Journal of Herbal Drug
journal homepage: www.jhd.iaushk.ac.ir

Study of antioxidant and mushroom Tyrosinase inhibitory activities of selected medicinal plants of Nepal

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ARTICLE INFO

Type: *Original Research*
Topic: *Medicinal Plants*
Received *January 05th 2020*
Accepted *May 12th 2020*

Key words:

- ✓ *DPPH antioxidant activity*
- ✓ *depigmentation*
- ✓ *Diploknema butyraceae*
- ✓ *mushroom tyrosinase inhibitory activity*

ABSTRACT

Background & Aim: Phytochemicals are naturally occurring chemical compounds present in plants. It includes glycosides, alkaloids, tannins, saponins, phenols, flavonoids, carbohydrates, etc. Among them, some of them possess antioxidant property, which is responsible for regulation of free radicals in the body and tyrosinase inhibitory activity that results in the reduction in melanin synthesis. In addition, phytochemicals provide different health benefits to humans. Thus, the aim of the study was to determine antioxidant and tyrosinase inhibitory activities of some selected medicinal plants of Nepal.

Experimental: Phytochemical screening of methanolic extracts of test samples was performed with various reagents and the phenol and flavonoid contents were also determined. Antioxidant activity was carried out by DPPH free radical scavenging method and tyrosinase inhibitory activity was performed by mushroom tyrosinase inhibitory method. The presence of phytochemicals was confirmed by the visual color change upon addition of test reagents. The higher phenol and flavonoid contents among the selected plants were found to have better antioxidant and tyrosinase inhibitory activities.

Results: Among the selected plants, *Diploknema butyraceae* had the highest phenol content (665.33 ± 0.0 mg GAE/g dry extract weight) and flavonoid content (728 ± 0.3 mg quercetin/g dry extract weight) and showed similar DPPH free radical scavenging activity (IC_{50} value 6.012 µg/ml) to standard ascorbic acid (IC_{50} value 4.73 µg/ml). In addition to this, mushroom tyrosinase inhibition was also found highest in the same plant (31.07 ± 2.13 %) followed by *Jatropha curcas* (17.51± 0.49 %), *Woodfordia fruticosa* (16.95 ± 2.24 %) and least in *Crateva unilocularis* (1.41 ± 2.13%).

Recommended applications/industries: The results above showed some probability of *Diploknema butyraceae* with potential tyrosinase inhibitory property. Therefore, further studies should be focused on isolation of active constituents responsible for tyrosinase inhibitory activity.

1. Introduction

Several pollutants, xenobiotics and chronic exposure to UV radiation generate reactive oxygen species (ROS) and reactive nitrogen species (NOS) inside the human body resulting in severe oxidative stress (Fisher *et al.*, 2002; Liyanaarachchi *et al.*, 2018). Consequently, such

reactive species oxidize biologically important molecules and induce oxidative damage to cellular membranes, tissues and enzymes, which in turn leads to several diseases including hyperpigmentation, skin cancer and other skin-related disorders (Halliwell *et al.*, 2015). Melanin; a complex Quinone/indole Quinone-derived mixture of biopolymers is produced in melanocytes from tyrosine (Rees, 2003). Tyrosinase is a multifunctional, glycosylated copper-containing enzyme which is widely distributed in both plants and animals that catalyzes the first two steps in the formation of the melanin. First, the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). Second,oxidation of L-DOPA to dopaquinone (Parvez *et al.*, 2007). Since the first step is the rate-limiting step in melanin synthesis, its inhibitors can act as depigmenting agents (Garcia *et al.*, 1989).Mushroom tyrosinase is commonly used to study the tyrosinase inhibitory activity. The enzyme is extracted from the mushroom *Agaricus bisporus* which is highly homologous with the mammalian tyrosinase and is commercially available (Solano, 2014; Chang, 2009).

Various ROS plays significant role in regulation of melanogenesis, as it is reported that melanogenesis produces hydrogen peroxide (H_2O_2) and other ROS. In addition to this, ROS scavengers and inhibitors of ROS generation are believed to down-regulate UV-induced melanogenesis (Yamakoshi *et al.*, 2003). Therefore, antioxidants like ascorbic acid derivatives have been used for inhibition of melanogenesis (Kumano *et al.*, 1998). Different natural products have been studied as skin photoprotectants and skin-whitening agents, like polyphenols (flavonoids, coumarins, catechins, tanins and stilbens), alkaloids and vitamins (Chiva-Blanch *et al.*, 2012; Adhami *et al.*, 2008; Giampieri *et al.*, 2012; Silveira *et al.*, 2013).

Nepal is tremendously rich in biodiversity and harbors variety of flora and vegetation which have several medicinal benefits (Manandhar, 2002). The traditional uses of medicinal plants used in this study is mentioned in Table 1. Therefore, in an effort to find new potential ingredients for skin whitening, five local and less exploited plants of Nepal were investigated for their antioxidant and *in-vitro* tyrosinase inhibition properties. The total phenol (TPC) and total flavonoid contents (TFC) were also investigated.

Table 1: Traditional uses of the medicinal plants used in this study

Plant name	Plant part	Traditional uses
Crateva unilocularis	Leaves, root and bark	Wound healing, laxative, anthelmintic, blood detoxifier, urolithiasis, urinary antiseptic (Khattar *et al.*, 2012)
Diploknema butyraceae	Bark and seeds	Treatment of hemorrhage, rheumatic pain, pimples, boils, antileprotic, antidiabetic (Khanka *et al.*, 2010)
Jatropha curcas	Leaves and stem	Curing skin diseases, cough, antiseptic, arrest bleeding from wounds, rheumatic pain (Manandhar, 2002)
Oxalis corniculata	Whole plant	Treatment of pimples, diarrhea, peptic ulcer, conjunctivitis, hemorrhoids, anemia (Manandhar, 2002; Sharma *et al.*, 2014)
Woodfordia fruticosa	Flowers and bark	Antileprotic, astringent, dysentry, toothache, stomatitis (Manandhar, 2002)

Their antioxidant and *in-vitro* tyrosinase inhibition properties. The total phenol content (TPC) and total flavonoid content (TFC) were also investigated.

2. Materials and Methods

2.1. Reagents

All the reagents used were of analytical grade and purchased from different suppliers such as Qualigens Fine Chemicals for methanol, quercetin, sodium nitrite, aluminium chloride, potassium dihydrogen phosphate; Thermofischer Scientific for dimethyl sulfoxide (DMSO), Folin's reagent, sodium carbonate, sodium hydroxide; Tokyo Chemical for DPPH; Nike Chemicals for ascorbic acid; Wako Pure Chemicals for gallic acid; Cambridge Isotype Laboratories for L-DOPA; Affymetrix for mushroom tyrosinase enzyme; Alfa Aesar for kojic acid.

2.2. Plant materials

The plant samples; *Crateva unilocularis* (leaves), *Diploknema butyraceae* (leaves), *Jatropha curcas* (twigs), *Oxalis corniculata* (whole plant), *Woodfordia fruticosa* (leaves) were collected from different places of Kaski district, Western Nepal. Herbarium of each

plant material were prepared and sent to National Herbarium and Plant Laboratories, Godawari, Lalitpur botanical identification.

The collected plant materials were cleaned and cut into pieces for shade drying at room temperature. After sufficient drying, electric blender was used for size reduction of the plantmaterials.

2.3. Extraction procedure

The plant materials were extracted by double maceration. In brief, 50 g of the each plant material was macerated using 250 ml of methanol and kept at room temperature for 24 hours. Then, it was filtered and the obtained filtrate (filtrate-I) was stored separately. The marc (residue) was again macerated using same volume of methanol and left for another 24 hours. After subsequent filtration, theobtained filtrate (filtrate-II) was mixed with filtrate-I and were concentrated in rotary evaporator under reduced pressure. The concentrated extract was further dried in vacuum desiccator for about 20 days and the yield value of each extract was calculated which is presented in Table 2.

2.4. Phytochemical analysis

Phytochemical analysis was carried out for flavonoids, tannins, carbohydrates, phenols (Kodangala *et al*., 2010) and alkaloids, glycosides, saponins, coumarins, steroids and terpenoids according to literature (Ch *et al*., 2013). Wagner's reagent was used for alkaloid test, ferric chloride for flavonoids, aqueous sodium hydroxide for glycosides, gelatin for tannins, sodium bicarbonate for saponins, α-naphthol for carbohydrates, alcoholic sodium hydroxide for coumarins, chloroform and sulphuric acid for both steroids and terpenoids.

2.5. DPPH antioxidant activity

DPPH (2, 2-diphenyl-1-picryl hydrazyl radical) antioxidant activity was performed according to the literature with some modifications (Kim *et al.*, 2007). DPPH solution was freshly preparedfor the assay. In brief, 2 ml of different extract solutions (1, 10 and 100 µg/ml) of each plant sample were mixed with 2 ml of DPPH solution (100 µM). Then, mixtures were allowed to stand for 30 minutes to perform the complete reaction. Finally, the absorbance of each plant samples was measured at 517 nm by using UV spectrophotometer. The percentage radical scavenging activity of each sample was calculated by using the following formula:

$$\text{Radical scavenging activity (\%)} = \frac{[A_o - A_s]}{A_o} \times 100\%$$

Where,

A_o = Absorbance of control

A_s = Absorbance of sample

Control was the test solution without the sample. Kojic acid solution (1000 µg/ml) was used as positive control.

2.6. Determination of total phenol contents

Total phenols were determined by Folin-Ciocalteu method according to Pourmorad *et al*. (2006). Overall, 1ml of sample was mixed with 5 ml of distilled water and 1ml of Folin reagent. After standing for 5 minutes, 1ml of 10 % sodium carbonate was added and stirred. The mixture was incubated for 1 hr at room temperature and the absorbance was measured at 725 nm against a blank. The total phenol content was expressed as milligrams of gallic acid equivalent per gram dry extract weight using the calibration curve of gallic acid (50-350 µg/ml) standards.

2.7. Determination of total flavonoid contents

Aluminum chloride colorimetric method was used for flavonoids determination according to the method of Chang *et al.*, (2002). In brief, 1 ml of sample solution was mixed with 4 ml of distilled water. Then, 300µl of sodium nitrite(5% w/v) was added. After 5 minutes, 300 µlaluminum chloride (20 % w/v) was added and allowed to stand for 6 minutes and 2 ml of NaOH (1 M) was added. The mixture was stirred and the absorbance was measured at 510 nm using UV spectrophotometer and compared with standard. The total flavonoids content was expressed as milligrams of quercetin equivalent per gram dry extract weight using calibration curve of quercetin (50-350 µg/ml) standards.

2.8. Mushroom tyrosinase inhibition assay

Mushroom tyrosinase inhibitory activity was performed according to Adhikari *et al.* (2008) with some modifications. For this, 2 ml of 1.5 mM L-DOPA solution, 0.2 ml of sample solution (1000 µg/ml) and 1.6 ml of phosphate buffer solution (pH 6.8) were mixed in test tube and the mixture was incubated at

room temperature for 10 minutes. Then, 0.2 ml of mushroom tyrosinase solution (1000 U/ml) was added and the absorbance was measured at 475 nm in UV spectrophotometer. The percentage tyrosinase inhibition was calculated using the formula:

$$\text{Tyrosinase inhibitory activity } (\%) = \frac{[A - B]}{A} \times 100$$

Where,

A= Absorbance difference of the control sample observed at 30 s and 60 s

B= Absorbance difference of the test samples observed at 30 s and 60 s

Control was the test solution without the sample. Kojic acid solution (1000 µg/ml) was used as positive control.

3. Results and discussion

3.1 Extraction Yield Value

In this study, we used methanol as solvent for extraction. The percentage yield of extracts ranged from 4.40 % to 23.06 % (Table 2). The highest yield value was observed in *Woodfordia fruticosa*(23.06 %) followed by *Diploknema butyraceae* (10.43 %), while the lowest value was observed in *Jatropha curcas* (4.82 %) and *Oxalis corniculata* (4.40 %).

Table 2: Extraction yield value

S.N.	Scientific name	Parts used	Common name	Local name	Extract yield (gram)	Yield value (%)
1	*Crateva unilocularis*	Leaves	Three-leaved capper	Siplican	3.27	6.55
2	*Diploknema butyraceae*	Leaves	Butter fruit	Chyuri	5.21	10.43
3	*Jatropha curcas*	Twigs	Curcas bean	Sajiwan	2.41	4.82
4	*Oxalis corniculata*	Whole plant	Creeping sorrel	Chari amilo	2.20	4.40
5	*Woodfordiafruticosa*	Leaves	Fire flame bush	Dhanyaro or dhairo	11.53	23.06

3.2. Phytochemical Analysis

The extracts were screened for the presence or absence of phytochemicals by observing the change in colour when treated with the corresponding test reagents. The qualitative analysis of extracts confirmed the presence of several constituents such as alkaloids, glycosides, flavonoids, tanins, carbohydrates, saponin, coumarins, terpenoids, phenols and steroids (Table 3) which are known to have various pharmacological activities (Jennifer *et al.*, 2012; Sharma *et al.*, 2014; Tyagi *et al.*, 2015).

All the plant extracts showed positive results for most of the phytochemicals. However, tannin in *Crateva unilocularis*, saponin in *Diploknema butyraceae* and coumarin in *Jatropha curcas* were absent (Table 3).

Table 3: Phytochemical screening of selected plant extracts

S.N.	Phytochemicals	*Crateva unilocularis*	*Diploknema butyraceae*	*Jatropha curcas*	*Oxalis corniculata*	*Woodfordia fruticosa*
1	Alkaloid	+	+	+	++	++
2	Glycosides	++	++	++	++	++
3	Flavonoid	++	++	++	++	++
4	Tannin	-	++	+	++	+
5	Carbohydrate	+	+	+	++	++
6	Saponin	++	-	++	++	++
7	Coumarin	+	++	-	++	++
8	Terpenoid	++	++	++	++	++
9	Phenol	+	++	+	++	++
10	Steriod	++	++	++	++	++

Note: ++: Strong intensity reaction, + : Weak intensity reaction, - : Not Detected

3.3. Total Phenol Content

The phenolic content in the plant extracts was determined using Folin-Ciocalteu method. The calibration curve of gallic acid as standard at different concentrations with R^2=0.971 was used to calculate total phenol content. The total phenol content varied widely from 138.67 ± 0.4 to 665.33 ± 0.0 GAE/g dry weight of sample, which represents a variation of about 5 folds (Table 4). The highest TPC was reported for *Diploknema butyraceae* (665.33 ± 0.0 mg GAE/g dry weight of sample) whereas the lowest was observed for *Oxalis corniculata* (138.67 ± 0.4 mg GAE/g dry weight of sample) as shown in Table 4.

Phenolics are among the largest groups of phytochemicals found in plants, which includes more than 8000 biologically active compounds (Marinova *et al*., 2005). *Diploknema butyraceae* showed the highest phenol contents among the selected plants followed by *Woodfordia fruticosa*. In a previous study, when fruit pulp of *Diploknema butyraceae* was extracted with 50% methanol: water, a phenol content of 37.1 ± 4.1 mg GAE/g dry weight of sample was measured (Prakash *et al*., 2012). Another study revealed that the methanolic extraction of *Woodfordia fruticosa* leaves and bark had the phenol content of 613±7.63 and 663±37.85 µg/ml, respectively, when tannic acid was used as standard equivalent (Chaturvedi *et al*., 2011). Moreover, phenolics-particularly hydrosable tannins 'Woodfruticosin' along with gallic acid, ellagic acid, isochiwalin and oenthein has been isolated from MeOH and water extracts of leaves (Chaturvedi *et al*., 2011; Kadota *et al*., 1990). Hence, leaves and bark of this plant are rich in phenolic compounds that can easily be extracted using methanol.

Table 4: Total phenol content

S.N.	Scientific name	Total Phenol Content (mg GAE/g dry weight of sample)
1	*Crateva unilocularis*	168.55 ± 0.1
2	*Diploknema butyraceae*	665.33 ± 0.0
3	*Jatropha curcas*	257.11 ± 0.2
4	*Oxalis corniculata*	138.67 ± 0.4
5	*Woodfordia fruticosa*	409.11 ± 0.1

Note: Data expressed as mean ± standard deviation (n=3). Total phenol content was calculated by the help of calibration curve of gallic acid as standard.

3.4. Total Flavonoid Content

Aluminum chloride colorimetric method was used to determine the flavonoid contents in the plant extracts. The calibration curve of quercetin as standard at different concentrations with R^2= 0.961 was used to calculate total flavonoid content. As with phenols, a substantial difference in TFC was observed among the plant extracts ranging from 292 ± 0.1 to728 ± 0.3 mg quercetin/g dry extract weight (Table 5). In brief, the maximum flavonoid content was observed in *Diploknema butyraceae* (728 ± 0.3 mg quercetin/g dry extract weight) followed by *Woodfordia fruticosa* (541 ± 0.2 mg quercetin/g dry extract weight). Both *Oxalis corniculata* and *Jatropha curca* showed similar flavonoid contents (355 ± 0.4 and 354 ± 0.1 mg quercetin/g dry extract weight, respectively) while *Crateva unilocularis* (292 ± 0.1mg quercetin/g dry extract weight) witnessed the lowest value as shown in Table 5.

Flavonoids, a group of polyphenolic compounds are one of the most studied secondary metabolites of the plants (Falcone *et al*., 2012). The total flavonoid content was calculated by aluminum chloride colorimetric method. Similar to phenols, the highest flavonoid content was also found in *Diploknema butyraceae*. Previous studies demonstrated the presence of flavonoids, quercetin and dihydro-quercetin from the nut-shell of this plant (Awasthi *et al*., 1962). Next to *Diploknema butyraceae*, *Woodfordia fruticosa* had the highest flavonoid content. *Woodfordia fruticosa* has also been reported to have flavonoids like quercetin, myricetin, kaempferol, naringenin, etc. (Chaturvedi *et al*., 2011). This further confirms the presence of flavonoids in these plant extracts.

Table 5: Total flavonoid content

S.N.	Scientific name	Total flavonoid content (mg quercetin equivalent/g dry weight of sample)
1	*Cratevaunilocularis*	292 ± 0.1
2	*Diploknemabutyraceae*	728 ± 0.3
3	*Jatrophacurcas*	354 ± 0.1
4	*Oxalis corniculata*	355 ± 0.4
5	*Woodfordiafruticosa*	541 ± 0.2

Note: Data expressed as mean ± standard deviation (n=3). Total Flavonoid content was calculated by the help of calibration curve of quercetin as standard.

3.5 DPPH Radical Scavenging Activity

The hydrogen atom or electron donation ability of each plant extract against DPPH free radical was measured from the bleaching of violet colored methanol solution of DPPH. The DPPH radical scavenging activity of different plant samples at different concentrations was measured in terms of IC_{50} value as shown in the Table 6. All the plant extracts showed DPPH free radical scavenging activity, but to varying degrees, ranging from IC_{50} value of 6.01 to 61.38 µg/ml.

Among the five selected plants, *Diploknema butyraceae* (IC_{50}:6.012 µg/ml), exhibited the maximum DPPH radical scavenging activity which was comparable with that of standard ascorbic acid (IC_{50} 4.73µg/ml) as shown in the Table 6. Whereas, the lowest scavenging activity was observed for *Oxalis corniculata* (IC_{50}: 61.38 µg/ml).

Table *6:* *DPPH radical scavenging activity (%)*

S.N.	Sample/conc	1 µg/ml	10µg/ml	100µg/ml	IC_{50}(µg/ml)
1	Ascorbic acid	8.9 ± 4.1	97.1± 0.2	98.3 ± 0.3	4.73
2	*Crateva unilocularis*	26.52 ± 4.28	14.48 ± 1.11	82.91 ± 0.64	46.65
3	*Diploknema butyraceae*	42.08 ± 3.10	57.98 ± 7.08	96.42 ± 0.00	6.01
4	*Jatropha curcas*	2.84 ± 2.11	26.40 ± 1.68	89.04 ± 0.21	50.21
5	*Oxalis corniculata*	0.51 ± 0.49	15.61 ± 1.20	78.76 ± 5.70	61.38
6	*Woodfordia fruticosa*	9.42 ± 1.23	59.11 ± 4.38	94.55± 0.30	30.53

Note: Data expressed as mean value ± standard deviation (n=3)

3.6 MushroomTyrosinase Inhibitory Activity

Mushroom tyrosinase inhibition by plant extracts is shown in Table 7. *Diploknema butyraceae* showed the highest tyrosinase inhibitory activity of 31.07±2.13 % followed by *Jatropha curcas* (17.51±0.49%) and*Woodfordia fruticosa* (16.95±2.24 %). On the other hand, the least activity was observed in *Crateva unilocularis* (1.41±2.13 %).

Melanin is the primary determinant of the skin color, which provides photoprotective function by absorbing UV radiation by 50% to 75% and scavenge the ROS (Brenner *et al.*, 2008). Tyrosinase is the key enzyme responsible for melanin synthesis. Overproduction of melanin in the skin may cause hyperpigmentation, melanoma and other pigmentory abnormalities (Draelos *et al.*, 2005). Therefore, tyrosinase inhibitors are becoming important in cosmetics as whitening agents and in treatment of several pigmentory disorders like melasma, lentigines and ephelides (Draelos *et al.*, 2005; Liyanaarachchi *et al.*, 2018). Due to the various drawbacks of the synthetic whitening agents like hyperpigmentation, inflammation, cytotoxicity, etc., tyrosinase inhibitors from plant sources as an alternative to current treatments is increasing day by day. In this study, extract of *Diploknema butyraceae* showed the maximum tyrosinase inhibition followed by *Woodfordia fruticosa*. Interestingly, these plants showed higher phenolic and flavonoid content as well as high DPPH free radical scavenging property. The high tyrosinase inhibitory activity may be due to the presence of higher concentration of phenols and flavonoids in these plant extracts, as both phenols and flavonoids have previously been reported as potent tyrosinase inhibitors (Jennifer *et al.*, 2012). Several active compounds have already been isolated from plants such as arbutin, aloesin, gentisic acid, flavonoids, niacinamide, etc. which inhibited tyrosinase enzyme and helped in skin whitening (Zhu *et al.*, 2008).

DPPH is stable free radical generating compound commonly used for in vitro assessment of antioxidant potency of different compounds, which shows maximum absorption at 517nm (Antolovich *et al.*, 2001). As mentioned before, the highest DPPH free radical scavenging property was found in *Diploknema butyraceae* followed by that in *Woodfordia fruticosa*. Other researchers have also reported that 50% methanolic extract of fruit pulp (1 mg/ml) of *Diploknema butyraceae* inhibited 19.6 ± 2.1 % of DPPH radical (Prakash *et al.*, 2012.). Likewise, methanolic extract of *Woodfordia fruticosa* leaves (250 µg/ml) showed 88.36±0.02 % DPPH inhibition (Chaturvedi *et al*., 2011). Previous studies have shown that phenolic compounds are mostly responsible for free radical scavenging activity of plant extracts which justifies the high antioxidant activity of *Diploknema butyraceae* and *Woodfordia fruticosa* in our study as well.

Table 7: Tyrosinase inhibitory activity

S.N.	Samples	Tyrosinase Inhibition (%)
1	*Kojic acid*	96.33±0.49
2	*Crateva unilocularis*	1.41±2.13
3	*Diploknema butyraceae*	31.07±2.13
4	*Jatropha curcas*	17.51±0.49
5	*Oxalis corniculata*	6.78±0.85
6	*Woodfordia fruticosa*	16.95±2.24

Note: Data expressed as mean value ± standard deviation (n=3)

4. Conclusion

Our results demonstrated that methanolic extract of *Diploknema butyraceae* and *Woodfordia fruticosa* have high phenol and flavonoid contents along with high free radical scavenging property. Furthermore, this is the first report to identify the antioxidant and tyrosinase inhibitory activity of leaves of *Diploknema butyraceae*. Hence, further study is necessary to identify the active components responsible for antioxidant property and safety of these plant extracts.

5. Acknowledgements

The authors are highly grateful to School of Health and Allied Sciences, Pokhara University, Nepal for giving us the platform and institutional facilities for the research work. We would also like to show our humble gratitude towards the whole team of NITEC for facilitating us with required chemicals and equipments. We are indebted to Dr. Nirmala Jamarkattel Pandit, Reader, School of Health and Allied Sciences, Pokhara University for their continuous encouragement and support during our research. Our sincere thanks goes to Dr. Suvadra Karki, BAMS, Mr. Prakash Bhandari, Ayurveda Practitioner.

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