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Research Article

PHYTOCHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES OF *Ficus racemosa* LINN.*, Myrica esculenta* BUCH-HAM. EX D. DON AND *Urtica dioica* LINN.

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ABSTRACT
Medicinal plants like *Ficus racemosa, Myrica esculenta*, and *Urtica dioica* were traditionally used to treat diabetes, cough, fever etc. These plants were collected from Kathmandu and Gorkha district of Nepal and subjected to various biological activities. Methanol extract of all three selected plants when subjected to phytochemical analysis showed the presence of various chemical constituents such polyphenols, flavonoids, tannins, saponins, and glycosides. The methanol extract of *F. racemosa* bark was found with strongest DPPH radical neutralization activity having IC_{50} value of 41.48 µg/mL, which was comparable with standard ascorbic acid (31.07 µg/mL). The bark of *F. racemosa* contained the highest level of total phenolic and total flavonoid contents with the values of 168.89 ± 1.08 mg GAE/g and 41.56 ± 1.83 mg QE/g respectively. The methanol extract of bark of *F. racemosa* showed high *α*-amylase inhibitory activity with IC_{50} value of 164.99 µg/mL. Brine shrimp bioassay showed that the methanol extracts of bark of *F. racemosa*, bark of *M. esculenta,* leaves of *M. esculenta* and *U. dioica* were lethal to brine shrimp nauplii with LC_{50} values of 70.79, 177.83, 190.54 and 269.15 µg/mL respectively. Methanol extract of leaves of *U. dioica* was least active in these biological activities.

Keywords*: α*- amylase, antioxidant, antidiabetic, brine shrimp, cytotoxicity, Nepalese medicinal plants, total phenol

INTRODUCTION
Medicinal plants serve as models for pharmacologically active compounds (Hussein & El-Anssary, 2019). Herbal drugs are often prescribed due to their effectiveness, lack of adverse effects and comparatively lower cost (Bhandari *et al*., 2008). *F. racemosa* is a tree of medium to large size, commonly known as Cluster Fig in English, Gular in Hindi and Dumri in Nepali. Decoction of bark is done to treat diabetes (Joseph & Raj, 2010). *M. esculenta* is a dioecious, subtemperate, small evergreen tree native to northern India, Nepal, China and Pakistan (Sood & Shri, 2018). It is also known as bayberry, box myrtle, Kaphal or Katphala and leaves are widely used to cure cough, throat disorders, and fever (Kabra *et al*., 2018). *Urtica dioica* or Sisnu, also called stinging nettle, is found in wild form in the forest of high hill and himalayas (Al-Tameme *et al*., 2015). Nettle leaves are used for treating various diseases such as eczema, digestion, joint pain and anaemia (Ghaima *et al*., 2013).

The biological activity of plants depends upon the types of phytochemical constituents present in them. Climates, genetic variation, and localities have a significant impact on the phytochemical constituents of the plant. As per available literature, *F. racemosa* grown in Gorkha district, *M. esculenta*, and *U. dioica* grown in Kathmandu district has not been evaluated scientifically for such biological activities. To fill this research gap, the current work is primarily concerned with the evaluation of the phytochemicals, antioxidant activity, total flavonoid content, total phenolic content, brine shrimp bioassay, and alpha-amylase inhibition assay of *F. racemosa, M. esculenta* and *U. dioica.*

MATERIALS AND METHODS
Plant collection and extraction
The plant samples were collected from Kathmandu and Gorkha district in the month of August to November 2018 A.D. Air dried and coarsely pulverized parts of sample plants (100 g) extracted with methanol (100%) for 72 hours. Obtained extract was concentrated to dryness in a rotatory evaporator (Buchi RE 111 with Buchi 461 water bath) at room temperature to obtain methanol extract. After removal of solvent, plant extract was stored in refrigerator at 4 °C until use.

Phytochemical analysis
The concentrated methanol extract of plant samples was used for preliminary screening of phytochemicals such as saponins, polyphenols, flavonoids, alkaloids, steroids, glycosides, tannin, reducing sugars, cardiac glycosides, anthraquinone, and carotenoids (Harbone, 1973).

Brine shrimp bioassay
The Brine shrimp bioassay cytotoxicity test was conducted following the standard protocol (Meyer *et al*., 1982). In brief, newly hatched Brine shrimp nauplii were introduced to each crude methanol plant extract and LC_{50} values of the crude extracts was determined in µg/mL. Each sample's 20 mg was dissolved in 2 mL of methanol to create the stock solution. Three test tubes were used for each dose level, totaling nine test tubes, from which 500

µL (or 1000 ppm), 50 µL, and 5 µL were extracted from each stock solution. The solvent used to dissolve the extract in the test was included as a control. To guarantee proper diffusion, 5 mL of sea water was added to each test tube with gentle shaking once the solvent had completely evaporated overnight. Ten fully grown prawns were then added to each test tube. In a similar manner, ten fully grown nauplii were placed into each of the three controlled vials. Using disposable pipettes, the number of survivors was counted after a 24-hour period. The entire experiment was carried out in a room with constant lighting and a temperature control of 28 °C (Meyer *et al*., 1982).

Antioxidant activity

In 100 mL methanol 0.2 mM DPPH solution was prepared. DPPH solution (2 mL) in methanol was mixed with different concentrations of 2 mL of test samples of 5, 10, 20, 40, 60, 80 and 100 µg/mL made from stock solution (1000 µg/mL). The reaction was allowed to run for 30 minutes at 37 °C, and the SpectraMax340 multiplate reader was used to measure the absorbance at 517 nm. The solution's colour disappears during reduction. The percentage of the DPPH free radical scavenging activity was calculated by using the following equation:

$$\% \text{ scavenging} = \left[\frac{\text{Ac} - \text{As}}{\text{Ac}}\right] \times 100$$

Where,

A_s = Absorbance of sample solution

A_c = Absorbance of control (DPPH solution + methanol)

Ascorbic acid of the same concentration was prepared as a standard (positive control). The IC_{50} values of compound was calculated (Jamuna *et al*, 2012).

Total phenol content (TPC) determination

The Folin-Ciocalteu Colorimetric technique was used to quantify the total phenolic content of the plant extract, adhering to established methodology. Gallic acid was taken as a reference. To express TPC, milligram of gallic acid equivalent (GAE) were used for every 100 g of extract. (Kim *et al*, 2003).

Total flavonoid content (TFC) determination

Total flavonoid content present in plants extract was calculated by Aluminium Chloride Colorimetric Assay. Quercetin was taken as a reference. TFC was expressed as milligrams of quercetin equivalent (QE) per 100 g of extract (Kalita *et al*, 2013).

Alpha amylase inhibition assay

Antidiabetic potential of selected plants was identified by using a slightly modified standard protocol of *a*-amylase inhibition assay. Acarbose was taken as a standard. Three duplicates of the experiment were run, and the percentage of enzyme inhibition was calculated (Kusano *et al*, 2011).

Analysis of phytochemicals

Phytochemical analysis showed that the methanol extract of plant samples is rich in secondary metabolites (Table 1) and can be used as the source of phytoconstituents for isolation of active constituents. Quinines was present only in methanol extract of *F. racemosa.*

Table 1. Result of phytochemical screening of selected plants.

S.N.	Group of compounds	*F. racemosa*	*M. esculenta* (bark)	*M. esculenta* (leaves)	*U. dioica*
1.	Alkaloids	-	+	+	+
2.	Polyphenols	+	+	+	+
3.	Flavonoids	+	+	+	+
4.	Saponin	+	+	+	+
5.	Glycosides	+	-	-	+
6.	Terpenoids	+	+	+	+
7.	Quinones	+	-	-	-
8.	Tannin	+	+	+	+
9.	Reducing sugar	-	+	+	-
10.	Coumarins	+	-	-	-
11.	Sterols	+	+	+	-

Where, + = Present and - = Absent

Evaluation of Brine Shrimp bioassay

Nauplii of newly hatched brine shrimp were kept at various concentrations of methanol extracts and methanol. LC_{50} values (µg/mL) was calculated to determine toxicity of the extracts. Pharmocologically active extracts have an LC_{50} value of less than 1000 µg/mL (Meyer *et al*., 1982). Methanol extracts of bark of *F. racemosa*, bark of *M. esculenta*, leaves of *M. esculenta* and leaves of *U. dioica* had LC_{50} values of 70.79, 177.83, 190.54 and 269.15 µg/mL respectively and were toxic to brine shrimp nauplii. Among

them, bark of *F. racemosa* was found to be most biologically active due to high toxicity towards the brine shrimp larvae and hence can be used in pharmaceutical industry. The in-vitro XTT bioassay of *F. racemosa* plant extract, which demonstrated cytotoxic action against HL-60 and HepG2 cancer cell lines and low toxicity against normal cell lines, is substantially linked with this conclusion (Sukhramani *et al*, 2013). The calculation of LC_{50} values of plant extracts is demonstrated in Table 2.

Table 2. Calculation of LC_{50} of different samples.

Sample	Z	x	y	xy	x^2	Σx	Σy	Σxy	Σ x^2	β	α	X	LC_{50}
F. racemosa	10	1	6.33	6.33	1	6	14.33	25.66	14	-1.5	7.78	1.85	70.79
	100	2	4.67	9.34	4								
	1000	3	3.33	9.99	9								
M. esculenta (bark)	10	1	6.67	6.67	1	6	16	29.33	14	-1.34	8.01	2.25	177.83
	100	2	5.33	10.66	4								
	1000	3	4	12	9								
M. esculenta (leaves)	10	1	7.66	7.66	1	6	16.65	29.3	14	-2	9.55	2.28	190.54
	100	2	5.33	10.66	4								
	1000	3	3.66	10.98	9								
U. dioica	10	1	8	8	1	6	18	31.33	14	-2.34	10.68	2.43	269.15
	100	2	6.67	13.34	4								
	1000	3	3.33	9.99	9								

where, Z = concentration in µg/mL, x = logZ, y = No. of alive larvae, $\beta = \frac{Sxy - Sx\ Sy/n}{Sx^2 - (Sx)^2/n}$, $\alpha = \frac{1}{n}[Sy - bSx]$, X = $(Y - \alpha)/\beta$, Y = constant having value 5, and LC_{50} = antilog X

Evaluation of DPPH free radical scavenging activity

Together with standard ascorbic acid, the percentage of DPPH free radical scavenging activity of plant extracts in methanol extract was evaluated at various doses and is shown graphically in Fig. 1. The methanol extract of bark of *F. racemosa* and *M. esculenta* showed the strongest DPPH radical scavenging activity and IC_{50} values were 41.48 and 48.39 µg/mL respectively which was near to standard ascorbic acid (31.07 µg/mL). The phytoconstituents of both plants, which include alcoholic groups, acids, polyphenols, flavonoids, and esters—may be responsible for their antioxidant properties. This result is in line with a review of the literature, which discovered that the best DPPH scavenging activities were shown by methanol extracts of *F. racemosa* stem bark (IC_{50} = 21.50 µg/mL). Extracts capacity for electron transfer and hydrogen donation may be enhanced by their ability to scavenge radicals, which may be linked to phenolics properties (Manian *et al,* 2008). Strong antioxidant activity was demonstrated by the ethanolic extract of the stem bark of *F. racemosa*, as per previous research (Veerapur *et al*, 2009).

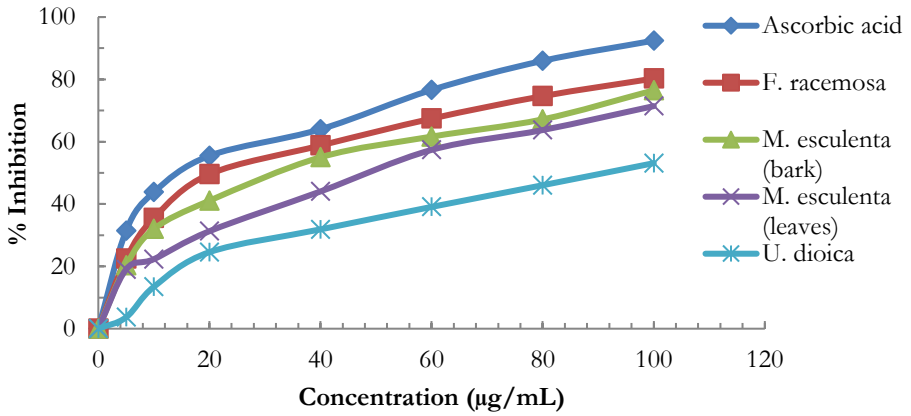

Figure 1. Graph showing % free radical scavenging activity vs. various concentration of different plant extracts as well as of ascorbic acid

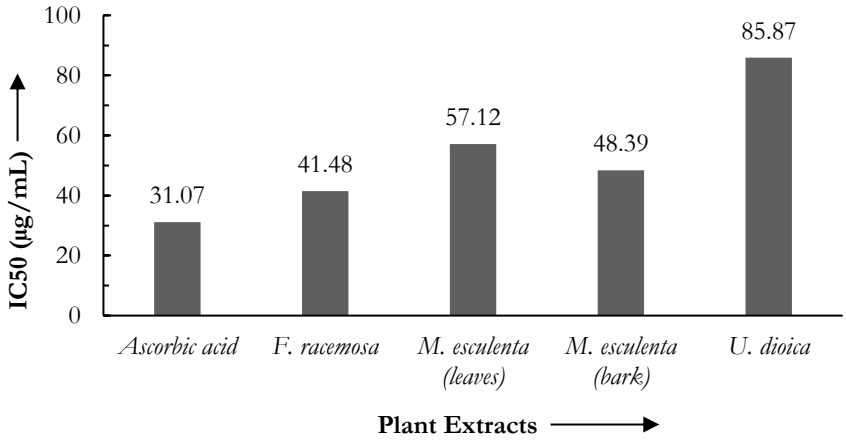

Figure 2. Bar graph indicating IC_{50} values of different methanol plant extracts.

Estimation of total phenolic content

The result revealed that the total phenolic content was highest in *F. racemosa* (168.89 ± 1.08 mg GAE/g) extract as shown in Fig. 3. Previous phytochemical investigation of the bark of *F. racemosa* led to the isolation of polyphenols like berginin, racemosic acid and quercetin which could be the reason for its higher phenolic content (Paarakh, 2009). Manian *et al.* (2008) had reported the phenolics compounds like pelargonidin and leucopelargonin derivatives, flavonoids and high molecular tannins in *Ficus* species.

Estimation of total flavonoid content

As seen in Fig. 4, the extract of *F. racemesa* had the highest total flavonoid concentration (41.56 ± 1.82 mg QE/g), followed by the bark of *M. esculenta* (34.17 ± 2.95 mg QE/g extract). This outcome is in harmony with the previous report (Keshari *et al*, 2016). The flavonoids were isolated from the bark of *F. racemosa* that were structurally similar to Kaempferol, Quercetin, Naringenin and Baicalein which exhibited remarkable antidiabetic properties (Keshari *et al*, 2016). Several compounds were reported in bark and leaves of *M. esculenta* which could have been attributed for the flavonoid content (Panthari *et al,* 2012).

Alpha amylase inhibition assay

The % inhibition of each plant extract at different concentrations was determined as shown in Table 3 and their IC_{50} values were compared with Acarbose taken as a reference as shown in Table 4.

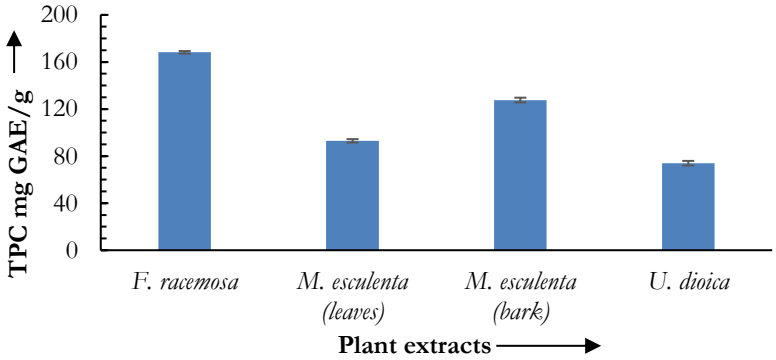

Figure 3. Total phenolic content in different methanol plant extracts.

The findings presented in Table 3 indicate that the alpha amylase inhibition activity was present in all of the chosen plant extracts, and that the inhibition percentage increased in a concentration-dependent manner. *F. racemosa* had the lowest IC_{50} value of 164.99 µg/mL which indicated high affinity for inhibition of *a*-amylase. The previous study stated that the inhibiting activity could be due to the phytochemical components detected in methanol bark extract of *F. racemosa* such as polyphenols, flavonoids, and glycosides (Deepa *et al*, 2018). Literature survey showed that *F. racemosa* has a variety of biological effects such as hepatoprotective, antidiabetic, anti-inflammatory, chemopreventive, antipyretic, and antidiuretic (Ahmed *et al.,* 2010). The isolated flavonoids from *F. racemosa* demonstrated good antidiabetic, hypolipidemic and antioxidant properties (Keshari *et al*., 2016). This could be

the reason for higher antidiabetic potential i.e., lower IC_{50} value of bark of *F. racemosa.* Analgesic, antidiabetic, antiallergic, antimicrobial, antiulcer, antioxidant, and anti-inflammatory properties of *M. esculenta* have been reported. These properties were assessed using a variety of animal models (Kabra A., *et al.,* 2018).

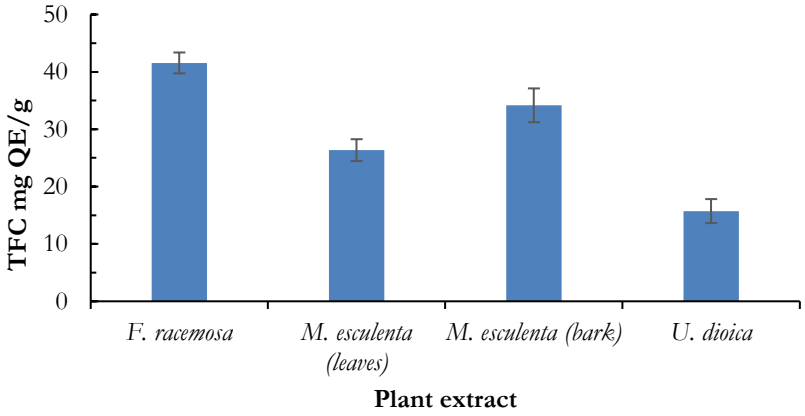

Figure 4. Bar graph showing total flavonoid content in different methanol plant extracts.

Table 3. % inhibition of *α*-amylase at different concentration of plant extracts and acarbose.

Sample	*F. racemosa*	*M. esculenta* (bark)	*M. esculenta* (leaves)	*U. dioica*	Acarbose
Concentration (µg/mL)	**Percentage Inhibition**				
1000	85.71 ± 0.91	79.21 ± 0.43	73.11± 0.20	71.14 ± 0.44	91.23 ± 0.31
640	79.19 ± 1.01	72.47 ± 0.84	69.75 ± 0.65	67.05 ± 0.94	85.69 ± 0.23
320	70.44 ± 0.23	66.74 ± 0.98	66.16 ± 0.54	62.21 ± 0.61	76.89 ± 0.34
160	65.41 ± 0.54	61.69 ± 0.44	61.45 ± 0.23	56.37 ± 0.13	69.43 ± 0.65
80	58.57 ± 0.25	51.04 ± 0.76	50.88 ± 0.48	44.87 ± 0.08	63.69 ± 0.54
40	49.79 ± 0.21	44.96 ± 0.13	43.67 ± 0.16	40.89 ± 0.25	56.60 ± 0.36

(Each value is a mean of triplicate data)

Table 4. Table showing IC_{50} values of different plant extracts along with standard Acarbose.

S.N.	Sample	IC_{50}(µg/mL)
1.	Acarbose	85.40
2.	*F. racemosa*	164.99
3.	*M. esculenta* (bark)	246.95
4.	*M. esculenta* (leaves)	272.63
5.	*U. dioica*	341.44

CONCLUSIONS

Phytochemical analysis showed that the selected plant samples have a large number of secondary metabolites. Among the four selected plant extracts, methanol extract of *F. racemosa* bark showed significant cytotoxicity and was found to be most biologically active. The total phenolic content as well as the total flavonoid content was also highest in the extract of *F. racemosa.* This result correlates well with the strongest DPPH radical scavenging activity and highest *a*-amylase inhibitory activity of bark of *F. racemosa.* Therefore, bark of *F. racemosa* showed strongest biological activities and can be used in medicinal industry. However, substantial clinical and pharmacological research should be conducted before *in-vivo* application.

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AUTHOR CONTRIBUTIONS

S. Maharjan carried out the laboratory work and wrote the manuscript under the supervision of B. Subba. Both authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Upon reasonable request, the data that supports this study will be made available by the authors.

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