African Journal of Biotechnology Vol. 8 (13), pp. 3084-3091, 6 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Antioxidant activity and quantitative estimation of azadirachtin and nimbin in *Azadirachta Indica* A. Juss grown in foothills of Nepal

Amal Kumar Ghimeray[1], Cheng-Wu Jin[1], Bimal Kumar Ghimire[2] and Dong Ha Cho[1]*

[1]School of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, South Korea.
[2]Bioherb Research Institute, Kangwon National University, Chuncheon, South Korea.

Accepted 17 June, 2009

The leaf and bark fraction extracts of *Azadirachta indica* A. Juss. (neem) grown in the foothills (subtropical region) of Nepal were evaluated for their antioxidant activity, total phenolic (TP) and total flavonid (TF) contents. HPLC method was employed to quantify the amount of azadirachtin and nimbin present in the seed, leaf and the bark extracts of neem. The result showed that the highest azadirachtin content was found in the methanolic extract of the seed (3300 µg/g dw). Similarly, the hexane fraction of bark showed the highest nimbin content (271 µg/g dw) followed by the methanolic extract (260 µg/g dw). Antioxidant activity was determined by measuring 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity, hydroxyl radical scavenging activity, DNA protection assay, metal chelating and the inhibition of peroxidation using linoleic acid system and their results were found at different magnitudes of potency. The results of TP content expressed in tannic acid equivalents ranged from 66.63 to 629.04 µg/mg in the bark extracts and 23.85 to 237.00 µg/mg in the leaf extracts. Likewise, the content of TF expressed in quercetine equivalents ranged from 12.87 to 17.07 µg/mg in the bark and 13.72 to 93.17 µg/mg in the leaf extracts.

Key words: *Azadirachta indica* A. Juss (neem), antioxidant, azadirachtin, flavonoid, foothills (subtropical region), HPLC, nimbin, polyphenol.

INTRODUCTION

Azadirachta indica A. Juss, known as neem in vernacular, belongs to the family meliaceae and is widely distributed in Asia, Africa and other tropical parts of the world (Sombatsiri et al., 1995). In Nepal, neem plants are distributed in the Terai (tropical) and the foothills (subtropical) of the country. Neem is a versatile medicinal plant, almost every part of which is being used in folklore and traditional systems of medicine for the treatment of a variety of human ailments. Traditionally, most of the Nepali people clean their teeth with neem twigs, take its juice as a tonic to increase appetite and use it in fever or to remove intestinal worms. Neem oil, bark and leaf extracts have been therapeutically used as folk medicine to control diseases like leprosy, intestinal helminthiasis, respiratory disorders, constipation, and skin infections (Biswas et al., 2002).

However, apart from these uses, there are several reports on the biological activities and pharmacological actions based on modern scientific investigations, such as antiviral (Gogati and Marathe 1989), antibacterial (Singh and Sastry 1997), antifungal (Kher and Chaurasia 1997), anti-inflammatory and antipyretic (Okpanyi and Ezeukwu 1981), antiseptic, antiparalitic (Allan et al., 1999), antioxidant (Bandyopadhyay et al., 2002; Sultana et al., 2007), etc.

Phenolic compounds and flavonoids are very important plant secondary metabolites. These compounds have numerous defense functions in plants, and thus several environmental factors, such as light, temperature, humidity, and internal factors, including genetic differences, nutrients, hormones, etc., contribute to their synthesis (Strack 1997). Similarly, other factors, such as germination, degree of ripening, variety, processing and storage,

*Corresponding author. E-mail: chodh@kangwon.ac.kr. Tel: 82-33-250-6475. Fax: 82-33-250-6470

also influence the content of plant phenolics (Bravo, 1998). It is reported that the phenolics are responsible for the variation in the antioxidant activity of the plant (Cai et al., 2004). They exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Pokorney, 2001; Pitchaon et al., 2007) or chelate metal ions and protect against pathogens and predators (Balasundram et al., 2006).

The Neem tree is also considered as a natural insecticide/pesticide plant and the quality of pesticide and pharmacological products depend upon the contents of azadirachtin and nimbin in the plant (Sidhu et al., 2004). Azadirachtin, a major compound of the neem has potent antifedent, growth and reproductive regulating properties. Likewise, nimbin, a limonoid from neem, is also involved in improving pesticide properties (Sidhu et al., 2004). Therefore, their quantification is important to understand the insecticidal/pesticidal property of the neem tree.

The neem tree from the different tropical parts of the world is reported to contain high level of polyphenolic compounds (Siddiqui et al., 1992, Sultana et al., 2007), but due to the wide range of geographical distribution, a large variety of morphological and biochemical characteristics have been reported (Ermel, 1995). It has also been re-ported that phenolic contents of neem can be influenced by geographical locations and other abiotic factors (Ermel et al., 1986; Kaura et al., 1998; Kaushik et al., 2007).

Therefore, taking into account of these pervious reports that the concentration of phenolic compounds can alter the antioxidant activity and also the concentration of azadirachtin and nimbin cause difference in the insecticidal-pesticidal property of neem, here the total polyphenol, total flavonoid and antioxidant activities of leaf and bark extracts of neem is investigated in a comparative way. Also the quantity of azadirachtin and nimbin content in the neem (seed, bark and leaf) using different solvents was estimated.

MATERIALS AND METHODS

Collection of plant materials

Neem (leaf and bark) was collected from the foothills (subtropical region) of the Eastern province (Mechi Anchal) of Nepal in the month of early June in 2008. The young leaves (eight to twelve leaflets) and old barks were collected, cleaned, washed under running tap water and sun dried. The seeds were collected in the end of August month (2008) from the same place and brought to the Kangwon National University (South Korea) for analysis.

Preparation of plant extracts

The powdered neem leaf and bark samples (30 g each) were suspended and extracted with 500 ml of 80% methanol (v/v) and kept for 2 days in a shaker at room temperature. The extracts were filtered through Advantec 5B Tokyo Roshi Kaisha ltd, Japan. The extraction of the residue was repeated twice using the same conditions. The methanolic extract was first dried using a vacuum rotary evaporator (EYLA N-1000, Tokyo, Japan) in a 37°C water bath and then successively fractionated with equal volumes of hexane, ethyl acetate and n-butanol, leaving behind residual water soluble fraction. The fractionated sample of Neem was evaporated under the reduced pressure using a vacuum rotary evaporator. Dried samples were weighed and kept at -20°C for further analysis.

Free radical scavenging activity

The antioxidant activity of Neem (leaf and bark) on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined according to the method described by Bracca et al. (2003) with slight modification. Briefly, an aliquot of 1 ml extract at the concentration of 0.1 mg/ml was added to 4 ml of 0.15 mM methanol solution (80% in water v/v) of DPPH. The mixture was shaken vigorously and left to stand for 30 min at room temperature in the dark. Vitamin C (0.1 mg/ml) was taken as positive control. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm and the percent inhibition activity was calculated.

Scavenging ability on hydroxyl radicals

The scavenging ability of the test extracts on OH was based on the deoxyribose method described by Aruoma (1994), with slight modification. The reaction mixture containing $FeSO_4$ (10 mM, 0.2 ml), EDTA (10 mM, 0.2 ml), H_2O_2 (10 mM, 0.2 ml) and 2-deoxy-D-ribose (10 mM, 0.2 ml) were mixed with 200 µl of extract at the concentration of 1 $mgml^{-1}$, followed by the addition of 1 ml phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.1M, pH 7.4). The mixture was incubated for 4 h at 37°C in a water bath. After incubation, 1 ml of 1% thiobarburitic acid (TBA) and 1 ml of ice cooled 2.8% TCA (trichloro acetic acid) were added to the resultant reaction mixture and boiled for 10 min at 100°C. Finally, the reaction mixture was cooled, centrifuged at 800 x g for 10 min and the absorbance of the supernatant was measured at 532 nm. Butylated hydroxytoluene (BHT) was used as a positive control at 0.1 mg/ml concentration.

DNA damage protection assay

The method of hydroxyl radical-induced DNA damage in plasmid pBR 322 was as described previously by Qian et al. (2008) with slide modification. To test for DNA damage induced by hydroxyl radicals, the reaction was conducted at a total volume of 15 µl containing 0.5 µl of the plasmid pBR 322 DNA in 4 µl of Neem extract (leaf and bark fraction extracts), 3 µl of 5 mM $FeSO_4$, and 4 µl of 50 mM PB buffer (pH 7.4), followed by the addition 4 µl 30% H_2O_2. The resulting mixture was incubated for 30 min at 37°C. The DNA (super-coiled, linear and open circular) was analyzed on 1% agarose gels and visualized using ethidium bromide staining.

Fe^{2+} chelating activity

The chelation of ferrous ions by the extract was estimated by the method of Decker and Welch (1990). In brief, 1 ml of extract at 1 mg/ml concentrations was mixed with 3.7 ml of methanol and 0.1 ml of $FeCl_2$ (1 mM). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine, followed by shaking vigorously and left to stand at room temperature for 10 min. EDTA (0.5 mg/ml) was taken as positive control in the assay. The absorbance was measured spectrophotometrically at 562 nm and the percent inhibition was calculated and compared.

Lipid peroxidation assay

The antioxidant activity of the leaf and bark extracts of neem was determined according to the peroxidation in the linoleic acid system mentioned previously by Sultana et al. (2007) with slight modification. The extracts (2 mg) of each treatment were added to a solution mixture of linoleic acid (0.056 ml), absolute ethanol (5 ml) and 0.2 M sodium phosphate buffer 5 ml (pH 6.9). The total mixture was diluted to 12.5 ml with distilled water. The solution was incubated at 40°C for 24 h and 5 ml of ethanol (75%), 0.1 ml of an aqueous solution of ammonium thiocyanate (30%), 0.1 ml of sample solution (incubated) and 0.1 ml of ferrous chloride (0.2 mM) solution in 3.5% HCl were added sequentially. After 3 min, the absorption values of the mixtures measured at 500 nm were taken as the peroxide contents. A control was tested with linoleic acid but without extracts. Butylated hydroxytoluene (BHT 0.2 mg/ml) was used as a positive control. The percentage inhibition of peroxidation was calculated to express antioxidant activity.

Estimation of total phenolic content

The total phenolic compound content was determined by the Folin-Ciocalteu assay, following the method described by Kim et al. (2007). A sample aliquot of 200 µl of each extract (1 mg/ml) was added to a test tube containing 200 µl of phenol reagent (1 M). The volume was increased by adding 1.8 ml of distilled deionized water and the solution was allowed to stand for 3 min for reaction after vortex. Further to continue reaction, 400 µl of Na_2CO_3 (10% in water, v/v) was added and vortexed and the final volume (4 ml) was adjusted by adding 1.4 ml of distilled deionized water. A reagent blank was prepared using distilled deionized water. The absorbance was measured at 725 nm after incubation for 1 h at room temperature. The total phenolic content was expressed as tannic acid equivalents (TAE) in µg/mg of sample.

Estimation of total flavonoid content

The total flavonoid content was determined according to Eom et al. (2007). Briefly, an aliquot of 0.5 ml of sample (1 mg/ml) was mixed with 0.1 ml of 10% Aluminum nitrate and 0.1 ml of potassium acetate (1 M). In the mixture, 4.3 ml of 80% methanol was added to make the total volume of 5 ml. The mixture was vortexed and the absorbance was measured spectrophotometrically at 415 nm and calculated. Quercetine was used as a standard.

HPLC analysis

To compare chemical profile of different extracts, we performed quantitative HPLC analyses of the azadirachtin and nimbin. An HPLC system (CBM-20A, Shimadzu Co, Ltd., Japan) with two gradient pump systems (LC-20AT, Shimadzu), an auto sample injector (SIL-20A, Shimadzu), a UV-detector (SPD-10A, Shimadzu) and a column oven (35°C CTO-20A, Shimadzu) were used for analysis. The separation was performed on a C_{18} column (Synergi 4u MA-RP 80A, 150 × 4.6 mm, 4 micron Phenomenex). HPLC conditions were as follows: solvent A (water) and solvent B (acetonitrile). A gradient elution used was 0-10 min, 30-40% B; 10-15 min, 40-45% A; 15-20 min, 45-50% B; 20-25 min, 50-60% B; 25-35 min, 60-70%. Flow rate of mobile phage solution was 1.0 ml/min, and detection was at 217 nm. 10 µl of each sample was injected in to the HPLC machine.

Statistical analysis

All data were expressed as mean value ± standard deviation (SD) of the number of experiments (n=3). Microsoft EXCEL program was used for data analysis.

RESULTS AND DISCUSSION

The effect of different solvents on the yields of Neem leaf and bark

A significant variation in the yields of neem bark was shown using various fraction solvents. The yields (%) of bark using water, butanol, ethyl acetate and hexane were 57.94, 21.57, 13.6 and 3.16, respectively. Likewise, the Neem leaf and seed also followed the same order as the bark. The yields (%) of leaf using water, butanol, ethyl acetate and hexane were 58.2, 18.76, 16.76 and 3.85% respectively and that of seed were 56.54, 21.66, 13.65 and 6.33% respectively. This variation in yield may be due to the polarity of the solvent used in the extraction process.

DPPH radical scavenging activity

The effects of phenolic compounds on DPPH radical scavenging are thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Siddaraju and Dharmesh 2007). It is reported that the decrease in the absorbance of DPPH radical caused by phenolic compound is due to the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radical by hydrogen donation and is visualized as a discoloration from purple to yellow (Meir et al., 1995). Table 1 shows the results of free radical (DPPH) scavenging activity of neem leaf and bark extracts, expressing the activity in percentage inhibition. The result revealed that the water fraction of the bark exhibited the highest radical scavenging activity with 93.11% followed by butanol with 91.3%, ethyl acetate 83.73% and hexane the lowest with 8.89%. The methanolic crude extract of bark also showed a higher scavenging activity with 82.45%. In contrast to bark, the leaf exhibited a poor scavenging activity. Among the leaf extracts, butanol fraction showed comparatively higher scavenging ability (nearly 33%) than other fractions. In overall comparison between the bark and the leaf, it can be conformed that the bark shows higher free radical scavenging activity at the concentration of 0.1 mg/ml than the standard (vitamin C, 92%) used in the experiment. However, in the result, the increase in the free radical scavenging capacity of the bark extracts can be explained by the presence of higher phenolic contents (Siddhuraju et al., 2002).

Scavenging ability on hydroxyl radicals

Hydroxyl radical is the most reactive among ROS (reac-

Table 1. DPPH free radical and Hydroxyl (OH) radical scavenging activity of bark and leaf fraction extracts of neem expressed in percentage and shown in comparison.

Solvent used	DPPH scavenging (%)		Hydroxyl (OH) radical scavenging (%)	
	Bark	Leaf	Bark	Leaf
Methanol crude	82.45 ± 1.06	12.56 ± 2.04	87.84 ± 0.09	36.47 ± 2.06
Hexane	8.89 ± 1.67	9.00 ± 1.15	84.89 ± 0.21	36.17 ± 2.32
Ethyl acetate	83.73 ± 1.90	15.00 ± 1.19	86.38 ± 0.08	38.72 ± 0.60
n-Butanol	91.03 ± 1.03	33.25 ± 0.95	87.87 ± 0.12	35.72 ± 2.36
Water	93.11 ± 0.96	5.83 ± 3.45	92.12 ± 0.13	43.86 ± 1.24
Vitamin C (standard)	92.00 ± 2.82	ND	ND	ND
BHT (standard)	ND	ND	88.89 ± 2.03	ND

All the samples were used at the concentration of 0.1 mg/ml for DPPH free radical and 1 mg/ml concentration was used for the OH radical scavenging activity. Vit. C at 0.1 mg/ml and BHT at 0.1mg /ml of concentration were used as standard. ND = Not determined.

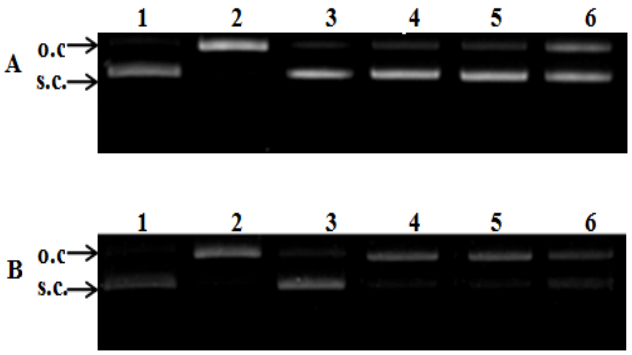

Figure 1. Effect of neem bark (A) and leaf (B) extracts on the protection of supercoiled DNA against ~OH generated by the H_2O_2. Lane 1: plasmid DNA (positive control); Lane 2: DNA+Fenton's reagent (DNA damage control); Lanes 3 – 6 of Figure 1 A and B: hexane, ethyl acetate, butanol and water fraction sample respectively of neem at the concentration of 1 mg/ml in presence of Fenton's reagent. S.C. and O.C. represents supercoil DNA and open circular DNA, respectively.

tive oxygen species) and can react with lipids, amino acid, polysaccharides and nucleotides in the body (Hsu et al., 2006) and the prevention of such harmful reactions is highly advantageous in terms of both human health and the shelf-life of foodstuffs, cosmetics and pharmaceuticals (Sasaki et al., 1996; Branen and Davidson 1997). The OH radicals scavenging ability, formed in the body is an effective antioxidant property. Neem extracts specially that from bark showed strong scavenging ability on OH radicals (Table 1).

The water fraction of bark scavenged at the highest percentage with 92.12% at the concentration of 1 mg/ml. Likewise, butanol (87.87%), ethyl acetate (86.38%) and hexane (84.89%) fractions also showed a higher percentage of scavenging activity than that of the leaf fractions (water 43.86%, ethyl acetate 38.7%, hexane 36.1% and butanol 35.6%). The crude methanolic extract of bark and leaf scavenged nearly 87.84 and 36.47% respectively. This scavenging activity of bark is comparable with the positive control (BHT, synthetic scavenger) which scavenged 88.41% of hydroxyl radical at the concentration of 0.1 mg/ml.

DNA damage protection assay

Oxidative DNA damage has been implicated in various degenerative diseases. The antioxidant effect of the extract was evaluated by examining the protective effect on hydroxyl radical induced plasmid pBR 322 DNA. The Plasmid pBR 322 DNA is made up in three forms, supercoiled (undamaged), open circular and linear forms (damaged). When plasmid DNA was treated with Fenton reagent for 30 min, the intact supercoiled DNA was broken into an open circular form compared with the untreated DNA (Figure 1). According to the result, DNA treated with neem (bark and leaf) fractions at the concentration of 1 mg/ml showed a protective effect on hydroxyl radical induced DNA damage.

Comparatively, all the fractions of bark (hexane, ethyl acetate, butanol and water of lane 3-6 respectively) showed a stronger protective effect (Figure 1A) than that of the leaf fractions (hexane, ethyl acetate, butanol and water of Figure 1B, lane 3-6 respectively). This result is consistent with the result of hydroxyl radical scavenging activity of the leaf and bark fractions extracts of Table 1. This result supports the antioxidant property (DNA protection) shown by Bandyopadhyay et al. (2002), in which they reported that the neem bark extract can protect human gastric mucosal DNA from OH mediated damage *in vitro*.

Metal chelating activity

Transition metal has played a pivotal role in the generation of oxygen free radicals in living organisms. Chela-

Table 2. Metal chelating activity and inhibition percentage of bark and leaf extracts of neem against linoleic acid peroxidation at 2 mg/ml of concentration.

Solvent used	Linoleic acid peroxidation inhibition (%)		Metal chelating activity (%)	
	Bark	Leaf	Bark	Leaf
Methanol Crude	55.46 ± 0.33	46.52 ± 0.86	74.71 ± 0.78	47.51 ± 1.14
Hexane	57.46 ± 0.65	51.59 ± 0.72	66.09 ± 1.71	56.91 ± 0.57
Ethyl Acetate	52.60 ± 0.43	53.04 ± 0.57	60.23 ± 2.21	59.08 ± 0.64
n-Butanol	54.56 ± 0.07	45.07 ± 1.44	60.69 ± 1.64	54.08 ± 2.99
Water	62.17 ± 0.86	47.52 ± 1.08	70.43 ± 0.57	56.96 ± 0.49
BHT (standard).	98.34 ± 0.78	ND	ND	ND
EDTA (Standard)	ND	ND	98.22 ± 0.45	ND

The result is expressed in percentage. BHT and EDTA were used as positive controls. ND = Not determined.

Table 3. Total polyphenol (TPF) and Total Flavonoid (TF) contents in the bark and the leaf extracts of neem in different solvents used. TPF and TF contents were expressed in tannic acid (TAE) and quercetin (QE) equivalent, respectively.

Solvent used	Total Polyphenol contents (TAE µg/mg)		Total Flavonoid contents (QE µg/mg)	
	Bark	Leaf	Bark	Leaf
Methanol crude	651.07 ± 1.30	126.72 ± 3.84	14.21 ± 1.22	32.50 ± 1.95
Hexane	66.63 ± 1.67	23.85 ± 0.37	13.41 ± 1.28	20.67 ± 2.32
Ethyl Acetate	500.33 ± 8.70	149.59 ± 5.74	17.07 ± 2.38	71.71 ± 3.35
n-Butanol	468.48 ± 2.78	237.00 ± 7.96	12.87 ± 1.22	93.17 ± 3.23
Water	629.04 ± 2.96	96.44 ± 2.22	14.94 ± 1.59	13.72 ± 2.20

Each value is expressed as the mean ± SD (n = 3).

ting agents may inactivate metal ions and potentially inhibit the metal-dependent processes (Finefrock et al., 2003). Transition elements like iron and copper are powerful catalysts of oxidation reactions because they contain one or more unpaired electrons that can enable to participate in electron transfer reactions (Lloyd et al., 1997). They can participate in the conversion of H_2O_2 to OH in the Fenton reaction and in the decomposition of alkyl peroxides to the heavy reactive alkoxyl and hydroxyl radicals (Hsu et al., 2006).

Due to this property, transition metal chelation to form low redox potential complexes can be an important antioxidant property (Halliwell et al., 1995). The metal chelating activity of bark and leaf fractions of neem at 2 mg/ml concentration is shown in Table 2. At the same concentration, the bark extracts showed a comparatively higher chelating activity than that of the leaf ones. The crude methanolic bark extracts chelate metal with 74.71% and the leaf extract with 47.51%. Among the fractions used, the water fraction showed a metal chelating activity of nearly 70%, followed by hexane (66%), ethyl acetate (60%) and butanol (60%).

Compared to the bark extracts, the leaf extracts showed relatively lower activities (water 56.96%, butanol 54.08%, ethyl acetate 59.08% and hexane 56.91%). Overall, both the neem bark and leaf possesses a better metal chelating property. This metal chelating property of the extracts may be attributed to their endogenous chelating agents, mainly phenolic compounds that have properly oriented functional groups, which can chelate metal ions (Thompson and Williams, 1976).

Lipid peroxidation assay

It has been reported that the lipid peroxidation is one of the causes of the occurrence of cardiovascular disease and cancer. Therefore, the inhibition of peroxidation by the plant extracts indicates its antioxidant potency. In this study, the peroxidation inhibition activity of the neem extract was measured and shown in Table 3. In statistical data, inhibition percentage of lipid peroxidation was high in the order of water (62.17%), hexane (57.46%), methanol crude (55.46%), butanol (54.56%) and ethylacetate (52.6%) at 0.2 mg/ml of concentration. Likewise, the leaf extracts inhibited 53, 51, 47 and 45% in ethyl acetate, hexane, water, methanol (crude) and butanol fractions, respectively. Our data revealed that the bark and the leaf extracts showed a good inhibition of lipid peroxidation formed during linoleic acid system.

The high peroxidation scavenging effect of neem may be due to the high contents of phenolic compounds or radical scavengers involved in the extracts (Duh et al., 1999) which can terminate the peroxidation chain reac-

Table 4. Estimation of azadirachtin and nimbin content in the seed, bark and leaf extracts of neem in different solvents.

Sample	Azadirachtin (µg/g dw)	Nimbin (µg/g dw)
Neem seed (methanol 80%)	3300	88
Neem seed Hexane	177	82
Neem leaf Hexane	ND	112
Neem leaf water	17	ND
Neem bark (methanol 80%)	ND	260
Neem bark Hexane	86	271
Neem bark butanol	ND	60

ND = Not determined.

tions easily (Soares et al., 1997) and quench reactive oxygen or nitrogen species, thereby inhibiting the oxidation of lipids and other biological molecules (Morton et al., 2000).

Determination of total phenolic contents

The total phenolic contents in the neem varied significantly between the bark and the leaf (Table 3). The methanolic crude extract showed the highest polyphenol content with 651.07 µg/mg in the bark and 126.72 µg/mg in the leaf. The water, ethyl acetate and butanol fraction of bark also showed higher phenolic content with 629.04, 500.33, 468.48 µg/mg (tannic acid equivalent) respecttively. However, the hexane fraction of bark showed weaker polyphenol content (66.63 µg/mg) among the solvents used. Likewise, the butanol and ethylacetate fractions showed higher phenolic contents in the leaf with 237 and 149.59 µg/mg respectively. The water and the hexane fraction showed the lowest polyphenol content (96.44 and 23.85 µg/mg, respectively). The above data revealed that the bark possesses higher amount of polyphenolic compounds compared to the leaf.

Determination of total flavonoid contents

The quantitative estimation of total flavonoid revealed that the leaf has higher flavonoid content than the bark (Table 3). The flavonoid contents of the leaf in the butanol and ethyl acetate fractions of leaf extracts were highest with 93.18 µg/mg and 71.71 µg/mg (quercetine equivalent), respectively. Likewise, the methanol crude extract contained 32.50 µg/mg, hexane with 20.67 µg/mg and water the lowest with 13.72 µg/mg. In contrast to the leaf, the bark exhibited lower flavonoid contents in all the fractions (ethyl acetate 17.07 µg/mg, water 14.94 µg/mg, methanol crude 14.21 µg/mg, hexane 13.41 µg/mg and butanol 12.87 µg/mg) used.

HPLC analysis

The quantitative analysis of azadirachtin and nimbin compounds in different extracts performed using HPLC is given in Table 4 and representative chromatograms are shown in Figure 2. These compounds were identified in solvents used by matching their retention times (RT) and spectra with those of the standards and the quantitative data were calculated on the basis of the peak area of each compound. The result revealed that the azadirachtin content was highest in methanolic extract of seed (3300 µg/g dw), followed by the hexane extract (177 µg/g dw). The hexane fraction of bark also showed the presence of azadirachtin (86 µg/g dw).

However, the leaf showed trace amount (17 µg/g dw) of azadirachtin content in water fraction. Likewise, the hexane fraction of bark showed highest amount of nimbin content (271 µg/g dw) followed by the methanolic extracts (260 µg/g dw) and butanol fraction (60 µg/g dw). Similarly, the hexane fraction of leaf also showed significant amount of nimbin content (112 µg/g dw). The methanolic crude extract and hexane fraction of seed showed 88 and 82 µg/g dw of nimbin content, respectively.

Overall, the HPLC result showed that the concentration of azadirachtin is concentrated in seeds than in the leaf and bark. And also, methanolic extract showed better azadirachtin content among the solvents used. In the above result, azadirachtin content in the methanolic extract is in agreement with the result (hot semi-arid with cold winter) of previous findings by Kaushik et al. (2007). This similarity in the result may be due to the fact that the foothills of Nepal has hot climate in the summer with cold winter. However, little variation in the azadirachtin and the nimbin content may be due to the origin (Ermel et al., 1986), genetic (Sidhu et al., 2004), climatic or geographical factors (Kaushik et al., 2007).

Conclusion

The results obtained in this study clearly demonstrate that all the tested leaf and bark extracts/fractions of neem grown in the foothills (subtropical region) have significant antioxidant properties. Though, in comparative study, bark was found to be more potent than the leaf in the entire assay with higher phenolic content, but, both the leaf

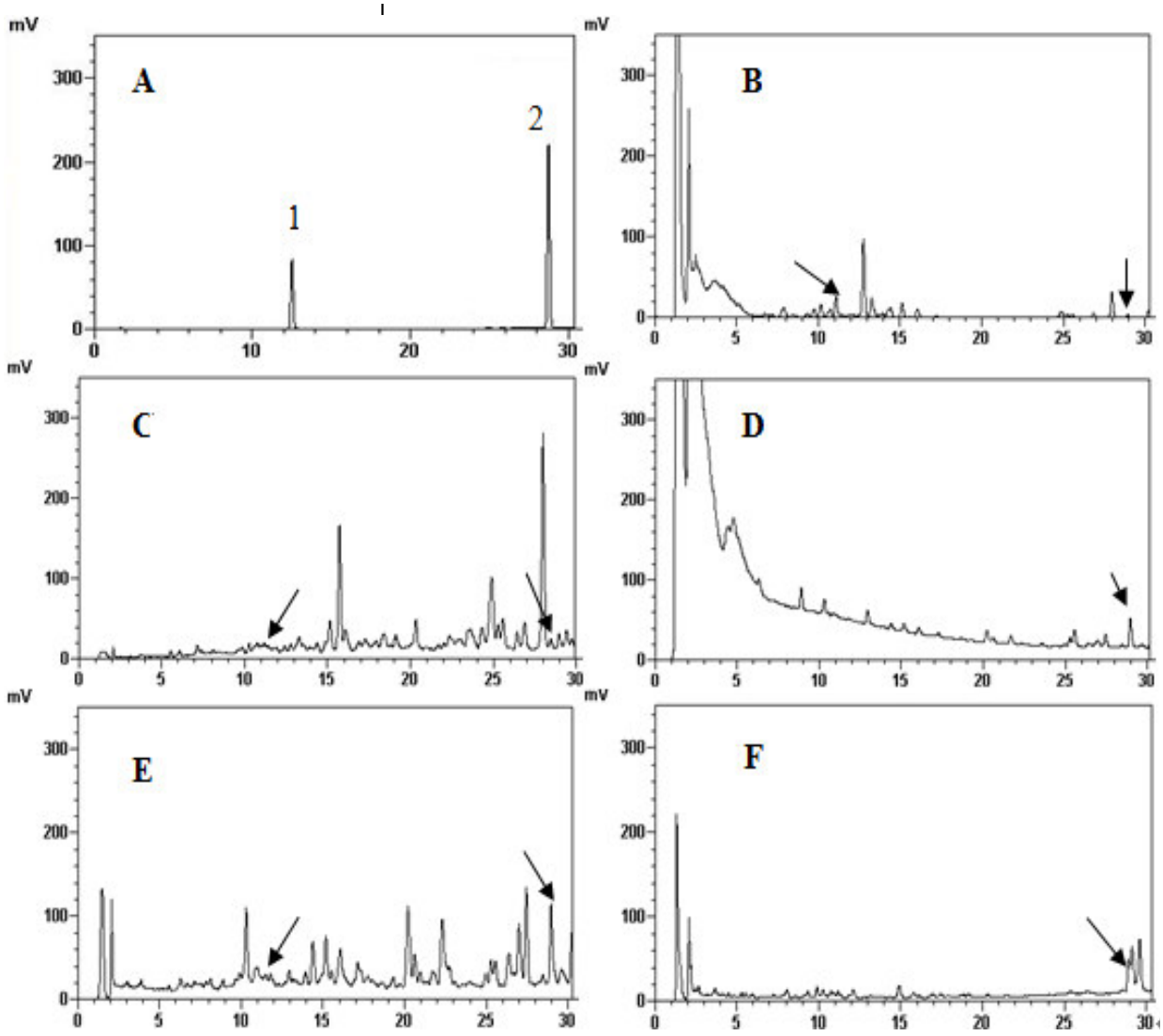

Figure 2. HPLC result of neem (seed, bark and leaf extracts) showing azadirachtin and nimbin. (A) Standard azadirachtin (1) and nimbin (2) with their retention time 12.76 min and 28.93 min, respectively. Arrow denotes the chromatogram of azadirachtin and nimbin in neem seed methanolic (B); seed hexane (C); bark methanolic (D); bark hexane (E); and leaf hexane extracts (F). Figures B, C and E show both the azadirachtin and nimbin content. Figures D and E show only nimbin content.

and the bark can be used in the pharmacological applications as a valuable antioxidant natural source and medicine. Likewise, the presence of significant amount of azadirachtin and nimbin in all the parts of neem grown in this region showed its potential use as natural insecticide.

ACKNOWLEDGMENTS

The author gratefully acknowledges financial support by the BK21 project, Korea. Prof. E. D. Morgan (Department of Chemistry, Keele University, Staffordshire, UK) is gratefully acknowledged for donating nimbin (standard sample) and also Mr. Hu Weicheng and Mr. Ram Chandra Kafle for helpful suggestions and manuscript corrections.

REFERENCES

Aruoma OI (1994). Deoxyribose assay for detecting hydroxyl radicals. Methods Enzymol. 233: 57-66.

Allan EJ, Stuchbury T, Mordue (Luntz) AJ (1999). *Azadirachta indica* A.Juss. (Neem tree): *In vitro* culture, micropropagation and the production of azadirachtin and other secondary metabolites. In: Bajaj YPS (Ed) Biotechnology in agriculture and forestry science series, Springer, Berlin Heidelberg NY, Medical aromatic plants, 43: 11-41.

Biswas KI, chattopadhyay R, Banerjee K, Bandyopadhyay U (2002). Biological activities and medicinal properties of Neem (*Azadirachta indica*). Curr. Sci. 82(11): 1336-1345.

Balasundram N, Sundram K, Samman S (2006). Phenolic com-pounds in plants and agri-industrial by-products: Antioxidant activity, occur-

rence, and potential uses. Food Chem. 99: 191-203.
Branen AL, Davidson PM (1997). Use of antioxidants in self pre-serving cosmetic and drug formulations. In: Preservative-Free and Self-Preserving Cosmetics and Drugs, Cosmetic Sci. Technol. Series, 16: 159-179.
Bracca A, Fico G, Morelli I, De Simone F, Tome F, De Tommasi N (2003). Antioxidant and free radical scavenging activity of flavonol glycosides from different *Aconitum* species. J. Ethnopharmacol. 86: 63-67.
Bandyopadhyay U, Biswas K, Chatterjee R, Bandyopadhyay D, Chattopadhyay I, Ganguly CK, Chakraborty T, Bhattacharya K, Banerjee RK (2002). Gastroprotective effect of neem (*Azadirachta indica*) bark extract: possible involvement of H^+-K^+-ATPase inhibition and scavenging of hydroxyl radical. Life sci. 71: 2845-2865.
Bravo L (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56: 317-333.
Cai Y, Luo Q, Sun M, Corke H (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74: 2157-2184.
Decker EA, Welch B (1990). Role of Ferritin as a lipid oxidation catalyst in muscle food. J. Agric. Food Chem. 38: 674-677.
Duh PD, Tu YY, Yen GC (1999). Antioxidant activity of water extract of Harn jyur (*Chrysanthemum morifolium* Ramat), Lebensmittel-Wissenschaft Technologie. 32: 269-277.
Eom SH, Cheng WJ, Hyoung JP, Kim EH, Chung MI, Kim MJ, Yu CY, Cho DH (2007). Far infrared ray irradiation stimulates antioxidant activity in *Vitis flexuosa* THUNB. Berries. Korean J. Med. Crop Sci. 15 (5): 319-323.
Ermel K (1995). Azadirachtin content of Neem seed kernels from different regions of the world. In: Schmutterer H (Ed.). The Neem tree, source of Unique Natural products for integrated pest management, medicine, industry and other purposes, VCH, Weinheim, pp. 89-92.
Ermel K, Pahlich E, Schmutterer H (1986). Azadirachtin contents of Neem kernels from different geographical locations and its dependence on temperature, relative humidity and light. In proceedings, international Neem conference, Nairobi, Kenya, pp. 171-184.
Finefrock AE, Bush AI, Doraiswamy PM (2003). Current status of metals as therapeutic targets in Alzheimer's disease. J. Am. Geriatr. Soc. 51: 1143-1148.
Gogati SS, Marathe AD (1989). Anti-viral effect of Neem leaf (*Azadirachta indica*) extracts on Chinkugunga and Measles viruses. J. Res. Edu. Ind. Med. 8: 1-5.
Hsu B, Coupar IM, Ng K (2006). Antioxidant activity of hot water extract from the fruit of the Doum palm, *Hyphaene thebaica*. Food Chem. 98(2): 317-328.
Halliwell B, Aeschbach R, Loliger J, Aruoma OI (1995). The characterization of antioxidants, Food Chem. Toxicol. 33(7): 601-617.
Kaura SK, Gupta SK, Chowdhury JB (1998). Morphological and oil content variation in seeds of *Azadirachta indica* A. Juss. (Neem) from northern and western provenances of India. Plant foods for human Nutr. 52: 293-298.
Kim KT, Yoo KM, Lee JW, Eom SH, Hwang IK, Lee CY (2007). Protective effect of steamed American ginseng (*Panax quinquefolius* L.) on V79-4 cells induced by oxidative stress. J. Ethnopharm. 111: 443-445.
Kher A, Chaurasia SC (1997). Antifungal activity of essential oils of three medical plants. Ind. Drug, 15: 41-42.
Kaushik N, Gurudev singh B, Tomar UK, Naik SN, Satya V, Bisla SS, Sharma SK, Banerjee SK, Thakkar P (2007). Regional and habitat variability in azadirachtin content of Indian Neem (*Azadirachta indica* A Juss.). Curr. Sci. 92(10):1400-1406.
Lloyd RV, Hanna PM, Mason RP (1997). The origin of the hydroxyl radical oxygen in the Fenton reaction. Free Rad. Biol. Med. 22(5): 885-888.
Morton LW, Abu-Amsha Caccetta R, Puddey IB, Croft KD (2000). Chemistry and biological effects of dietary phenolic compounds: relevance to cardiovascular disease. Clin. Exp. Pharm. Phys. 27: 152-159.
Meir SJ, Kanner B, Akiri SPH (1995). Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. J. Agric. Food Chem. 43: 1813-1817.
Okpanyi SN, Ezeukwu GC (1981). Anti-inflammatory and antipyretic activities of *Azadirachta indica.* Planta media, 4 (1): 34-39.
Pitchaon M, Suttajit M, Pongsawatmanit R (2007). Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. Food Chem. 100: 1409-1418.
Pokorney J (2001). Introduction. In Pokorny J, Yanishlieva N, Gordon MH (Eds.), Antioxidants in food: practical applications, Cambridge: Woodhead Publishing Limited. pp. 1-3.
Qian ZJ, Jung WK, Byun HG, Kim SK (2008). Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, *Crassostrea gigas* against free radical induced DNA damage. Biores. technol. 99: 3365-3371.
Sultana B, Anwar F, Przybylski R (2007). Antioxidant activity of phenolic components present in barks of *Azadirachta indica, Terminalia arjuna, Acacia nilotica,* and *Eugenia jambolana* Lam. trees. J. Food Chem. 104: 1106-1114.
Sombatsiri K, Ermel K, Schmutterer H (1995). Other Meliaceous plants containing ingredients for integrated pest management and further purpose. In H. Schmutter (Ed.), The Neem tree *Azadirachta indica* A. Juss and other meliaceous plants. Germany: VCH pp. 585-97.
Siddiqui BS, Ghiasuddin Faizi S, Siddiqui S (1992). Triterpenoids from the fresh fruit coats of *Azadirachta indica*. Phytochem. 31(12): 4275-4278.
Singh N, Sastry MS (1997). Antimicrobial activity of Neem oil. Ind. J. Pharmaciol. 13: 102-106.
Sasaki S, Ohta T, Decker EA (1996). Antioxidant activity of water-soluble fractions of salmon spermary tissue. J. Agric. Food Chem. 44: 1682-1686.
Sidhu O, Vishal Kumar P, Hari MB (2004). Variability in triterpenoids (Nimbin and Salanin) composition of neem among different provenances of India. Ind. crops products, 19: 69-75.
Siddhuraju P, Mohan PS, Becker K (2002). Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): A preliminary assessment of crude extracts from stem bark, leaves, flower and fruit pulp. Food Chem. 79: 61-67.
Siddaraju MN, Dharmesh SM (2007). Inhibition of Gastric H^+, K^+ATPase and Helicobacter pylori Growth by Phenolic Antioxidants of *Curcuma amada*. J. Agric. Food Chem. 55(18): 7377-7386.
Soares JR, Dins TCP, Cunha AP (1997). Antioxidant activity of some extracts of *Thymus zygis*, Free Radical Res. 26: 469-478.
Strack D (1997). Phenolic metabolism. In Plant Biochemistry, Dey PM, Harborne JB Eds, Academic Press, San Diego, pp. 387-416.
Thompson M, Williams CR (1976). Stability of flavonoids complexes of copper (II) and flavonoid antioxidant activity. Anal. Chem. Acta. 85: 375-381.