidative stress in the cells. The same plant extract showed high phenolic and flavonoid content. The plant extract of *Clerodendrum infortunatum* displayed moderate α-amylase enzyme inhibition activity and could be used as the source to isolate the active compounds as the drug candidate in the future drug discovery process. So these plant parts could be the greater significance in preventing several harmful human diseases especially to manage oxidative stress and diabetes. Further studies should be directed towards the isolation and characterizations of natural compounds from these plant extracts and to perform *in vivo* and *in vitro* biological activities with their mechanism of action for validation of traditional uses of these plants in several medicinal practices.

## Data availability

All data generated of analyzed during this study are included in this manuscript.

## Conflicts of interest

All the authors declare that they have no conflicts of interest.

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