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Antioxidant potential of the ethanol, ethyl acetate and petroleum ether extracts of *Kyllinga nemoralis*

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Abstract

Kyllinga nemoralis has been used in traditional folk medicine. Despite ethnobotanical reports, there are only limited studies on its antioxidant properties. This study determined the antioxidant properties of the ethanolic (KnE), ethyl acetate (KnEA) and petroleum ether (KnP) extracts of *K. nemoralis* using total antioxidant capacity (TAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and reducing power assays. Results showed that the antioxidative property of *K. nemoralis* extracts is extraction solvent-dependent. The TAC of KnP (94.60 mg AAE/g) and KnEA (72.08 mg AAE/g) were significantly higher than KnE (70.25 mg AAE/g). However, the EC_{50} (effective concentration) of KnE (15.02 mg/L; 112.43 mg/L) and KnEA (62.19 mg/L; 123.13 mg/L) indicates stronger DPPH free radical scavenging activity and reducing power than KnP (765.32 mg/L; 514.85 mg/L). Thus, *K. nemoralis* is a potential source of natural antioxidant compounds. Moreover, ethanol and ethyl acetate are recommended as a relatively good solvent for the extraction of antioxidant compounds from *K. nemoralis*.

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Introduction

Antioxidants are substances that help prevent or limit the damage to cells caused by free radicals. They can be synthesized *in vivo*, such as reduced glutathione (GSH) and superoxide dismutase (SOD) or taken as dietary antioxidants. The use of plants as a source of dietary antioxidants has long been established. It is believed that two-thirds of the world's plant species have medicinal importance, and almost all of these have excellent antioxidant potential (Krishnaiah *et al.* 2011). Plant-derived antioxidants are primarily phenolics synthesized by fruits, vegetables, nuts, seeds, leaves, roots, and barks (Asif, 2015). Recent epidemiological studies have revealed that frequent consumption of natural plant antioxidants is associated with a lower risk of cardiovascular diseases and cancer (Anbudhasan *et al.*, 2014). Thus, awareness of the beneficial properties of plant-based antioxidants has received a great deal of attention. With this, more studies nowadays are geared towards finding plants that can be promising sources of antioxidant compounds.

Whitehead spike sedge (*Kyllinga nemoralis)* grows in tropical regions of the world. It is a perennial creeping hedge, glabrous, arising from long-creeping rootstocks (Bhragual *et al.*, 2010). It is dominant in waste places and open grasslands throughout the Philippines. In traditional folk medicine, its root decoction is used as a sudorific for malaria with chills, refrigerant, tonic, demulcent, and relief of thirst for diabetics (Raju *et al.*, 2011). The rhizomes of the plant are reported to have been used for fever and cough in India (Karthikeyan *et al.*, 2009). Moreover, essential oils from the aerial parts of the *K. nemoralis* have been found to possess significant antimalarial activity against *Plasmodium falciparum*, anticancer activity against NCI-H187 (small cell lung cancer) (Pyne *et al.*, 2011), analgesic activity (Amor *et al.*, 2009), and hypoglycemic activity (Quanico *et al.*, 2008). However, to our knowledge, no study has reported its antioxidant properties as affected by the use of different extraction solvents. Extraction is a technique employed to separate desired secondary metabolites from the raw material. Extraction is carried out to maximize the obtained concentration of target compounds with potential biological activity. The extraction yield and biological activity of the resulting extract are not only affected by the extraction technique but also by the extraction solvent (Ngo *et al.*, 2017). Many solvents such as methanol, ethanol, ethyl acetate, petroleum ether, hexane, and water have been used for the extraction of bioactive compounds from plant materials; however, due to the variety of compounds in plants and their differing solubility properties in various solvents, a suitable extraction solvent for individual plant material needs to be identified. In this study, the antioxidant potential of the petroleum ether, ethyl acetate and ethanolic extracts of *K. nemoralis* were evaluated via total antioxidant capacity, DPPH radical scavenging activity, and reducing power assay.

Materials and methods

Sample collection, preparation and extraction

Five (5) kilograms of the fresh and healthy whole plant samples of *K. nemoralis* were collected from Central Mindanao University (CMU), University Town, Musuan, Maramag, Bukidnon. Identification and authentication of the sample were done by Dr. Fulgent P. Coritico of the Center for Biodiversity Research and Extension in Mindanao (CEBREM), CMU.

The collected samples were washed with tap water, rinsed with distilled water, air-dried, and homogenized. A 125 g-portion of the homogenized *K. nemoralis* was soaked separately in enough petroleum ether, ethyl acetate and ethanol for 72 h. After 72 h, the mixture was filtered. The residues were discarded and the filtrate was concentrated under *vacuo* at 40°C to give concentrated petroleum ether extract (KnP), ethyl acetate extract (KnEA) and ethanolic extract (KnE).

Total antioxidant capacity determination

The total antioxidant capacity (TAC) of the KnP, KnEA and KnE extracts was determined by employing the method described by Prieto *et al.* (1999) with slight modification. For each extract, a

1000 mg/L in ethanol sample solution was prepared. A 200 µL aliquot of the sample solution was then placed in an Eppendorf tube followed by the addition of 600 µL of the reagent solution (0.6 M sulfuric acid; 28 mM sodium phosphate; and 4 mM ammonium molybdate). The reaction mixture was then incubated at 95 °C for 90 min and was allowed to cool at room temperature. After cooling, the mixture was centrifuged for 3 min at $11x10^3$ rpm. A 200 µL reaction mixture was placed in each of the three wells of a 96-well plate. Then the absorbance was measured at 695 nm using a microplate reader (Spectramax 250). The same procedure was done for the blank (absolute ethanol) and the various concentrations (0 to 300 mg/L) of standard L-ascorbic acid for the calibration curve. Total antioxidant activity, expressed as mg L-ascorbic acid equivalent (AAE) per gram extract, was calculated using equation (1):

$$\text{TAC relative to L-ascorbic acid, mg AAE/g extract} = \frac{A}{B} \quad (1)$$

where: A = L-ascorbic acid concentration of the test sample solution derived from the calibration curve, mg AAE/L; and

B = concentration of the test sample stock solution, g extract/L

DPPH radical scavenging activity assay

The DPPH radical scavenging activity was carried out in a 96-well microplate employing the method described by Miser-Salihoglu *et al.* (2013). Various concentrations (10 to 1500 mg/L) of KnE, KnEA, and KnP test solutions were prepared.

In a well containing 150 µL of test solution, 50 µL of 0.1 mM DPPH in absolute ethanol were added. The reaction mixture was incubated for 30 min in the dark at room temperature and the absorbance of the reaction mixture (A_{sample}) was measured at 517 nm using a microplate reader (Spectramax 250). The same procedure was followed for the absolute ethanol (blank) and the various concentrations (0.40 to 4.00 mg/L) of the standard ascorbic acid . The absorbance readings were recorded as $A_{control}$ and $A_{standard}$, respectively. The DPPH radical scavenging activity of the test solutions, expressed as percentage DPPH inhibition was obtained using equation (2).

$$\%\text{DPPH Inhibition} = \frac{A_{control} - A_{sample\ or\ standard}}{A_{control}} x\ 100 \quad (2)$$

where: $A_{control}$ = Absorbance of the control

A_{sample} = Absorbance of the sample test solution

$A_{standard}$ = Absorbance of the ascorbic acid test solution

To determine the EC_{50} value, a graph was plotted with the % DPPH radical scavenging activity as the ordinate value and the log values of the concentration of the test solutions as the abscissa. The Least Square's Method was then employed to obtain the equation of the line. From the equation, the EC_{50} value was calculated by solving for the corresponding log concentration value (x-value) at 50% DPPH radical scavenging activity and taking its antilog.

Reducing power assay

The reducing power was determined employing the method adapted for a 96-well plate assay (Murugan and Lyer, 2012). Various concentrations (20-750 mg/L) of the test solutions were prepared for KnE, KnEA, and KnP. In an Eppendorf containing 1 mL of the sample test solution, 200 µg/mL of 0.2 M phosphate buffer (pH 6.6) and 200 µL of 1% (w/v) solution of potassium ferricyanide were added. The mixture was then incubated at 50°C for 30 min, cooled to room temperature, and added with 200 µL of 10% (w/v) trichloroacetic acid. The resulting mixture was centrifuged at 11000 rpm for 3 min. After centrifugation, an aliquot (150 µL) of the solution was transferred into a 96-well plate and 20 µL of 0.1% (w/v) solution of ferric chloride was added. The absorbance of the solution was measured at 620 nm using a microplate spectrophotometer (Spectramax 250). The same procedure was done for the negative control (absolute ethanol) and the ascorbic acid test solutions (7.5 to 225 mg/L). The reducing power was calculated using equation (3).

$$\%\text{Reducing Power} = \frac{A_{sample\ or\ standard} - A_{control}}{A_{sample\ or\ standard}} x\ 100 \quad (3)$$

where:

$A_{control}$ = Absorbance of the control
A_{sample} = Absorbance of the sample test solution
$A_{standard}$ = Absorbance of the ascorbic acid test solution

The EC_{50} value for the reducing power were then determined as mentioned above.

Statistical analysis

For each determination, three (3) replicates and three (3) trials per replicate were carried out. The results obtained were subjected to statistical analysis using One-Way Analysis of Variance (ANOVA) at 0.05 level of significance. Significant differences among the means were determined using Post hoc Tukey's test at 0.05 level of significance.

Results and discussion

Total antioxidant capacity

The results of the total antioxidant capacity (Fig. 1) determination imply that *K. nemoralis* contains antioxidant compounds that contribute to its antioxidant activity. This may be accounted to the lipid-soluble antioxidants such as carotenoids, flavonoids and cinnamic acid derivatives (Prieto *et al.* 1999) which contribute to the overall lipid-soluble antioxidant capacity of the *K. nemoralis*.

Table 1. EC_{50} for the reducing power and DPPH radical scavenging activity of the *K. nemoralis* extracts.

Sample/Standard	EC_{50}, mg/L (±SD)	
	DPPH radical scavenging activity	Reducing power
KnE	15.02(±2.39)[b]	112.53 (5.46)[b]
KnEA	62.19 (±2.25)[b]	123.13 (4.17)[b]
KnP	765.32 (±28.10)[a]	514.85 (16.67)[a]
Ascorbic Acid	1.55 (±1.66)	<7.50

[a,b]-Means (n=9) of the same letter superscript are not significantly different by Tukey's Test at 0.05 level of significance.

The findings are consistent with the results of the study conducted by Sindhu *et al.* (2014), wherein *K. nemoralis* was found to exhibit high total antioxidant capacity comparable to that of butylated hydroxytoluene (BHT). Studies on related plant species, like *Cyperus rotundus* and *Cyperus brevifolius,* under the same family Cyperaceae have reported total antioxidant activity of their acetone and ethanolic extracts (Yazdanparast *et al.* 2007).

Among the plant extracts, KnP exhibited the highest total antioxidant capacity, followed by KnEA then KnE. The one-way analysis of variance (ANOVA) revealed significant differences among the TAC values of KnE, KnEA and KnP. Subsequent, Post Hoc Tukey's test at 0.05 level of significance showed that KnP exhibited significantly higher TAC than KnE but statistically similar activity to KnEA. The results of the study are in agreement with the findings of Zahin *et al.* (2013) on the antioxidant capacity of *Murraya koenigi* extracted with petroleum ether and sequentially fractionated with various solvents. In the study, the total antioxidant capacity of the solvent extracts was found to decrease in the order of petroleum ether > ethyl acetate> ethanol. It can be inferred then that the extractability of antioxidant phytochemicals is influenced by the type of extraction solvent.

Considering the chemistry and uneven distribution of antioxidant compounds in the plant matrix, recovery of antioxidants from plant materials is generally performed through different extraction techniques. In general, the extract yields and antioxidant activities of the plant materials are strongly dependent on the nature of extracting solvent because of the varied chemical characteristics and polarities of the

antioxidant compounds present in plant samples (Yeasmen *et al.*, 2015).

Petroleum ether, as a nonpolar solvent, is capable of extracting a high concentration of lipid derivatives, resin, oils, steroids, and terpenoids (Anosike *et al.* 2012). Reports have shown that the petroleum ether extract of *K. nemoralis* contain triterpenoid, glycosides (Somasundaram *et al.* 2010), phytosterols, oils and fats (Rajagopal *et al.* 2016). Twenty-three (23) compounds, i.e., mainly oxygenated sesquiterpenes and particularly sesquiterpene hydrocarbons, have been identified in the essential oils from fresh aerial parts of *K. nemoralis*. The essential oil from *K. nemoralis* was found to exhibit significant antimalarial, anticancer and antimicrobial activities (Pyne *et al.*, 2011). Moreover, triterpenoids and sesquiterpenes, which are readily soluble in a nonpolar organic solvent such as petroleum ether (Jiang *et al.*, 2016), have been reported to demonstrate high antioxidant activity (Gulcin *et al.*, 2006; Zengin *et al.*, 2014).

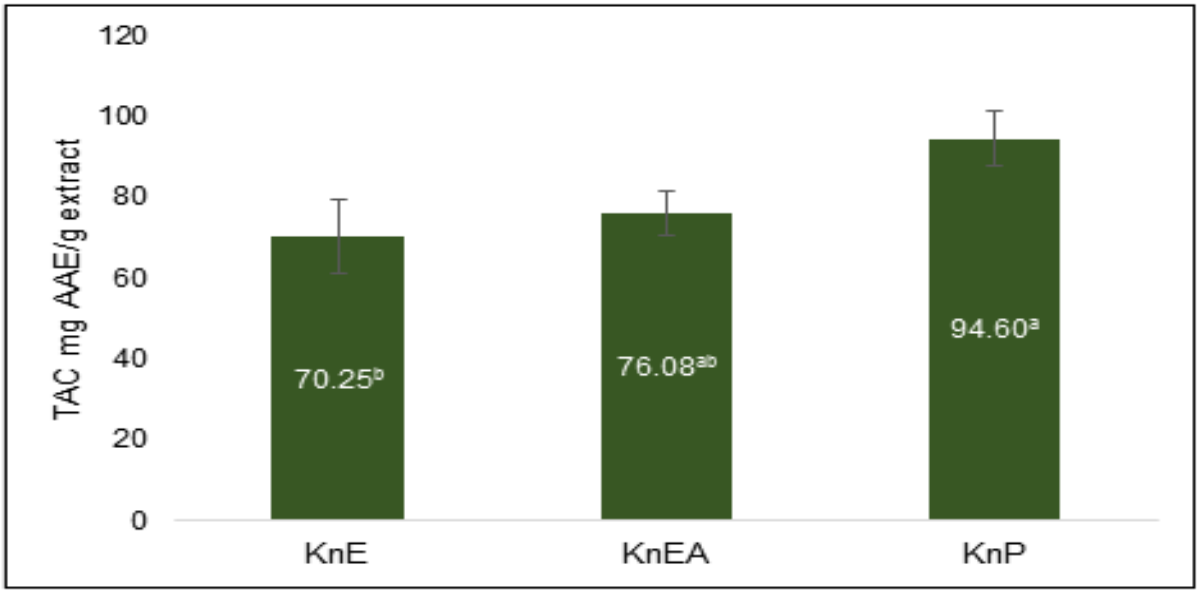

Fig. 1. Total Antioxidant Activity (TAC) of *K. nemoralis* extracts. Error bars are standard deviation (n=9). [a,b]- Means of the same letter superscript are not significantly different by Tukey's Test at 0.05 level of significance.

Ethanol with the highest polarity index (5.2) is more effective in extracting phytochemicals of relatively high polarity, such as phenolic compounds. In many studies, ethanol has been proven as an effective solvent for the extraction of phenolics (Siddhuraju *et al.* 2003). According to Karthikeyan *et al.* (2009), saponins, glycosides, carbohydrates, tannins and phenolic compounds, flavonoids, and triterpenoids were detected in the ethanolic extract of *K. nemoralis*. Despite the presence of phenolic compounds, which are known antioxidants, TAC of KnE was still found significantly lower compared to KnP. Thus, the overall total antioxidant capacity of *K. nemoralis* may be more attributed to its lipid-soluble antioxidant (nonpolar) rather than the water-soluble (polar) antioxidant components.

DPPH free radical scavenging activity

Table 1 and Fig. 2A summarize the EC_{50} values and the DPPH radical scavenging activity of the KnE, KnEA and KnP, respectively. **T**he EC_{50} for the DPPH radical scavenging activity of *K. nemoralis* extracts increases in the order of KnE< KnEA< KnP. The radical scavenging activity of the various *K. nemoralis* extracts is dose-dependent (Fig. 2A), i.e., radical scavenging activity increases with increasing concentration.

KnE, the most potent radical scavenger among the extracts, exhibits antioxidative property via DPPH free radical scavenging mechanism. The result is consistent with the findings of Sindhu *et al.* (2014), where the alcoholic extract *K. nemoralis* shows

efficient DPPH free radical scavenging activity. Studies on *Cyperus rotundus* (Yazdanparast *et al.* 2007) *and Cyperus brevifolius* (Rani *et al.*, 2012) which belong to the same family as *K. nemoralis* have also reported their antioxidant activity by scavenging DPPH free radical.

The results of one-way ANOVA and the subsequent Post Hoc Tukey's test at 0.05 level of significance showed that KnE exhibited significantly higher scavenging activity than KnP but statistically similar activity with KnEA.

The DPPH radical scavenging assay is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine (Contreras-Guzman *et al.*,1982).

Accordingly, phenolic compounds have the ability to quench DPPH radical in a concentration-dependent manner (Bendary *et al.*, 2013). DPPH free radical scavenging activity has been used to evaluate antioxidant ability of phenolic compounds (Sanchez-Moreno *et al.*, 1998) and is based on the ability of compounds to act as free radical scavengers or hydrogen donor (Shalaby and Shanab 2013). More importantly, significant linear correlations were found between DPPH antioxidant potentials of the plant materials and the concentration of phenolics and flavonoid compounds (Kicel *et al.*, 2013).

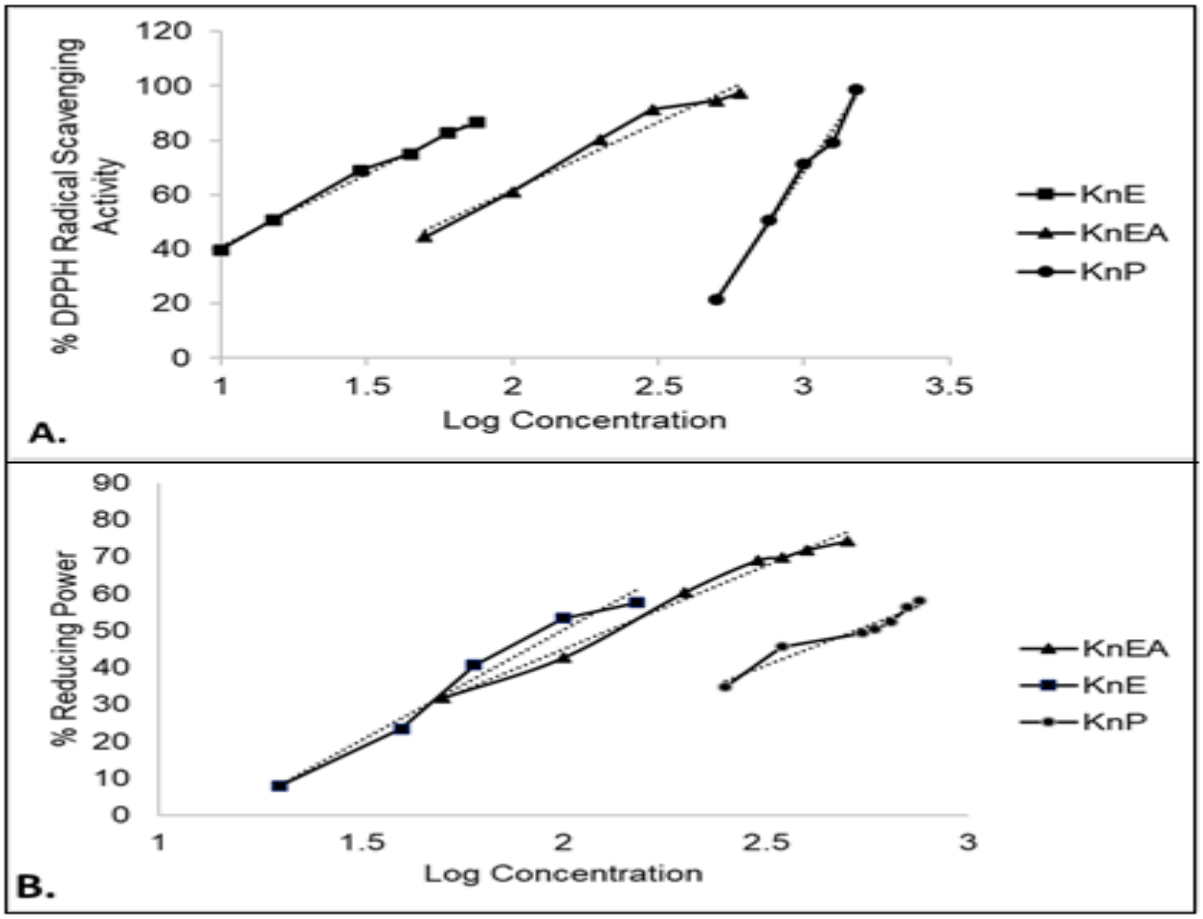

Fig. 2. Plot of the A. DPPH free radical scavenging activity; and B. reducing power as a function of the logarithm of KnE, KnEA, and KnP concentrations.

The extractability of the phenolic compounds depends on the type of the solvent, nature and preparation of material to be extracted, the chemical structure of phenolic compounds, and temperature, among others (Bucic-Kojic *et al.*, 2011). In a study on propolis extracts, results revealed that

extracts with higher phenolic composition gave higher antioxidant activities than the propolis extracts with lower content of phenolics (Sun *et al.* 2015). Furthermore, methanol, ethanol, or propanol and their mixtures in water, as well as acetone, ethyl acetate and dimethylformamide are so far the most commonly used solvents in the extraction of phenolic compounds from the plant materials (Altemimi *et al.* 2017). Considering that polarity indices of ethanol (5.2) and ethyl acetate (4.4) are very close, this may explain why KnE and KnEA have statistically comparable DPPH radical scavenging activity. Nevertheless, the antioxidant activity of KnE is relatively more potent than that of KnEA. The higher polarity of solvents usually means better solubility of polyphenols (Aires, 2017).

This is supported by the study of Boeing *et al.* (2014), where ethanolic extracts of olive tree wood yield around 6-fold higher amounts of extracts with the highest radical scavenging activity than those of ethyl acetate extracts. In addition, phytochemical studies have also shown the detection of phenolics, steroids, carbohydrates, saponins, flavonoids, alkaloids, anthocyanin, quinine steroids and coumarins, cardiac glycosides, and acid terpenoids in the ethanolic extracts of *K. nemoralis* (Muthu *et al.*, 2018). Thus, extraction employing ethanol must have resulted in high phenolic content in KnE and eventually high DPPH radical scavenging activity. On the other hand, KnP exhibited the lowest ability to scavenge free radicals. The nonpolar nature of petroleum ether hinders it from extracting the polar phenolic compounds. Non-polar solvents will extract only very small amounts of phenolic compounds (Yadav *et al.*, 2018), making petroleum ether being least effective compared to ethanol and ethylacetate. Lastly, the probable absence or very low concentration of phenolics and flavonoids in the petroleum ether extract of *K. nemoralis* may led to the low DPPH radical scavenging activity of KnP.

Reducing power

The EC_{50} value for the reducing power of *K. nemoralis* extracts increases in the order of KnE <KnEA <KnP. Since reducing ability of plant extracts is associated with antioxidant activity (Sindhu *et al.* 2014), then *K. nemoralis* extracts exhibits antioxidant potential. As reported previously, the methanolic extract of *K. nemoralis* has demonstrated a reducing power (Sindhu *et al.*, 2014). Moreover, the reducing power of the various *K. nemoralis* extracts is found to increase with concentration (Fig. 2B), i.e. dose-dependent.

KnE exhibited significantly higher reducing power than KnP but statistically similar activity with KnEA as indicated by one-way ANOVA and Tukey's test at 0.05 level of significance. The reducing power can be associated with the presence of polyphenols, which can donate electrons and scavenge free radicals by converting them into more stable products leading to a termination of the radical chain reaction (Rajamanikandan *et al.*, 2011). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in neutralizing free radicals, quenching singlet and triplet oxygen species, or decomposing peroxides (Rice-Evans *et al.* 1996). Thus, extracts with a high concentration of phenolic compounds will more likely exhibit high reducing power.

As mentioned, solvent polarity is among the factors that affect the extractability of phenolics. Among the common extraction solvents, phenolic compounds have shown high solubility in ethanol (PI=5.2) (Aires 2017), methanol, and ethyl acetate (PI=4.4) (Babbar *et al.* 2014). In addition, phenolics and flavonoids have been detected in the ethanolic extracts of *K. nemoralis* (Arumugan *et al.*, 2010; Babu *et al.*, 2018). These accounts the significantly high reducing power of KnE and KnEA.

However, it is noteworthy that the results of the reducing power and DPPH radical scavenging activity have similar trend. Results indicated that the order of the rank based on the activity is KnP<KnEA<KnE. The similarity in the trend may be attributed to the mechanism involved for both assays. The DPPH and Reducing Power assays both rely on the reducing

ability of the antioxidants. The assays analyzed the ability of the antioxidant to reduce radical ion and metal cation (Fe^{3+}), respectively (Schlesier *et al.*, 2002). Moreover, the polarity of extracting solvents has been shown to significantly influence the antioxidant properties of organic solvent extracts (Nawaz *et al.*, 2020). It has been reported that reducing properties and free radical scavenging activity are dependent on the polarity of the extraction solvent. The reducing properties of the extracts increased with the increasing polarity of solvent extracts. This indicates that strong antioxidant compounds with high reducing power and DPPH radical scavenging activity are efficiently extracted with polar solvents (Nawaz *et al.*, 2020).

Conclusion

K. nemoralis can be a potential source of natural antioxidant compounds. However, the antioxidant property of *K. nemoralis* extract is solvent-dependent. The polarity of the extraction solvent affects the antioxidant potential of the extracts. Among the solvents, ethyl acetate and ethanol are relatively better solvents for the extraction of antioxidant compounds from *K. nemoralis*.

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