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Full Length Research Paper

Effects of hydrolytic conditions on recovery of antioxidants from methanolic extracts of *Nigella sativa* seeds

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***Nigella sativa* (NS) has health promoting beneficial properties including antioxidant activity. In this study, the impact of acid and alkaline hydrolysis on phenolic (TPC), flavonoid (TFC) contents and antioxidant activity of methanolic extracts from *N. sativa* (NS) seeds powder is evaluated. Total phenolic and flavonoid contents were evaluated according to Folin-Ciocalteu reagent and aluminium chloride colorimetric assays, respectively. The TPC of extracts varied from 67 to 73 mg/100g NS powder, expressed as Gallic acid equivalents (GAE), while TFC concentrations varied from 96 to 113 mg rutin equivalents (RE)/100g NS powder. Positive correlations were found between TPC and TFC in NS hydrolyzates and their 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (r^2 = 0.975 and 0.978 respectively). The alkaline hydrolyzed extract (BHE) showed the highest TPC and TFC (730 µg GAE/g and1130 µg RE/g NS powder respectively) as well as the highest antioxidant activity ($DPPH^{\cdot}$ =167±0.133 mg TEAC /100g dry NS, $ABTS^{\cdot+}$=112 ± 0.023 mg TEAC /100 g dry NS and FRAP=28 ± 0.159 mg GAE/100g dry NS) compared to acid hydrolyzed (AHE) and non-hydrolyzed extracts (NHE). Non-hydrolyzed extracts showed the lowest TPC and TFC content (670 µg GAE and 960 µg RE/g NS powder respectively) through the assays ($p < 0.05$). Findings of the study reveal that hydrolysis has profound effects on recovery of antioxidants from NS extracts. Overall, BHE has the highest antioxidant activity compared to AHE or NHE.**

Key words: *Nigella sativa*, methanolic extracts, hydrolysis, total phenolic content, total flavonoid content, antioxidant activity.

INTRODUCTION

Since long time ago, herbs have been used in many fields such as medicine, nutrition, flavoring, foods, and others. Moreover, herbs have been the source for nearly all medicinal therapies until synthetic drugs were developed during the nineteenth century (Djeridane et al., 2006). *Nigell sativa* Linn. (Ranunculaceae) commonly known as black seed or black cumin is an annual plant and has been particularly used in the traditional Arab and Ayurvedic medicines for the treatment of many diseases and ailments, e.g. diabetes, hypertension, asthma, inflammation, cough, bronchitis, headache and many others (Ali and Blunden, 2003; Khader et al., 2010). In Palestine, NS seeds are used to treat many diseases like dizziness and high blood pressure. The fixed oil of it's

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seeds is used against lung diseases and arthritis, also soap from NS fixed oil is used for improving skin glossiness and for blood circulation (Khader et al., 2010). The main component of NS is oil (~33%), besides the presence of other nutrients like carbohydrates (~34%), proteins (~17%) and fiber (~5.5%). The major content of oil is the fixed oil, while the volatile oil ranges between 0.4-0.7% (Abu-Al-Basal, 2009; El-Tahir and Bakeet, 2006).

The volatile oil of NS seeds and its main active constituent, that is, thymoquinone, are extensively reported to be exhibiting protective effects against many diseases (Mariod et al., 2009; Al-Naqeep and Ismail, 2009). Due to safety concerns associated with the use of synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and *t*-butyl hydroquinone (TBHQ) as preservatives during processing of lipid-containing foods; there is an increasing interest in developing natural and safer antioxidants from plant sources (Maisuthisakul et al., 2007; Iqbal et al., 2007).

However, the complexity of phenolic mixtures present in plant materials requires a preliminary clean-up and fractionation of crude extracts. In addition, some of the phenolic compounds are present in the form of glycosides in plants and are rarely present in the free form. Therefore, several hydrolytic procedures like acid and alkaline hydrolysis have been used to hydrolyze glycosides to a glycones (Annegowda et al., 2010; Chirinos et al., 2008). Many researchers have reported that *N. sativa* has a promising antioxidant activity, reducing power and inhibition of peroxidation (Ismail et al., 2010; Khattak et al., 2008; Thippeswamy and Naidu, 2005). Our laboratory also (Mariod et al., 2009) concluded that the antioxidant activities of crude methanolic extracts of *N. sativa* seeds cake and its fractions using ethyl acetate, hexane and water are rich in antioxidants.

To our knowledge, no report has, so far, been reported demonstrating the importance of acidic and alkaline hydrolysis of methanolic extracts of NS seeds on recovery of phenolics antioxidant activity. Hence, our objective is to quantify the effects of acid and alkaline hydrolysis on TPC, TFC and antioxidant activities of methanolic extracts of NS.

MATERIALS AND METHODS

Samples and reagents

N. sativa seeds were purchased from a local herbal grocery in Taiz, Republic of Yemen. The seeds were cleaned and kept at 4°C in the Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia. All the chemicals used in this study were of analytical grade. Methanol (Prolabo); ammonium thiocyanate (Sigma Chemical Co., St. Louis, MO, USA) and sodium hydroxide (HmbG chemicals, Hamburg, Germany). 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) were obtained from Sigma–Aldrich, Deutschland, Germany while Gallic acid and Folin–Ciocalteu's phenol reagent were obtained from Fluka Biochemica, Buchs, Switzerland. All other chemicals and organic solvents were procured from Merck, Darmstadt, Germany.

Preparation of methanolic extracts

N. sativa seeds were cleaned and dried overnight in an oven at 40°C, and were then ground to powder for 3 min using an electrical grinder (Waring Blender, USA). This procedure was performed just before the extraction procedure initiated. Fifteen grams of NS seeds powder were extracted in 3 Erlenmeyer flasks, each containing 5 g of sample and extracted in 80% methanol (50 ml in each flask) by placing the flasks in an electrical shaker at 40°C at 180 rpm for 2 h and filtered through filter paper, the first flask was left as non-hydrolyzed extract (NHE), while the second flask as acid hydrolyzed extract (AHE) by addition of 200 µl of 0.1 M HCl and the third flask as base hydrolyzed extracts (BHE) by addition of 200 µl of 0.1 M NaOH.

The residue was further extracted twice using same media as described above. The filtered extracts were combined, concentrated under reduced pressure at 50°C using rotary evaporator (Rotavapor R210, Buchi, Postfach, Flawil, Switzerland) and freeze dried (VirTis benchtop K, Bieleveld, Germany). The dried extracts were diluted with methanol (up to 5 ml with 80% methanol), making 1 ml of extract solution equivalent to 1 g NS powder.

Determination of total phenolic content (TPC)

Total phenolic content was estimated by spectrophotometric assay using Folin–Ciocalteu phenol reagent, based on the procedure of Qiu et al. (2010) with slight modifications, using gallic acid as standard.

Briefly, stock solutions from three different extracts (NHE, AHE, and BHE) were prepared by 40 folds dilution of the original samples by mixing 75 µl of each extract with 2.925 ml of 80% methanol followed by filtration using 0.45 µm filter paper. A 500 µl aliquot of the samples were placed in a tube, and then 2.5 ml of Folin-Ciocalteu reagent (10-fold diluted) and 2.0 ml of 7.5% sodium carbonate were added and incubated at 40°C for 1 h. After incubation, absorbance of the resulting blue-colored solution was measured at 765 nm spectrophotometrically. Quantitative measurements were performed based on a standard calibration curve of six points: 3.125, 6.25, 12.5, 25, 50 and 100 ppm of gallic acid ($y = 0.012X + 0.0213$; $r^2 = 0.9996$; $P < 0.05$). The total phenolic contents were expressed as gallic acid equivalents (GAE) as mg GAE/ 100 g of dry NS powder. Data were reported as mean ± SD for three replicate measurements.

Determination of total flavonoid content (TFC)

Total flavonoid contents were estimated following colorimetric assay based on the procedure of Ismail et al. (2010), with slight modifications. Briefly, stock solutions from three different extracts were prepared by 20 folds dilution of the original samples by mixing 150 µl of each extract with 2.850 ml of 80% methanol and then filtered using 0.45 µm filter.

A 100 µl of 2% $AlCl_3$ were added to 100 µl of diluted sample solution and incubated for 10 min in the dark at room temperature. After incubation, absorbance of the resulting yellow-colored solution was measured spectrophotometrically at 405 nm. Quantitative measurements were done based on standard calibration curve of six points of rutin ($y = 0.0077X - 0.0779$, $r^2 = 0.9992$; $P < 0.05$) as described in case of TPC, where "y" represents absorbance (nm) and "x" represents rutin equivalent (RE) per 100 g of dry NS powder. All the samples were analyzed thrice and results were averaged. Data is reported as mean ± SD.

Table 1. Levels of total phenolic, flavonoid, and FRAP in NS methanolic extracts (Mean± SD).

Methanolic extract	TPC (mg GAE)	TFC (mg RE)	FRAP (mg GAE)
NHE	67±0.201	96±0.452	24 ±0.262
AHE	71±0.442	103*±0.020	26* ±0.096
BHE	73*±0.280	113**±0.178	28** ± 0.159

Values are means of three replicate determinations per 100g *Nigella sativa* powder, GAE=Gallic acid equivalent, RE= Rutin equivalent and SD=standard deviation. Means in the same column with an asterisk are significantly different from the control (NHE) ($p < 0.05$).

PPH radical scavenging activity

The DPPH radical scavenging activity of extracts was measured according to procedure described by Chan and Ismail (2009). Briefly, 50 µl of each extract (NHE, AHE, and BHE) was placed in 96-well microplate and 195 µl of 80% methanolic solution of $DPPH^{\bullet}$ (0.1 mM) was added. After 60 min incubation in darkness at ambient temperature (25°C), the decrease in absorbance was recorded at 540 nm using a microplate reader (Opsys MR, Thermo Labsystems, Franklin, MA, USA). Absorbance of the $DPPH^{\bullet}$ without extracts, that is control was measured. All the determinations were performed in triplicate. Lower absorbance of the reaction mixture indicated higher scavenging activity of extracts. Methanolic solution (80%) was used as blank, while control sample contained 195 µl of above $DPPH^{\bullet}$ solution and 50 µl of 80% methanol instead of standard or extracts. Trolox, the water-soluble Vitamin E analog, served as standard, and the results were expressed relative to Trolox in terms of TEAC (Trolox equivalent antioxidant capacity).

$ABTS^{\bullet+}$ radical scavenging activity

The $ABTS^{\bullet+}$ scavenging activity of NS extracts was measured according to procedure described by Kim et al. (2010). The stock solutions included 7 mM $ABTS^{\bullet+}$ solution and 2.45 mM potassium-persulfate solution. The working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 16 h at room temperature in the dark. To obtain an absorbance of 0.700 ± 0.02 at 734 nm, the stock solution was diluted with as much ultra-pure water as necessary. A 100 µl of twenty fold diluted NS extracts were added to 900 µl of diluted $ABTS^{\bullet+}$, and the absorbance at 734 nm was determined after 2 min of initial mixing. Methanolic solution (80%) was used as blank, while control contained 0.9 ml of above $ABTS^{\bullet+}$ working solution and 0.1 ml of 80% methanol instead of standard or extracts. Trolox served as standard, and the results were expressed relative to Trolox in terms of TEAC.

Determination of ferric reducing antioxidant power (FRAP)

The reducing powers of three extracts from NS seeds were determined following the method of Berker et al. (2007). Briefly, five hundred microliters of different NS extracts (20-fold dilution) were mixed with 2.5 ml of distilled water, 0.75 ml of 1 M HCl, 0.75 ml of potassium ferricyanide solution ($K_3Fe(CN_6)$) (1%), 0.25 ml of SDS (1%) and finally 0.25 ml of $FeCl_3.6H_2O$ (0.2%) so that the final volume would be 5 ml. The mixture was incubated at 50°C for 20 min, allowing to cool room temperature, and absorbance of resulting solution was measured at 750 nm against a reagent blank (80% methanol). Control was prepared by adding all the reagents except extracts. Increased absorbance of the reaction mixture indicated an increase of reduction capability. Gallic acid was used as standard compound with the final concentration ranging from 3.125-100 ppm. And the calibration curve was Y (absorbance) = 0.0369X (GA content)-0.0936 (r^2=0.9932). The results were expressed as gallic acid equivalents (GAE) in mg/100g of dry NS powder. All of the samples were analyzed in triplicate.

Statistical analyses

All the experiments were conducted in triplicate; results were averaged. The data were recorded as mean ± standard deviation and analyzed by SPSS (version 19, SPSS Inc, Chicago, IL). One-way ANOVA was conducted followed by Least Significant Difference (LSD). A value of $p < 0.05$ was deemed to be statistically significant.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

Since extraction is an important step in the recovery of antioxidants from the plant materials. It is very clear that no single method may be the ultimate choice for the hydrolysis of plant materials; hence we in Table 1. In comparison to other extracts, BHE contained higher TPC (73 ± 0.280 mg GAE/100g NS powder) followed by AHE (71 ± 0.442 mg GAE/100g NS powder) and NHE (67±0.201mg GAE/100g NS powder). The BHE had significantly higher ($p<0.05$) TPC as compared to NHE, while the increase in AHE was non-significant. The increase in TFC exhibited the same pattern as shown in case of TPC with significant differences among the extracts as shown in Table 1. A positive correlation was observed between TPC and TFC (Figure 1, r^2 = 0.9108) and also between TFC and $DPPH^{\bullet}$ (Figure 2, r^2 =0.9787), thus indicating a significantly positive relationship between TPC, TFC and scavenging of DPPH radical.

Radical scavenging activity

Plants play an important role in health promotions through free radical scavenging activity (Sanchez-Moreno, 2002). The relatively stable DPPH radical has been widely used as substrate to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate the antioxidant activity (Rajaram and Nazeer, 2010). As shown in Figure 3, the

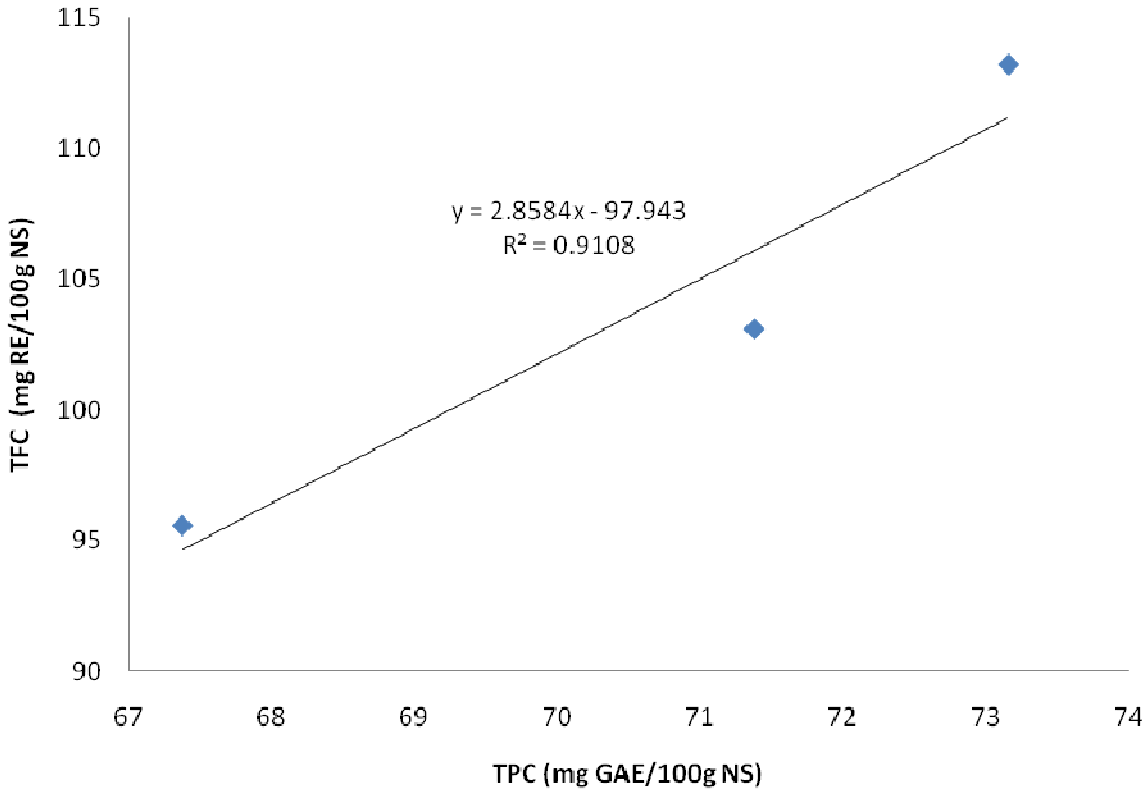

Figure 1. Correlation between TFC and TPC for NS extracts.

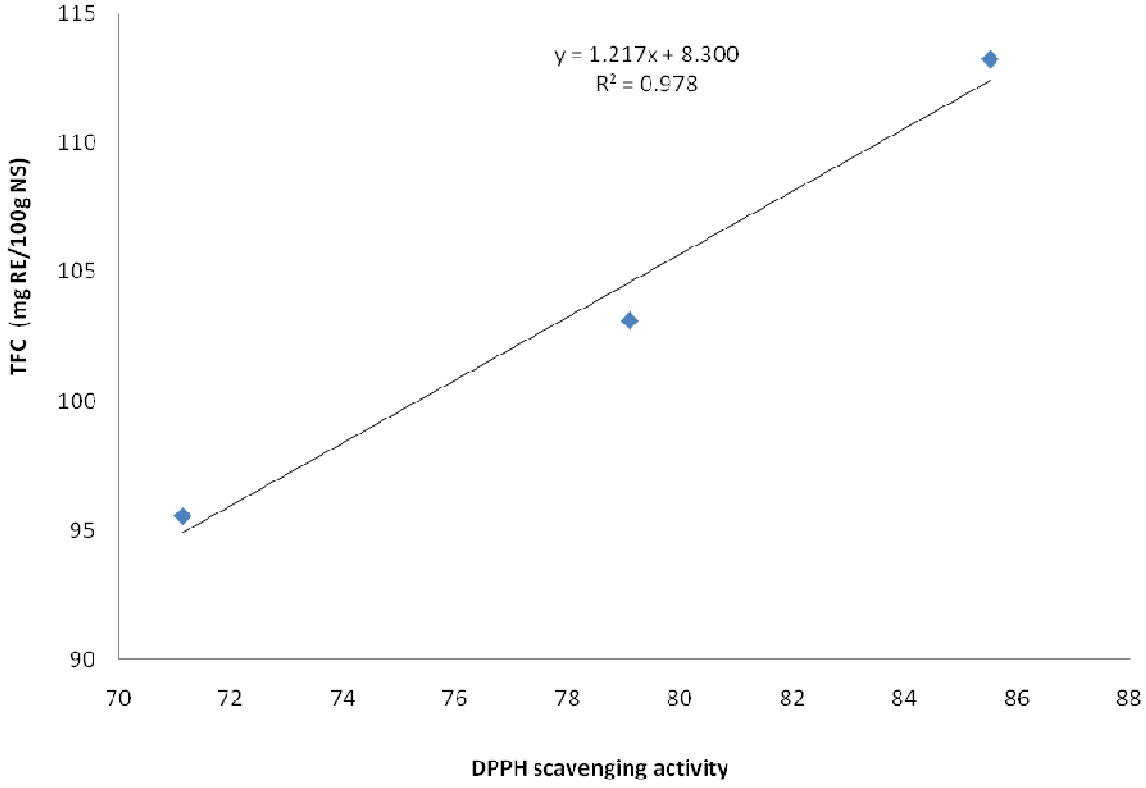

Figure 2. Correlation between TFC and DPPH˙ for NS extracts.

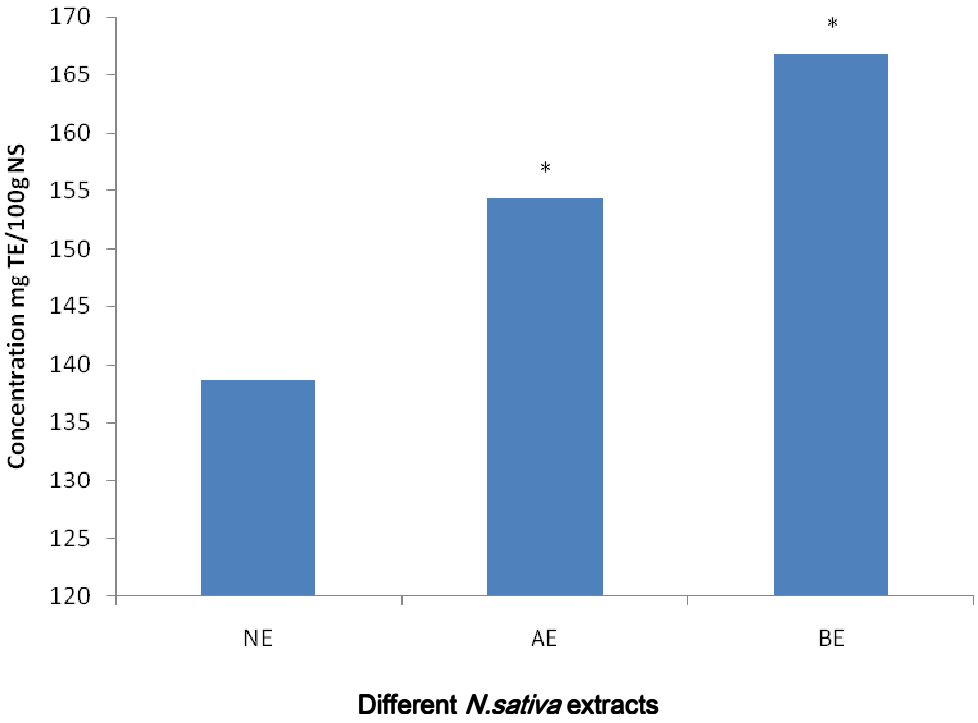

Figure 3. Radical scavenging activity using DPPH$^{\bullet}$ of NS extracts. Values are means of three replicate determinations per 100 g *Nigella sativa* powder. Asterisks denote significance at a P < 0. 05 compared to control (NHE).

results of DPPH radical scavenging assay indicate that BHE and AHE showed significantly ($p < 0.05$) higher antioxidant activity as compared to NHE as a control (167 ± 0.133, 154 ± 0.455, and 139 ± 0.960 mg TEAC/100 g NS respectively), while no significant differences could be observed between BHE and AHE. ABTS$^{\bullet+}$ scavenging assay is an excellent means to determine the antioxidant activity of hydrogen donating and chain breaking antioxidants.

The increase in ABTS$^{\bullet+}$ was of the same pattern as shown in case of DPPH$^{\bullet}$ with highly significant differences among all the extracts as compared to control (112 ± 0.023, 109 ± 0.099, and 103 ± 0.110 mg TEAC/100 g NS respectively). Moreover, BHE was found significantly higher in activity than AHE (Figure 4). In both the assays, BHE as well as AHE exhibited higher antioxidant activity ($p<0.05$). The rise in TPC and TFC of BHE may be due to hydrolysis of some high molecular weight components, and changing these components from insoluble to soluble ones in extraction medium. The high scavenging ability of BHE may be attributed to its higher phenolic and flavonoid contents (Khattak et al., 2008).

The increases in antioxidant activity in alkaline hydrolyzed extracts of some medicinal plants have also been reported by Madhujith and Shahidi (2009). Similarly, Qiu et al., (2010) found an increase in the antioxidant activity after alkaline hydrolysis of wild rice whole grain.

Ferric reducing antioxidant power

Ferric reducing antioxidant assay is appropriate to measure the total antioxidant capacity and state of medicinal herbs before use in phytotherapy (Szollosi and Varga, 2002). There is a positive relationship reported between TPC and antioxidant activity of many plant species. So, NS extracts could act as electron donors and may react with free radicals and convert them to stable products, hence terminating the radical chain reaction (Oktay et al., 2003). As shown in Table 1, the antioxidant capacities of NS extracts varied significantly ($p<0.05$) among the extracts. BHE showed the highest FRAP expressed as mg GAE / 100 g NS powder (28 ± 0.159) followed by AHE (26 ± 0.096) and the control (24 ± 0.262). Hence, order of FRAP of the extracts was as follows: BHE > AHE > NHE. The good correlation (Figure 5) between two methods employed for the determination of antioxidant capacity (FRAP and DPPH$^{\bullet}$) suggests that the antioxidant compounds from analyzed extracts possessed both reducing power and radical scavenging capacity.

Conclusion

From the present study, it may be concluded that alkaline hydrolysis can improve the quality of NS seed extracts, in

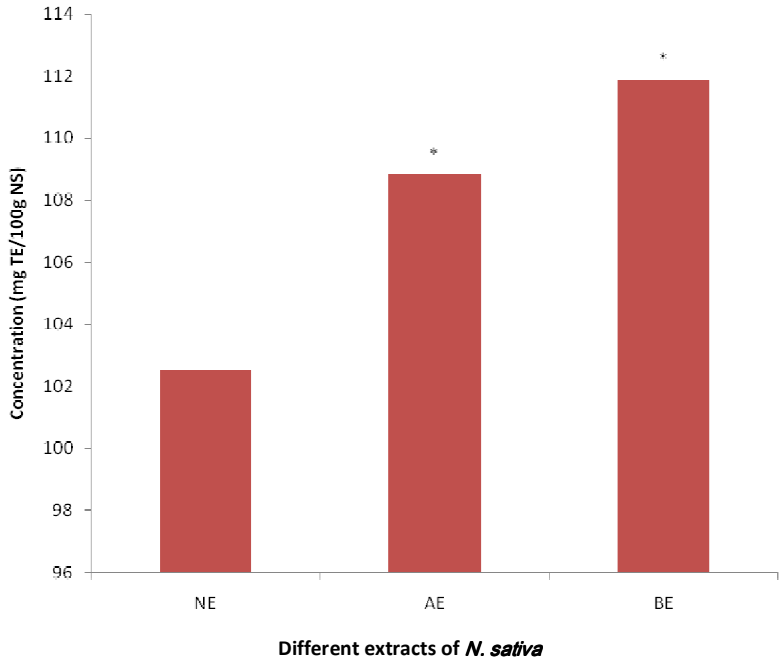

Figure 4. Radical scavenging activity using $ABTS^{\bullet+}$ of NS extracts. Values are means of three replicate determinations per 100 g *Nigella sativa* powder. Asterisks denote significance at a P < 0. 05 compared to control (NHE).

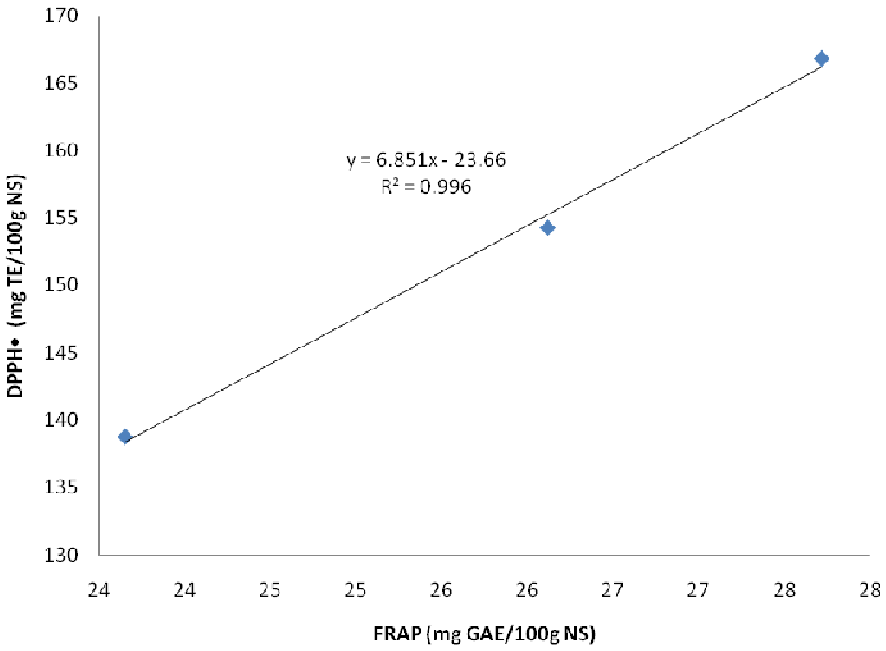

Figure 5. Correlation between $DPPH^{\bullet}$ and FRAP for NS extracts

addition to enhancement of scavenging activity, and increase in phenolic and flavonoid contents. Therefore this study supports alkaline hydrolysis during NS extraction. It is well documented that the quantity of phenolic compounds in foods is influenced by genotype, agronomic practices, maturity at harvest, post-harvest storage and climatic conditions (Luthria and Pastor-Corrales, 2006; Hakkinen and Torronen, 2000). Accordingly, this study cannot be generalized to all plants as different plants contain different phytoconstituents in

varying amounts. Further investigations are needed to be done for isolation and identification of phenolic compounds and *in vivo* studies are also needed for better understanding of their mechanism of action as antioxidant.

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