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Full Length Research Paper

Phytochemical and antioxidant studies of methanol and chloroform extract from leaves of *Azadirachta indica* A. Juss. in Tropical region of Nepal

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This research was carried out with the aim of phytochemical analysis and determining antioxidant activity present in methanol and chloroform leaf extracts of *Azadirachta indica*. Due to its potential in curing various ailments as well as wide spread application of antioxidant activity such as in the field of cosmetology, the plant was selected for the study. The total phenolics contained in the plant extracts were also studied which are responsible for the antioxidant activity. Antioxidant activity of the extracts were evaluated by diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method using ascorbic acid as standard in the concentration of 100, 50, 25 and 12.5 µg/ml. Phytochemical analysis were done with the established procedure and total phenolic content (TPC) was determined by using Folin-Ciocalteu colorimetric method. Phytochemical screening revealed the presence of similar constituents in both methanol and chloroform extracts such as alkaloids, glycosides, carbohydrate, phenol, flavonoid, steroids, protein, and amino acids. Total phenolic content in methanol and chloroform extracts were 207.39 ± 8.77 and 58.08 ± 4.41 mg gallic acid equivalent (GAE)/g, respectively. The inhibitory concentration (IC_{50}) value for methanol and chloroform extracts of *A. indica* were calculated and found to be 80.28 and 439.60 µg/ml, respectively. The finding suggests that methanol extract of the plant has significantly more antioxidant activity than the chloroform extract as clarified by total phenolics contained in the plant.

Key words: Phytochemical screening, antioxidant activity, total phenolic content, *Azadirachta indica*.

INTRODUCTION

Among various sources of medicine, plants have been known to contribute a crucial role in the health service as three quarters of world population relies on it and its extract for health care (Kunwar et al., 2006; Thomson, 2010). Although, Nepal is a small country, it has got many plants with medicinal and aromatic values due to geographical diversity. Most of the plants are being used in traditional medicine; however, some are not explored scientifically for their medicinal value yet (Lin et al., 2007).

Preliminary screening of phytochemicals is a valuable step for detecting various bioactive principles present in

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the plants which paved the way for drug discovery (Yadav et al., 2014). The presence or absence of such bioactive principles depends largely on the extent of accumulation, geographical location, method of collection, extraction procedure, amount of plant material used, and the analytical method employed (Yusuf et al., 2014). *Azadirachta indica* primarily comprised of several secondary metabolites including steroids, triterpinoids, reducing sugars, alkaloids, phenolic compounds, flavonoids, and tannins (Vinoth et al., 2012). Free radicals which have one or more unpaired electrons are produced as a result of metabolism in normal or pathological cell which role is crucial in cell injury accompanied by ageing and wide range of degenerative diseases including inflammation, cancer, atherosclerosis, diabetes, liver injury, Alzheimer, Parkinson and coronary heart pathologies (Halliwell, 1995; Erdemoglu et al., 2006; Gutteridge, 1994). Antioxidants prevent occurrence of these diseases by inhibiting the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress (Durackova, 2010; Reuter et al., 2010). Thus, there is a need for isolation natural antioxidants having less or no side effects in order to displace synthetic antioxidants which are possible promoter of carcinogenesis (Kaur and Arora, 2009; Newman and Cragg, 2007).

MATERIALS AND METHODS

Study species

A. indica A. Juss (widely known as Neem in vernacular name), is a versatile plant belonging to the family Meliaceae which is inhabitant to tropical and subtropical parts of the world (NEEM, 2016; Sombatsiri et al., 2005). Mainly, two species of *Azadirachta* have been reported, *A. indica* A. Juss, native to Indian subcontinent and *Azadirachta excelsa* kack, confined to Philippines and Indonesia (Pankaj et al., 2011). The leaves of *A. indica* are imparipinnate, alternate, exstipulate; leaflets are alternate or opposite, very shortly stalked, ovate-lanceolate, attenuate at the apex, unequal at the base and are medium to dark green in colour (Ali, 2012). Almost every parts of neem is being used in traditional medicine for treating variety of human ailments as it possess manifold of biological activities such as antiallergenic, antidermatic, antifeedant, antifungal, anti-inflammatory, antioxidant, antipyorrhoeic, antiscabic, diuretic, etc (Biswas et al., 2002).

Plant and chemicals

The leaves of *A. indica* were collected from Paklihawa, Siddharthanagar Municipality, Rupandehi district of Nepal in the month of August, 2015. The leaves were identified from Department of Environmental Sciences, Institute of Agriculture and Animal Sciences, Tribhuvan University. All the chemicals used in the experiment were of analytical grade and were purchased from S.d. Fine-Chem Ltd; Himedia Laboratories Pvt. Ltd. and Qualigens Fine Chemicals.

Preparation of extracts

The leaves of the plant were washed with distilled water, dried at room temperature in the laboratory for 3 weeks to obtain consistent weight and were powdered using mechanical grinder. About 200 g of the crushed leaves were extracted by maceration using pure methanol as solvent and other 200 g crushed leaves via chloroform for 7 days with frequent agitation. The extracts were filtered using Buckner Funnel and Whatmann No. 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure by Rotary vacuum evaporator below 40°C. Extract was stored at 4°C in air tight container with proper labeling.

Phytochemical screening

Phytochemical analysis was carried out for alkaloids, glycoside, saponin, steroid, phenol, flavonoid, tannin, protein, and amino acids and performed as mentioned by the authors (Yusuf et al., 2014; Tiwari et al., 2011). Mayer's and Hager's reagents were used to detect the presence of alkaloids; Molish's and Fehling's reagents were used for carbohydrate; Legal's test was used for glycosides; Froth and foam test were used for saponin; Salkowski's test was used for steroid; Ferric chloride test was used for phenol; Alkaline reagent and Lead acetate test were used for flavonoid; Ferric chloride and bromine water test were used for tannins and xanthoproteic test was used for the proteins and amino acids.

Total phenolic content

Preparation of standard

The total phenolic content in plant extracts was determined by using spectrophotometric method based on oxidation-reduction reaction with some modifications (Stanković, 2011). Various concentrations of gallic acid solutions in methanol (0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.025, and 0.0125 mg/ml) were prepared. In a 20 ml test tube, 1 ml gallic acid of each concentration was added, 5 ml of Folin-Ciocalteu reagent (10%) and 4 ml of 7% Na_2CO_3 were added to get a total volume of 10 ml. The blue coloured mixture was shaken well and incubated for 40 min at 40°C in a water bath. Then, the absorbance was measured at 760 nm against blank. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

Preparation of sample

Two different concentrations of the extracts (1, 0.1 mg/ml) were prepared. Following the procedure described for standard, absorbance for each concentration of extract was recorded. Total phenolics content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). The total phenolic contents in all samples were calculated the using the formula $C = c \times V/m$; where C = total phenolic content mg GAE/g dry extract, c =concentration of gallic acid obtained from calibration curve in mg/ml, V = volume of extract in ml, and m = mass of extract in gram.

Antioxidant activity by diphenyl-β-picrylhydrazyl (DPPH) scavenging

The antioxidant activity was accessed by standard protocol, that is, spectrophotometric method (Subedi et al., 2012). Stock solution of 100 μM DPPH in methanol was made. Test sample of the extract were made at different concentration (12.5, 25, 50, and 100 μg/ml) in methanol. Similarly, reference sample of ascorbic acid were made at similar concentration. Two milliliters of 100 μM DPPH was

Table 1. Phytochemical screening of methanol and chloroform extract of *A. indica*.

S/N	phytochemical tests	Reagents used/Test performed	Result	
			Methanol	Chloroform
1	Alkaloid test	Mayer's reagent	+	-
		Hager's reagent	+	+
2	Carbohydrate test	Molish's reagent	+	+
		Fehling's reagent	-	+
3	Glycoside test	Legal's test	+	-
4	Saponin test	Froth test	-	-
		Foam test	+	-
5	Steroid test	Salkowski's test	+	+
6	Phenol test	Ferric chloride test	+	+
7	Flavonoid test	Alkaline reagent test	+	-
		Lead acetate test	+	+
8	Tannin test	Ferric chloride test	+	+
		Bromine water test	-	+
9	Protein and amino acid	Xanthoproteic test	+	-

+: Present; -: Absent.

added to 2.0 ml of each methanol and chloroform extract of *A. indica* at different concentration and kept in dark. Similarly, 2.0 ml of 100 µM DPPH was mixed with 2.0 ml of methanol and ascorbic acid and kept in dark for 30 min in incubator at 37°C. The absorbance was measured at 517 nm by UV spectrophotometer after 30 min and % scavenging was calculated by the following equation:

$$\text{Percentage scavenging} = (Ao - A_T) / A_0 \times 100\%$$

where AO = Absorbance of DPPH solution and AT = Absorbance of test or reference sample. The % scavenging was then plotted against concentration and regression equation was obtained to calculate IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%) values.

Statistical analysis

All the data were expressed as mean value ± standard error of mean (SEM) of the number of experiments (n=3). Microsoft EXCEL program 2010 and Statistical Package for Social Sciences, Version 16.0 (SPSS V.16.0) were used for data analysis.

RESULTS

Phytochemical screening

The preliminary phytochemical screening of the extracts in methanol and chloroform extract revealed the presence of different phytochemicals which are presented in Table 1. The two extracts of plant showed the presence of similar phytochemicals such as alkaloids, glycosides, carbohydrate, phenol, flavonoid, steroids, protein, and amino acids.

Total phenolic content

The content of total phenol (TPC) was determined by using Folin-Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: $y=0.013x+0.252R^2$, 0.991). Total phenolic content in methanol extract was 207.39 ± 8.77 mg GAE/g which is significantly higher than chloroform extract and was found to be 58.08 ± 4.41 mg GAE/g of sample of dry weight as shown in Figure 1.

Antioxidant activity

Free radical scavenging activity of all the extracts and standard ascorbic acid increased with the increase in concentration. The maximum percentage inhibition of DPPH free radical at 517 nm is exhibited by standard ascorbic acid followed by methanol extract and chloroform extract of *A. indica* as shown in Figures 2 to 4.

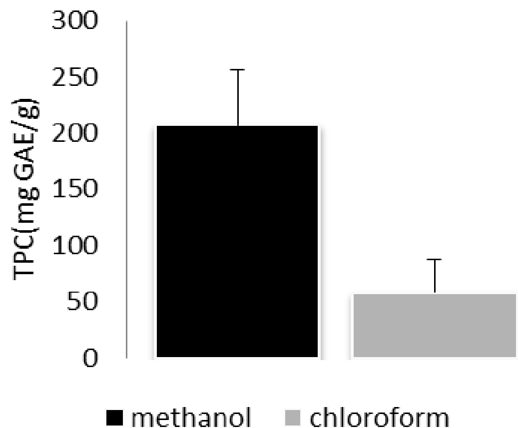

Figure 1. Total phenolic content (TPC) in different neem extract.

Figure 3. Antioxidant activity before absorbance measurement in UV spectrophotometry.

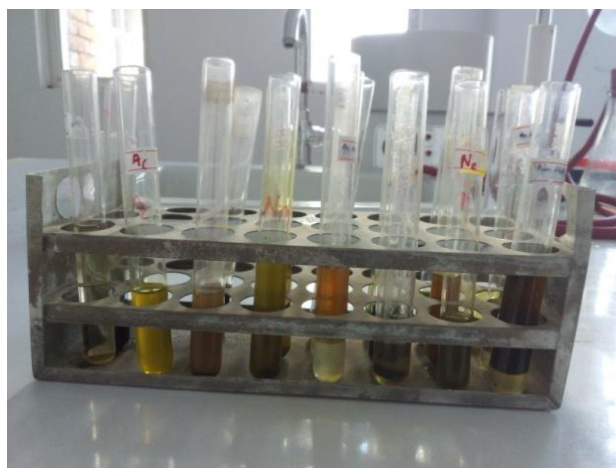

Figure 2. Phytochemical screening of extracts.

The inhibitory concentration IC_{50} value was compared which was found to be 80.28 and 439.60 μg/ml for methanol and chloroform extract of *A. indica*, respectively.

DISCUSSION

In recent years, the use of plants in herbal medicine possessing antioxidant property has been on the rise due to its potential in ameliorating various diseases. Various experimental studies have showed oxidative cellular damage arising due to imbalance between free radical generating and scavenging systems ultimately being the cause of cardiovascular diseases, cancer, aging, etc. Methanol and chloroform were the solvent system used for extraction as both the polar and non- polar components present in the plant can be extracted.

Phytochemicals such as phenol, flavonoid, alkaloid, glycoside, tannin, etc., were present in the extract which may be responsible for antioxidant activity as per the previous similar study conducted (Govindappa and Poojashri, 2011). Total phenolic content on methanol and chloroform extract is 207.39 ± 8.77 mg and 58.08 ± 4.41 mg GAE/g, respectively which is proportional to the antioxidant activity, that is, TPC exhibits positive correlation with the antioxidant activity (Karamian and Ghasemlou, 2013).

It is implied that plant extract contain compounds such as phenols, flavonoids, etc., which can donate hydrogen to a free radical in order to remove odd electron indicating its usefulness in various radical related pathological condition. Antioxidant activity test based on measurement of absorbance at 517 nm where all the extracts showed positive radical scavenging activity suggesting plant selected for the study was potently active (Aiyegoro and Okoh, 2010). Ascorbic acid is used as positive control which showed high percentage inhibition of free radicals about 95.65 to 96.66%; pattern of inhibition being similar at variable concentration due to the fact that it possesses high radical scavenging activity, that is, this concentration is sufficiently high to scavenge free radicals. Methanol extract of *A. indica* has shown the highest activity followed by its chloroform extract which coincide with the previous study (Sri et al., 2012). The antioxidant activity shown by methanol extract was much higher than that of chloroform extract which may be due to the presence of polar compounds like phenols, flavonoids, etc., that are soluble in methanol (Bhusal et al., 2014).

Conclusion

Thus, it can be concluded that methanol extract of neem possess better antioxidant activity than that of the

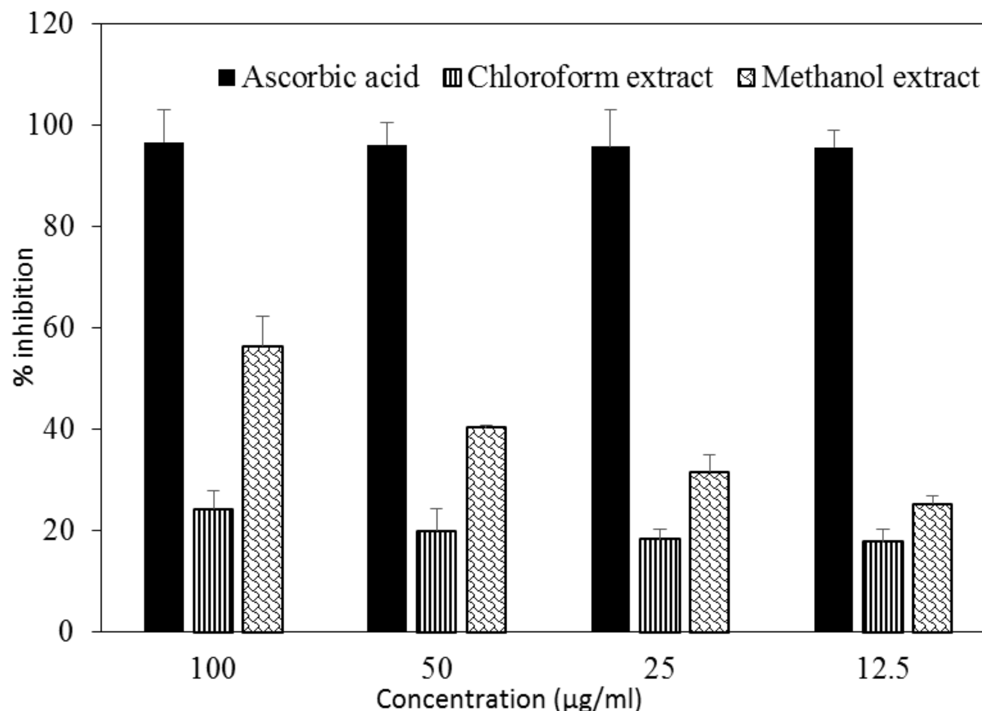

Figure 4. Percentage inhibition of DPPH free radical by Standard and extracts at 517 nm.

chloroform extract under similar condition, due to the presence of variable type and quantity of phytochemicals supporting its medicinal and cosmetic use in Nepal and globally. Further studies are required to evaluate *in vivo* antioxidant potential and also isolation and characterization of active compounds for commercialization in the field of pharmaceuticals.

Conflict of Interests

The authors have not declared any conflict of interests.

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