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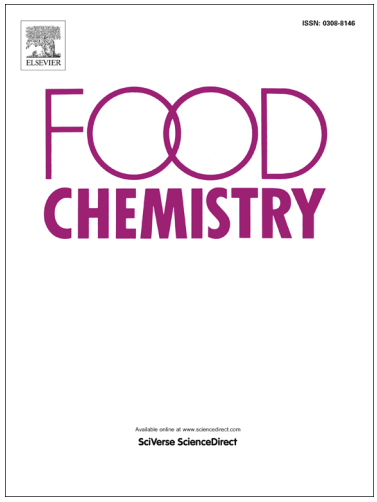

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Determination of Sinapine in Rapeseed Pomace Extract: its antioxidant and acetylcholinesterase inhibition properties

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Sinapine is the main secondary metabolite present in rapeseed pomace (RSP) with its concentration being dependent on rapeseed processing, growing conditions, extraction parameters and the country of origin. Here we report, the concentration of sinapine from an extract of defatted RSP harvested in the North East of Scotland. Using liquid chromatography tandem mass spectrometry, the most abundant phenolic compound in the RSP extract was, as expected, sinapine (109.1 mg/g RSP extract). Additionally, sinapic, caffeic, ferulic and syringic acids were identified (0.159-3.91 mg/g RSP extract). Sinapine together with the phenolics at the concentration present in the RSP extract, exhibited ≥ 50% activity relative to the extract in antioxidant assays. Furthermore, sinapine provided plasmid DNA (pBR322) protection, from 2,2'-azobis(2-amidinopropane) dihydrochloride and inhibited acetylcholinesterase activity by 85 %. Molecular docking was utilised to explain the inhibitory activity. RSP can be an excellent source of bioactive compounds for pharmaceuticals, food additive and nutraceutical applications.

Keywords
Rapeseed pomace; canola; sinapine; phenolic acids; antioxidant assays; plasmid DNA (pBR322); LC-MS/MS; acetylcholinesterase (AChE)

1. Introduction

Cultivation of *Brassica napus* (rapeseed/canola) has been on the increase for the last decade due to the high demand for edible rapeseed oil and its use as a biofuel (Kortesniemi et al., 2015). Rapeseed pomace (RSP) is a by-product obtained after production of edible oil from rapeseed. As a result, of increasing demand the amount RSP produced is accumulating and as such can cause storage issue although some has been used as animal feed (Kasprzak et al., 2016). There has been much interest to find ways in revalorizing this food by-product (also known as meal or cake) for example, to extract high quality protein (Campbell, Rempel, & Wanasundara, 2016; Thiyam, Pickardt, Ungewiss, & Baumert, 2009) and its valuable natural antioxidant contents (Thiyam, Stöckmann, Zum Felde, & Schwarz, 2006). The use of plant by-products in the diet has become a subject of great interest, to the food industry with the aim of finding and applying naturally-sourced, exogenous antioxidants in processed food. Some agricultural by-products including fruit and vegetable waste (Wijngaard, Rößle, & Brunton, 2009), olive pomace (Palmieri et al., 2012) and grape seed pomace (Jara-Palacios et al., 2013) have already been shown to contain well known phenolic antioxidants.

Rapeseed belongs to the *Brassicaceae* family which includes many edible plants and vegetables. These plants are a good source of bioactive compounds, including phenolics like flavonoids, hydroxycinnamic acid derivatives and glucosinolates (Cartea & Velasco, 2008; Vallejo, Tomás-Barberán, & Ferreres, 2004). Among the bioactive compounds in *Brassicaceae*, the most abundant phytochemicals are the hydroxycinnamic acid derivatives. The seeds of *Brassica oleracea* var. *acephala* (Kale) and *Brassica oleracea* var. *costata* (cabbage) contain, sinapine and 1,2-disinapoylgentibiose as the most prevalent phenolics and they were suggested to play a role in the inhibition of acetylcholine esterase (AChE) enzyme (Ferreres et al., 2009). AChE enzyme is

responsible for the breakdown of acetylcholine in the synaptic cleft of the brain. Hence by applying AChE inhibitors, acetylcholine is retained in the synaptic cleft for neurotransmission resulting in reducing the symptoms of Alzheimer's disease (Cheung et al., 2012). Therefore, sinapine is a viable AChE inhibitor for many diseases including Alzheimer and muscle disease e.g. *Myasthenia gravis* (Nićiforović & Abramovič, 2014).

Recently extracts of *Brassica chinensis* (Tsai Tsai) had exhibited strong antioxidant activity together with *in vivo* anti-aging properties conducted *in C. elegans* (Chen et al., 2016). Furthermore, sinapine had shown promise in the area of cancer chemotherapy where a combination of sinapine with the known anticancer agent, doxorubicin, demonstrated synergistic activity with enhanced *in vitro* anti-cancer properties against colon cancer Caco-2 cells (Guo et al., 2014).

In many Asian countries, sinapine has received much attention due to its significant biological *in vitro* and *in vivo* activity. Brassica plants such as *Brassica rapa var.rapa L.*, or parts of the plants have been shown to contain significant amounts of sinapine and are used in traditional Chinese medicine. The presence of sinapine had shown hepatoprotection in CCl_4-induced hepatotoxicity mice models (Fu, Zhang, Guo, Peng, & Chen, 2016). Neuroprotective properties of sinapine were also reported in a PC12 (rat pheochromocytoma) hypoxia cell model from $Na_2S_2O_4$-induced apoptosis and mitochondrial transmembrane potential disruption. Furthermore, it has been shown to decrease malondialdehyde (MDA) production and lactate dehydrogenase (LDH) leakage (Yang & He, 2008).

The phytochemical analysis of RSP ethanolic extract, together with their potent antioxidant and DNA protective properties was recently reported by us (Pohl et al., 2018), however, the analysis of the main component, sinapine could not be undertaken due to limited access to a reference compound. In addition to the various potential uses of the RSP extract by industry, there is good prospect for the application of purified sinapine to the pharmaceutical industry, for example by providing an accessible source of pharmacophore and biochemical tools in the discovery of novel cellular functions. Therefore we propose the RSP extract can provide a natural and economic source of sinapine.

The aim of this study is to determine the concentration of sinapine in an ethanolic extract of RSP originating from the North East of Scotland using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The antioxidant properties of sinapine using ferric ion reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays at the concentration present in the RSP extract and also in combination with other phenolic compounds (syringic, ferulic, caffeic and sinapic acids) present in the RSP extract will be studied. Furthermore, their ability to protect plasmid DNA from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) induced oxidative damage and inhibit acetylcholinesterase will be tested. To understand the inhibitory activity of AChE enzyme with sinapine, molecular docking methodology was applied and compared with known AChE, inhibitor, neostigmine.

2. Materials and Methods

2.1 Chemicals

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), methanol (HPLC grade), gallic acid, Trolox, Folin & Ciocalteu's phenol reagent, sodium acetate trihydrate, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), hydrochloric acid (HCl), ferric chloride, sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), AAPH, KH_2PO_4, EDTA, Sodium fluorescein, ferulic, caffeic and sinapic acids were obtained from Sigma-Aldrich, Dorset, UK. Syringic acid was provided from Lancaster synthesis, part of Alfa Aesar, Lancashire, UK. Glacial acetic acid, ethanol, HPLC grade methanol and acetonitrile, Tris-base, pBR322 Plasmid DNA (0.5 μg/μL), petroleum ether (bp 40-60°C), agarose, sodium sulphate (anhydrous), Amplex™ Acetylcholine/Acetlycholinesterase Assay Kit (A12217, Invitrogen™) and Phosphate Buffered Saline (PBS) Tablets (Dulbecco A, OXOID Limited) were supplied by Fisher Scientific, Loughborough, UK. GelRed™ Nucleic Acid Gel Stains 10000x in water (Biotium) was purchased from VWR, Lutterworth, UK. Sinapine as sinapine thiocyanate was from ChemFaces, Wuhan, China.

2.2 Plant Material

The RSP used throughout this study was provided by Mackintosh of Glendaveny (Mains of Buthlaw, Glendaveny, Peterhead), Scotland and stored at - 80^{o}C until used. Before extraction, the pomace samples were individually ground in a coffee grinder (De Longhi KG39) to a particle size between 710 and 125 µm and then freeze dried (Edwards, Freeze Dryer Modulyo). Ground dried samples were kept at -80 °C until extraction (Pohl et al., 2018).

2.3 Rapeseed Pomace Soxhlet Extraction

The automated Soxhlet (Gerhardt; Soxtherm SE 416) extraction of the RSP was carried out in replicate (n=9) according to our method previously reported using 95% ethanol from RSP defatted with petroleum ether (Pohl et al., 2018). Subsequently the ethanolic RSP extracts were freeze dried, pooled together and kept at -80 °C until further use.

2.4 LC-MS/MS Analysis

Chromatographic separation was performed, according to Neacsu *et al.* (Neacsu et al., 2013) with minor modifications, on an Agilent 1200 Infinity Series HPLC (Cheshire, UK) using a Zorbax Eclipse Plus C18 Rapid Resolution column (100 x 4.6 mm; 3.5 µm) maintained at 25 °C. The mobile phase conditions consisted of 0.1 % acetic acid in water (A) and 0.1 % acetic acid in acetonitrile (B) operated under gradient conditions at a flow rate of 0.4 mL/min over 69 min and an injection volume of 10 µL. The gradient programme was: 90% A – 10% B initially, changed to 45% A – 55% B over 45 min, then to 20% A – 80% B over 15 min and held for 3 min. This was further varied to 90% A – 10% B in 1 min and finally kept for 5 min. The system was allowed to equilibrate after each run. The HPLC was coupled to an Agilent 6420 MS/MS triple quadrupole. Electro-spray ionization (ESI) was utilised in both negative and positive ionisation modes. Sinapine was analysed in positive ionisation mode whilst syringic, ferulic, caffeic and sinapic acids were determined in the negative ionisation mode. The capillary voltage for both negative and positive ionisation modes was set at 4 kV. The desolvation temperature was kept at 350 °C with a gas flow rate of 12 L/min and nebulising pressure of 50 psi. Nitrogen gas was used as the nebulising, desolvation and collision gas. Full-scan

MS spectra were obtained by scanning from 40 – 1000 m/z. Optimized MS/MS transitions for each analyte are given in Table S1. Retention times, two multiple reaction monitoring (MRM) transitions and ion ratios were monitored for each analyte (The commission of the European Communities, 2002) for quantification and confirmation purposes.

Linearity was established *via* a six point calibration curve ranging in concentrations from 0.2 to 1.5 μg/mL (syringic and ferulic acids); 0.025 to 2.5 μg/mL (caffeic acid); 0.25 to 7.5 μg/mL (sinapic acid) and 0.025 to 2.5 μg/mL (caffeic acid); and 0.05 to 1 μg/mL (sinapine) all in mobile phase (90:10% acetonitrile:water). Intra-day and inter-day precision and accuracy was determined by triplicate injection of 0.1 and 1 μg/mL (0.8 μg/mL for sinapine) standards over two different days, respectively.

2.5 *In Vitro* Antioxidant Activity

All the antioxidant assays (FRAP, DPPH and ORAC) were carried out according to our previous work (Pohl et al., 2018). Additional information on the preparation of sinapine and the phenolic acids solutions, are given below, together with their analysed concentrations analysed. In all the assays the extract and relative concentrations of sinapine and phenolic acids in the extract: sinapine solution (10.9%), a mixed phenolic acid solution containing sinapic acid (0.39%), syringic acid (0.019%), caffeic acid (0.019%) and ferulic acid (0.016%) were used (Table 1) as well as a mixture of sinapine and the phenolic acids.

For the FRAP assay, the RSP extract (at 1mg/mL) and all other solutions were prepared in ethanol/water (40:60, v:v) and analysed at 593 nm (BioTek μQuant). Results were expressed as trolox equivalent (TE) per gram of dry weight RSP extract.

For the DPPH assay, the RSP extract and all other solutions were prepared in methanol and analysed at 517 nm (BioTek μQuant). Concentration dependent curves for the radical scavenging activity, from the RSP extract, phenolic acid mixture, sinapine and phenolic acids with sinapine mixture were obtained. Results were expressed as % radicals scavenged.

The ORAC was carried out for the extract, sinapine, phenolic acids and the phenolic acids with sinapine mixture in PBS. The assay was run at 20 µg/mL of extract and relative concentrations of sinapine and phenolic acids in the extract (Table 1). The reaction was monitored at excitation and emission wavelength of 480/20 and 525/20 nm, respectively (BioTek µQuant). Results were expressed as trolox equivalent (TE) per gram of dry weight RSP extract.

2.6 Inhibition of Supercoiled Plasmid DNA Strand Breakage

The inhibition of supercoiled plasmid DNA strand breakage was performed as previously described (Pohl et al., 2018). Briefly, pBR322 plasmid DNA (0.5 µg/µL) were incubated with PBS, AAPH (10mM) and RSP extract (20 µg/mL). Sinapine, the phenolic acids mix and, the phenolic acids mix with sinapine were analysed at their respective extract concentration. In addition, sinapine was analysed at 0.2 and 20 µg/mL. After one hour incubation, gels were loaded and electrophoresed (70 mins; 80V (Life Technologies Horizon 58 gel tank and Thermo EC 105 power pack) in TAE buffer and then visualized and photographed using Peqlab Fusion FX7 (Fusion 15.11 software) under UV-light. ImageJ software was used to analyse the band intensity.

2.7 Acetylcholinesterase inhibition activity

The Amplex™ Acetylcholine/Acetlycholinesterase Assay Kit (A12217, Invitrogen™) was used according to manufacturer instructions with minor modifications. The extract was dissolved in 1X reaction buffer to final well concentrations of 1-0.001 mg/mL. In addition, sinapine, the phenolic acids mix and a mixture of both at 0.25 mg/mL RSP extract was prepared and analysed. As the positive control, neostigmine was dissolved and diluted in 1X reaction buffer to 0.02 mg/mL giving a final well concentration of 0.005 mg/mL (22.39 µM). 50 µL of 0.2 U/mL AChE (in 1X reaction buffer) was used in each well, together with 50 µL sample to determine the inhibition activity. The buffer was used as negative control. A 10 µM hydrogen peroxide working solution in 1X reaction buffer (100 µL into the well) was used as a second positive control. To this, Amplex Red reagent (400 µM

Amplex Red, containing 2 U/mL HRP, 0.2 U/mL choline oxidise and 100 µM acetylcholine), were added to start the reaction. The plate was then transferred into a preheated (37°C) fluorescence plate reader (excitation wavelength 530/25 nm and emission wavelength 590/35 nm (Biotek, UK & Gen5 software) and a gain of 35 for 150 minutes).

2.8 Molecular docking experiments

The ligand structures were drawn using the ACD ChemSketch 2015 software and were then optimized by molecular mechanics by means of the Avogadro 1.1.1 software (Hanwell et al., 2012) using the MMFF94s force field. The PDB files from the Avogadro software were converted to the PDBQT format using AutoDockTools 1.5.6 software (Morris et al., 2009).

The acetylcholinesterase models were prepared for molecular docking using the AutoDockTools 1.5.6 software and the experimental data (file code 4EY7) for the complex of human acetylcholinesterase with *R*-donepezil (Cheung et al., 2012). The grid box size of approximately 60×40×60 Å was specified to cover the entire tentative binding site. Molecular docking was performed using the AutoDock Vina 1.1.2 software (Trott & Olson, 2010) with default scoring function parameters.

2.9 Statistical Analysis

Data are shown as mean ± standard deviation and all experiments were run at least in triplicate. Significant differences were determined using one-way ANOVA and Bonferroni multiple comparison using Prism6 (GraphPad Software, San Diego, CA, USA).

3. Results and Discussion

3.1 LC-MS/MS Analysis of RSP Extract

The predominant phenolics in our previous study (Pohl et al, 2018) along with sinapine were identified using their characteristic MRM transitions, ion ratios and retention times as shown in

Table S1 and Figure 1. Instrumental limits of detection (LODs) and quantification (LOQs) were determined as the minimum detectable amount of a phenolic in MRM mode with a signal-to-noise ratio of 3 and 10, respectively (Table 1). A six-level calibration curve was prepared and the majority of studied phenolics exhibited linearity between the limit of quantification (LOQ) and 7.5 µg/mL. Correlation coefficients (r^2) of the calibrations were ≥ 0.996 for all the phenolics studied. The LODs and LOQs ranged from 0.21 to 28 ng/mL and from 0.70 to 91 ng/mL, respectively (Table 1). Intra- and inter-day precision over 2 different concentrations levels (0.1 and 1 µg/mL for all phenolics; 0.1 and 0.8 µg/mL for sinapine) were <15 % for all studied phenolics (Table 1). Both within and between days accuracy was within 76-100 % for all the phenolics. Pohl *et al.* (2018) had previously reported the concentrations of syringic (224.2 µg/g RSP extract), ferulic (182.7 µg/g RSP extract), caffeic (110.8 µg/g RSP extract) and sinapic acids (4896.9 µg/g RSP extract) in RSP extract from the North East of Scotland which were similar to those found in this work (Table 1); however, the levels of sinapine were not determined.

Genetic factors, cultivation and growth conditions are considered to typically influence the phenolic contents and profile in rapeseed (Quinn et al., 2017). Sinapine was found to be the most dominant phenolic within the RSP extract at 10.91 ± 0.97%. Preliminary MS scans (Figure S1) of the extracts had also shown the presence of other compounds which were putatively identified based on literature (Clauβ et al., 2011; Ferreres et al., 2009; Oszmiański, Kolniak-Ostek, & Wojdyło, 2013; Thiyam et al., 2009; Yang et al., 2015) as derivatives of sinapic acid or benzoylcholine, cyclic spermidine conjugate and feruloyl choline (5-8') guaiacyl (Table S2). Due to unavailability of authentic standards, we have not quantified these additional compounds. The concentrations determined in this study (Table 1) were similar to those previously reported (Pohl *et al.* 2018).

3.2 Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay determines the capacity of compounds to reduce the ferric-tripyridyltriazine complex to the ferrous-tripyridyltriazine complex by electron transfer reaction. The reduction leads to a colour change of the solution, which is measured at 593 nm (Huang, Ou, Prior, & Rior, 2005). As

previously described (Huang, Ou, Prior, & Rior, 2005), the FRAP assay determines the capacity of compounds to reduce the ferric-tripyridyltriazine complex to the ferrous-tripyridyltriazine complex by electron transfer reaction. In this assay, the RSP extract showed a mean activity of 163 ± 7.91 mg TE/g RSP extract (Figure 2). Compared to this, the sinapine, at the extract specific concentration of 109.1 µg/mL, only shows an activity of 78.8 ± 4.54 mg TE/g RSP and the phenolic acids mix 10.6 ± 2.45 mg TE/g RSP (Figure 2). One – way ANOVA with Bonferroni's multiple comparison analysis showed statistical significant difference (p<0.001) for all the studied solutions in comparison with the extract. This only explains about 55% of the RSP extract activity. The mixture of the sinapine with the phenolic acids shows an additive effect (87.3 ± 14.1 mg TE/1 g RSP) implying the absence of any synergistic effect between the phenolic acids and sinapine. Therefore, 45% of the extract activity must be due to other compounds found in the extract (Table S2) or unknown compounds not detected. Statistical analysis between the results obtained for the extract in this study and the extract in our last study (Pohl et al., 2018); same extraction technique and harvest year of pomace, 172.4 ± 2.18 mg TE/1g RSP) showed no significant differences (p=0.1175).

3.3 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The radical scavenging activity of RSP extracts was carried out following the DPPH assay, using the stable DPPH radical. The scavenging is associated with a colour change of the solution from purple to yellow. An IC_{50} value of 19.2 ± 2.4 µg/mL was determined for sinapine which is lower than the IC_{50} for the RSP extract reported in Pohl *et al.* (2018). A lower IC_{50} value indicates stronger radical scavenging activity. At a concentration of 166.7 µg/mL the RSP extract was able to scavenge 78% of the radicals present (Figure 2). At the concentrations of sinapine and phenolic acids in the RSP extract, 73% and 8 % of radicals are scavenged respectively, relative to the extract, inferring that 81% of the scavenging capacity of the extract is due to sinapine and the four phenolic acids. One – way ANOVA with Bonferroni's multiple comparison analysis showed statistical significant difference (p<0.001) for all the studied solutions in comparison with the extract. The remaining activity as in the case of FRAP assay must be due to additional compounds (Table S2) within the extract with

radical scavenging activity. Thiyam *et al.* (2006) had previously studied the DPPH activity of various rapeseed as well as mustard extracts. By comparing three major components of rapeseed, they found the following order of scavenging activity: sinapic acid>sinapoyl glucose>sinapine. Interestingly sinapoyl glucose was one of the peaks identified in the RSP extract, although it was not quantified due to the absence of a reference standard. Furthermore, there is no synergistic effect between the phenolic acids and sinapine, only an additive effect was observed. In the extracts from Thiyam *et al.* (2006), sinapine was found to cause between 30-50% of the radical scavenging activity, which is lower than what was found in our study (73%). This could be due to the different extraction methods, ways of calculating the activity, the unit used to define activity, as well as the concentration at which the extract and sinapine were tested.

3.4 Oxygen Radical Absorbance Capacity (ORAC) assay

In the ORAC assay, AAPH is used to create radicals, which react with the fluorescent probe fluorescein, by causing oxidation of the later, to produce a non-fluorescent product. As previously described in Pohl *et al.* (2018), the RSP extract inhibited and/or delayed the probes (fluorescein) oxidation induced by AAPH. The latter produces a peroxyl free radical upon thermal decomposition which is commonly found in the body, making this reaction more relevant to biological systems (Isa et al., 2012). Here, sinapine and the phenolic acids delayed the decay of the fluorescence probe at 20 µg/mL RSP extract (Figure 2). However, sinapine only contributes to about 35% (681.3 ± 11.95 µmol TE/g RSP extract) while the phenolic acids only account for about 15% (288.4 ± 91.44 µmol TE/g RSP extract) when compared with the RSP extract alone. One – way ANOVA with Bonferroni's multiple comparison analysis showed statistical significant difference ($p<0.001$) for all the studied solutions in comparison with the extract. About 50% of the RSP extracts activity is unexplained, which is the highest among the three antioxidant assays studied. This can be explained by the fact that the three antioxidant assays have different reaction mechanisms (either based on electron- or hyrdrogen atom- transfer) and conditions (pH, solvent and temperature). The phenolic acids and sinapine tested did not show the same level of activity in all the assays.

3.5 Inhibition of Supercoiled Plasmid DNA Strand Breakage by RSP extracts

The introduction of AAPH to supercoiled plasmid DNA led to the complete cut of supercoiled DNA into the circular shape, which was visible by the lag of the DNA band in the gel. We previously showed that the RSP extract has the ability to protect plasmid DNA from AAPH induced damage (Pohl et al., 2018). Here we study the DNA protective capacity of the phenolic acids and/or sinapine at the concentration these compounds were present at in the RSP extract together with relevant controls i.e. sinapine at 0.2 and 20 µg/mL (Figure 3). The phenolic acids mix was not able to protect the plasmid DNA from damage, the band intensity was determined to be significantly different ($p<0.001$), to both the DNA control as well as the extract treated DNA. However, sinapine (at 2.18 µg/mL) showed protective activity, there was no significant difference ($p>0.05$) to the DNA control or extract treated DNA in band intensity (open circular DNA; Figure 3). Interestingly, the same level of DNA protection was observed for the mix of sinapine and the phenolic acids as for sinapine at RSP extract concentration ($p<0.05$). It is noteworthy to mention that a low concentration of sinapine (0.2 µg/mL) provided no DNA protection. In contrast, when the concentration of sinapine was increased to 20 µg/mL, complete DNA protection was achieved. Therefore, sinapine at RSP extract concentration was most likely responsible for the protection of the pBR322 plasmid DNA from AAPH damage.

3.6 Acetylcholinesterase inhibition activity

Patients with neurodegenerative diseases, for example, Alzheimer's disease have low concentrations of acetylcholine, which is a neurotransmitter responsible for the communication of signals between neurons and other cells. Within the brain, acetylcholine function has been associated with attention, cue detection and memory (Picciotto, Higley, & Mineur, 2012). The use of cholinesterase inhibitors such as Donepezil, Galantamine or Rivastigmine improves cognitive functions since inhibition of this enzyme will increase the concentration of acetylcholine in synapses. Therefore, the inhibition of acetylcholine esterase can be used as a strategy for the treatment of Alzheimer's disease. The RSP extract demonstrates concentration dependent AChE inhibition activity down to 0.01mg/mL over

time (Figure S2). Higher concentrations of RSP extract (0.75 or 1 mg/mL) showed almost complete AChE inhibition similar to the positive control, neostigmine (5 µg/mL).

Figure 4 showed that the RSP extract (0.25 mg/mL), sinapine and the phenolic acids and sinapine mixture (Table 1) after 40 min, all had the same level of AChE inhibitory activities at ~85% which was similar to the positive control neostigmine (5 µg/mL). However, the phenolic acids mixture only showed low AChE inhibitory capacity (~25%). Therefore, we can conclude that sinapine was the main contributor of AChE inhibition in the RSP extract.

Previous research on AChE inhibition by sinapine is limited. He *et al*. (2008) showed its AChE inhibition activity in cerebral homogenate and blood serum of rats. Extracts from kale and trochunda cabbage (*Brassica* family) had previously been shown to have *in vitro* AChE inhibition activity which was associated with the sinapine content (Ferreres et al., 2009). Our study shows similar evidence of inhibition acitivty *in vitro*.

3.7 Molecular docking experiments

In order to explain and shed further light on the inhibitory activity of sinapine, molecular docking studies were carried out. The AChE enzyme model was developed and optimised with the Alzheimer's disease drug Donepezil (binding score -11.9). The crystal structure of AChE and Donepezil are available from the Protein Data Bank (PDB). The root mean square deviation of the calculated and experimental X-ray data for Donepezil was less than 1 Å. The AChE active site for donepezil was used to dock both sinapine and neostigmine (Figure 5 (Figure 5 A, B). It is worth also mentioning that crystal structure of AChE with either neostigmine or sinapine is not currently available.

Although both sinapine and neostigmine share the same enzyme active site, the way they bind to the active site was found to be different from each other (Figure 5 C). The binding of sinapine to the AChE enzyme (Figure 5 A) involved amino acid residues Trp 286, Val 294, Tyr 337, Trp 86 and Tyr 341 giving a binding score (BS) of -7.4. For neostigmine, amino acids Phe 295, Phe 338, Tyr 337, Trp 86

and Tyr 341 contributed to its binding to AChE enzyme with a score of -7.1 (Figure 5 B). Interestingly both sinapine and neostigmine have a quaternary nitrogen in their side chain in close proximity to amino acid residues Tyr 337, Trp 86 in the active site of AChE enzyme. Those amino acids are known to form cation-π interactions with the quaternary protonated nitrogen as previously discussed by Lu et al. (2011). Hence, they can contribute to the overall binding capacity of sinapine and neostigmine. However, their lower binding capacities (-7.4, -7.1) when compared with donepezil (-11.7) may be attributed to the trimethylammonium group in sinapine and neostigmine being too big to form effective cation-π interaction. Furthermore, both sinapine and neostigmine lack a secondary amide functionality (O=C-NH) which is known to form strong hydrogen bond interactions with the amino acid residues in the active site of AChE enzyme (Lu et al, 2011). However, the similarity of the binding scores for both compounds is due to the presence of the aromatic ring in neostigmine and sinapine molecules. The binding scores of sinapine and neostigmine corroborated well with the inhibitory characteristic of both compounds observed in the Amplex™ Acetylcholine/Acetlycholinesterase assay (Figure 4) discussed above.

4. Conclusion

With the increasing interest in circular economy and zero waste, there has been intense effort to revalorise food by-products. Here we quantitatively determined sinapine as the most abundant bioactive compound in the RSP (95% ethanol) extract at a concentration of 10.9% of dry solid extract. At the concentration of sinapine present in the RSP extract, it exhibited strong antioxidant properties, had the ability to protect DNA from damage in the presence of a radical inducer and inhibited AChE enzyme activity. Sinapine showed the highest contribution to the extract activity in the DPPH assay, followed by the FRAP and then the ORAC assay. To our knowledge, this is the first evidence of antioxidant activity of sinapine determined by the ORAC and FRAP assays. In contrast, the mixture of phenolics at the concentration present in the RSP extract showed little activity in either of the assays. Taken together our findings from this work demonstrate the value added to

RSP. Furthermore, RSP can provide a viable and economical source of bioactive compound while the extract can be developed as nutraceutical supplement, natural preservative for the food industry or in pharmaceutical applications.

Conflicts of interest

There are no conflicts of interest to declare.

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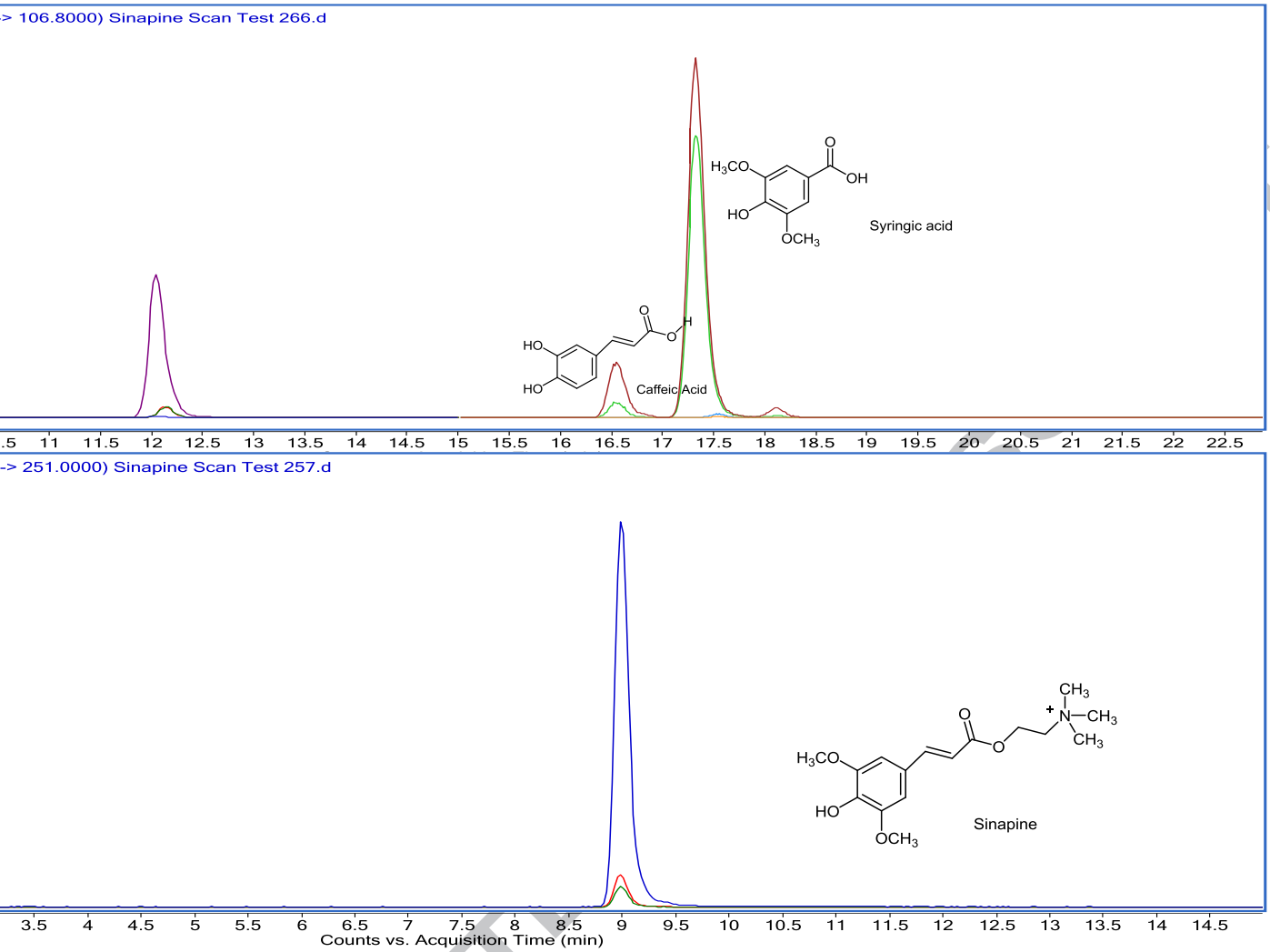


Figure 1| LC-MS/MS MRM chromatograms of phenolics studied in 5 µg/mL RSP extract (Bottom: sinapine - 9.3 min; positive mode) and 1 mg/mL RSP extract (Top: syringic – 12.1 min, ferulic – 17.5 min, caffeic – 12.0 min and sinapic – 17.3 min acids; negative mode)

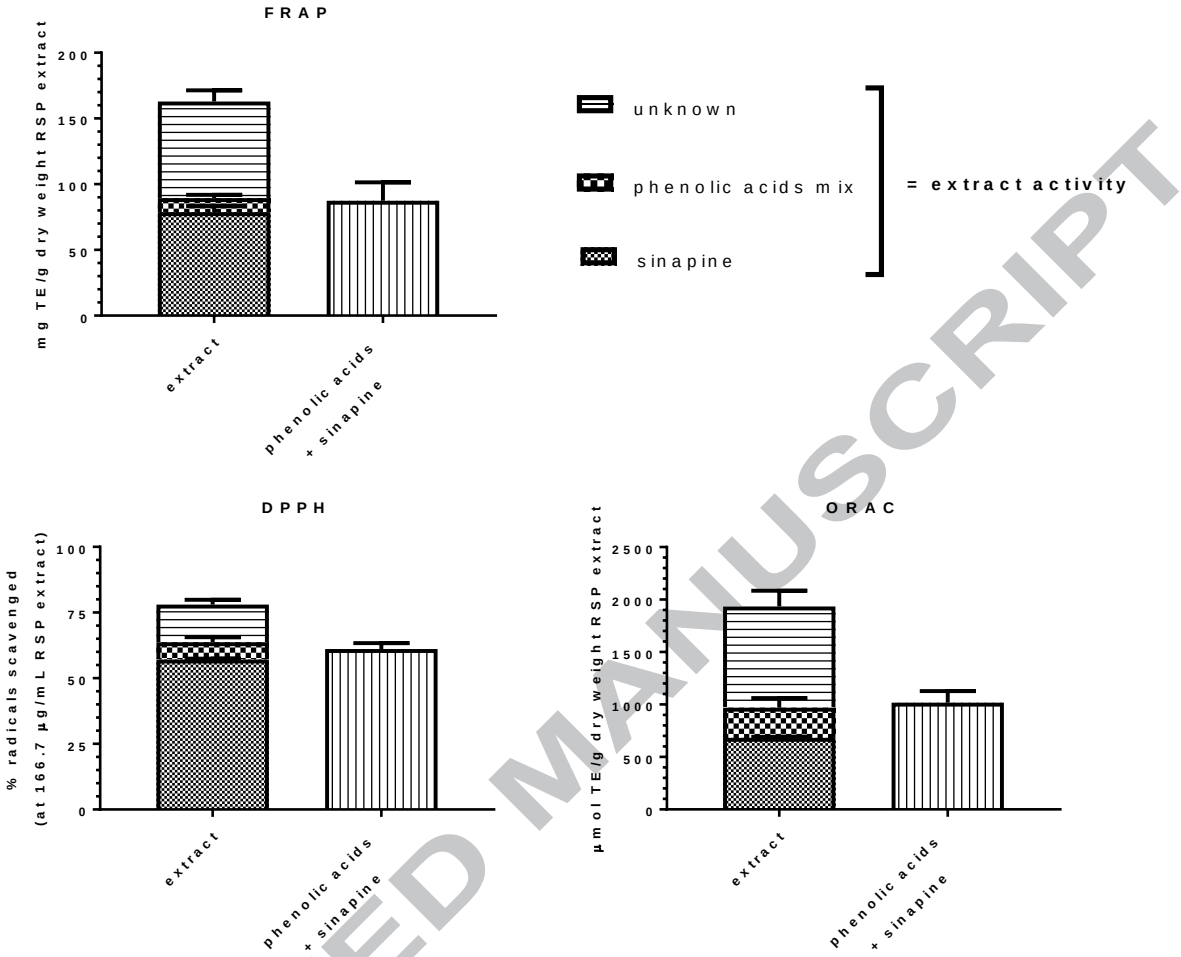


Figure 2| Antioxidant results obtained for the extract (1.0 mg/mL-FRAP, 0.1667 mg/mL) 0.02 mg/mL-ORAC) and sinapine/phenolic acids mix (at extract specific concentration), and a mixture of phenolic acids and sinapine

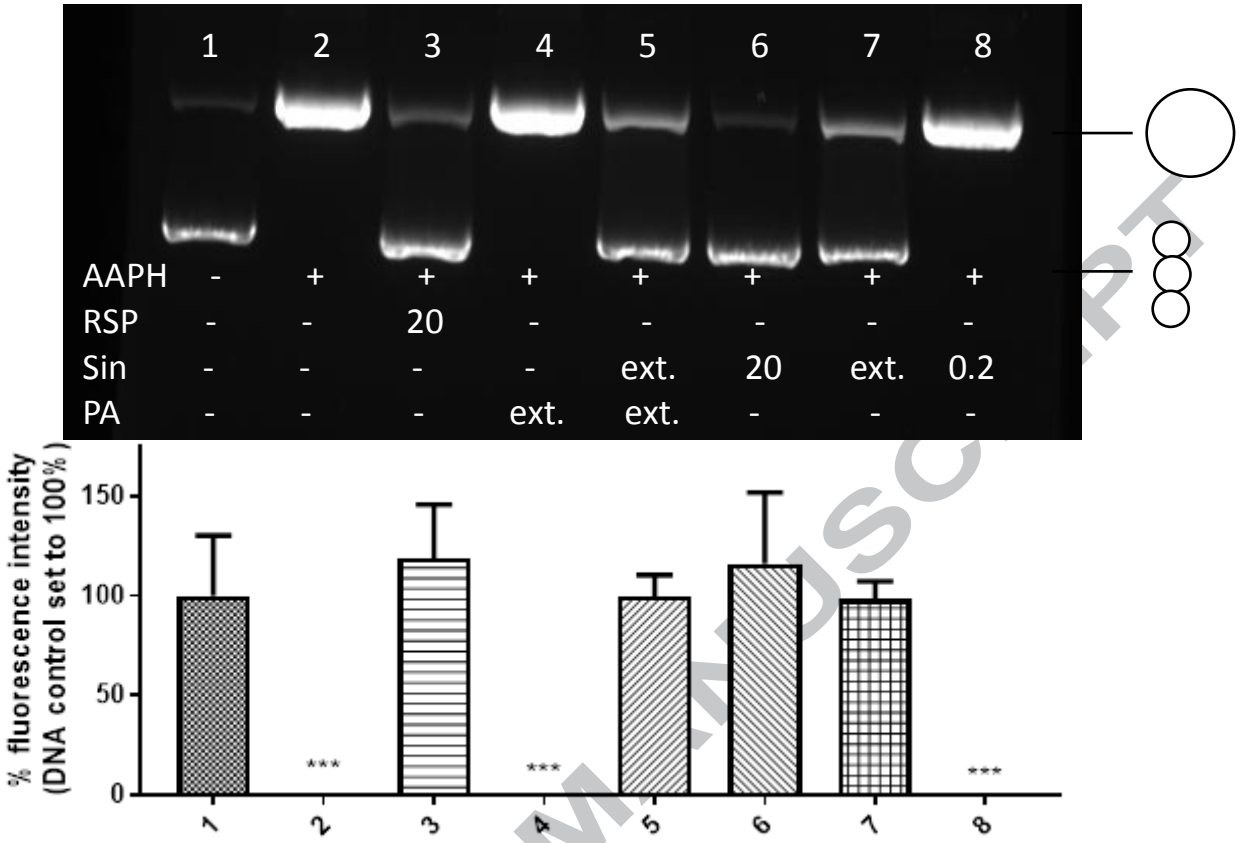


Figure 3|pBR322 DNA protection by RSP extract (20 µg/mL), phenolic acids (PA) mix, and sinapine (Sin – 20, 2.18 [ext] and 0.20 µg/mL). Band intensity of the circular DNA was analysed (n=3) to obtain graphical results, 1: DNA control, 2: DNA plus AAPH control, 3: RSP extract (20 µg/mL) + AAPH, 4: PA (at concentration found in RSP extract at 20 µg/mL) + AAPH, 5: Sin and PA (at concentration found in RSP extract at 20 µg/mL) +AAPH, 6: Sin (20 µg/mL) + AAPH, 7: Sin (at concentration found in RSP extract at 20 µg/mL) + AAPH, 8: Sin (0.2 µg/mL) + AAPH. Statistical significance observed when compared to DNA control and the RSP extract, ***p<0.001

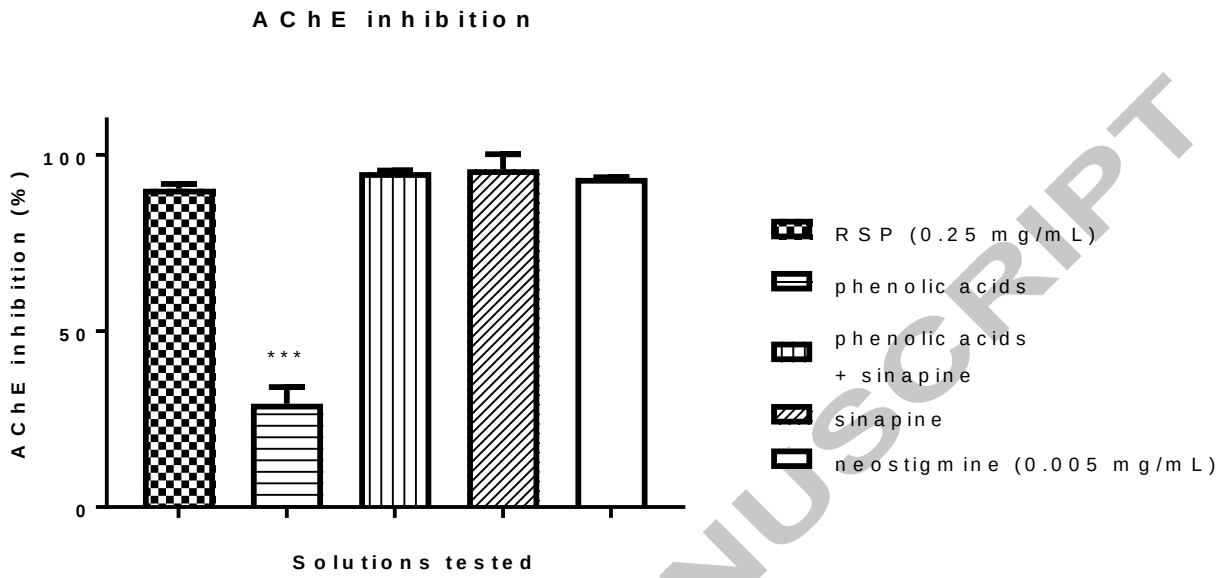
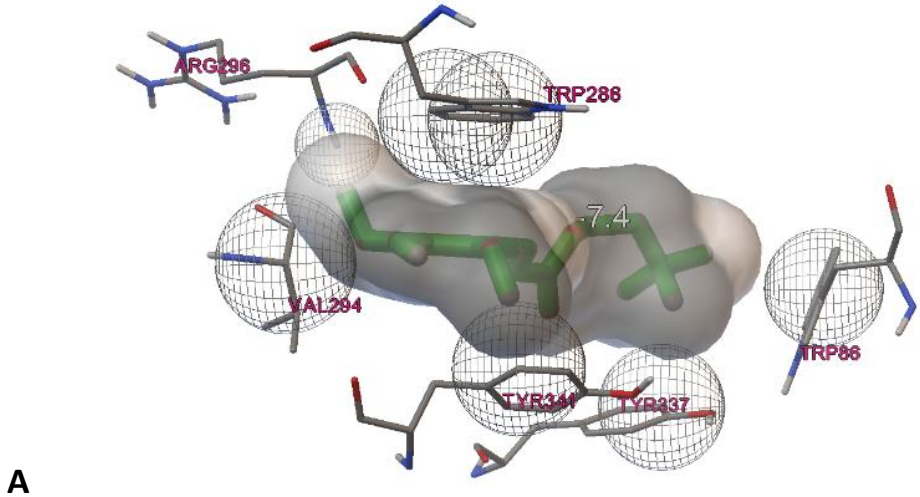
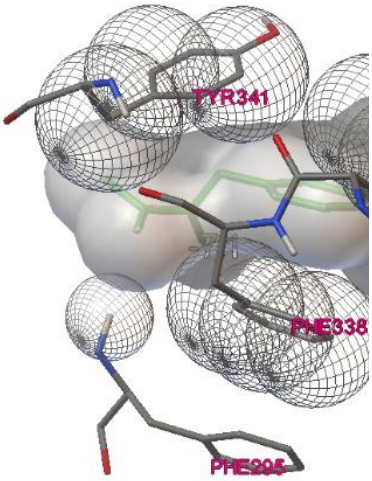


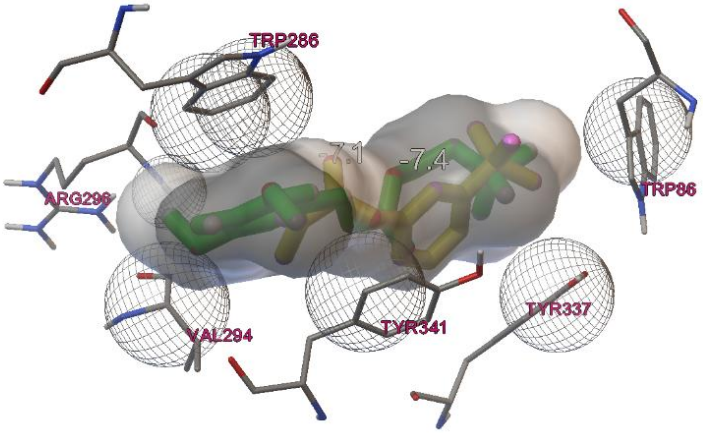
Figure 4| AChE inhibition (at 40min) activity of RSP extract (0.25 mg/mL), compared to phenolic acids, phenolic acids + sinapine, sinapine, and neostigmine (5 µg/mL). Statistical significant difference observed when compared to the RSP extract, ***p<0.001



A

B





C

Figure 5| Composite images of AChE binding with binding scores (BS). A Sinapine (BS = -7.4), B Neostigmine (BS = -7.1) and C Sinapine and Neostigmine together

Table 1| LC-MS/MS instrument performance and concentration of studied phenolics in RSP extract

Phenolic	*Rt* (min)	Linearity		Intra-day performance[a]		Inter-day performance[a]		$LOD_{S/N}$	LOQ S/N	Mean (±SD)[b] Concentration (µg/g RSP Extract)
		Range (µg/mL)	r^2	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	(ng/mL)	(ng/mL)	
Sinapine	9.3	0.050-1.000	0.9961	99.7	4.2	98.1	2.1	0.21	0.70	109091 (9698)
Caffeic acid	12.0	0.025-2.500	0.999	99.9	5.04	99.8	0.14	3.74	12.5	182.8 (9.6)
Syringic acid	12.1	0.200-1.500	0.999	83.9	7.90	85.7	10.0	17	57	191.5 (43)
Sinapic acid	17.3	0.250-7.500	0.992	99.2	0.59	96.8	3.64	19	63	3842 (426)
Ferulic acid	17.5	0.200-1.500	0.998	76.2	14.5	85.3	1.36	28	91	159.9 (29)

Key: R_t, retention time; LOD, limits of detection; LOQ, limits of quantification; S/N, signal to noise ratio;

[a]Mean of 2 concentration levels (100 and 1,000 ng/ mL for ferulic and syringic acid; 62.5 and 6,250 ng/ mL for caffeic and sinapic acid and 100 and 800 ng/ mL for sinapine

[b]n = 3

Highlights

- Quantitative determination of bioactive sinapine in rapeseed pomace (RSP) extract
- Anti-oxidant/radical scavenging properties of sinapine at RSP extract concentration
- DNA protective properties of sinapine against free radical inducer
- Sinapine inhibited AChE, a target for treatment of neurodegenerative diseases
- RSP is a source of bioactive compounds for nutraceutical & pharmaceutical industry