Optimized Production of Canolol Using Microwave Digestion as a Method of Pre-Treatment

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Abstract

Canola is the major oilseed crop of Canada. The de-oiled material is an important by-product due to its rich phenolic profile and high protein content. This co-processing stream from canola is primarily utilized as animal feed but represents an invaluable source of nutraceuticals. Microwave-assisted solvent extraction (MAE), as a green extraction method, has received considerable attention in recent times. The ease of use and application of many solvents at the same time makes the MAE one of the best methods for studying multiple solvents at the same time. The formation of canolol, from sinapine and sinapic acid, is primarily dependant on temperature which favors the decarboxylation reaction. Hence, MAE using green extractants can be used to enhance the yield of canolol. This study examined the effects of different pre-treatment temperature-time combinations of 140, 150, 160, and 170℃ for 5, 10, 15, 20 and 30 minutes on the extraction of canolol and other canola endogenous phenolic compounds. Three antioxidant assays assessed the antioxidant activity of the different extracts obtained by MAE confirming the microwave as a novel and versatile instrument for enhancing the yield of canolol. Improvements in the antioxidant activity of the different extracts further established the efficacy of the current method for isolating important natural phenolic derivatives for utilization by the nutraceutical industry.

Keywords – microwave assisted extraction (MAE), canolol, sinapine, high temperature, de-oiled canola, processing

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1. Introduction

Green chemistry and its associated technologies have gained considerable attention in recent years by both Federal and Provincial governments of North America. The Canadian government favors green technology and its associated applications for industrial use [1]. In Chapter 3 of their 2022, under the section dealing with clean air and a strong economy**,** the Canadian government has prioritized green technology as the future direction for industries and associated organizations [1]. This technology can be applied to the oilseed industry as a method for reducing the use of harmful chemicals and extraction solvents and minimize detrimental environmental effects. Traditional processing methods utilize large amounts of solvents, for example 1 g meal requires 70mL ethanol, which are considered to be uneconomical and environmentally undesirable [2,3].

During the oil refining process, most of the phenolic compounds are eliminated from the oil but retained in the meal fraction [4]. Hence, the meal is an important material for further processing as it is a rich source of valuable phenolic compounds including sinapate derivatives [5]. Sinapine is the choline ester of sinapic acid and the most abundant phenolic compound present in canola meal (up to 80% of the total phenolic compounds) [6]**.** Many reports have shown that sinapine undergoes structural changes. The first step involves the hydrolysis of sinapine in which choline ester is removed and sinapic acid is formed. This is followed by decarboxylation of sinapic acid with the formation of canolol (2,6-dimethoxy-4-vinylphenol), a potent antioxidant compound [5,7]. As described by Khattab *et al. (*2014) and Terpinc *et al.* (2011), [7,8], canolol stands out for its anti-carcinogenic and anti-mutagenic properties attributed to its radical scanveging capacity. Recent works revealed that canolol contributes to protect lipids and proteins of oxidation [9,10].

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Apart from the major sinapates, other thermo-generative phenolic compounds including canolol [5] and its derivatives**,** have been shown to exhibit illustrate greater antioxidative properties during thermal processing[11,12].

The application of more energy efficient techniques and the reduction in the use of harmful chemicals/ingredients by the industry has increased in recent years. Many novel energy efficient techniques have been applied to reduce the environmental impact by creating sustainable processing techniques. Hexane, the primary extraction solvent used in the oilseed industry, is being eliminate to reduce its presence in the residual oils and from the pressed cake [13]. The removal of hexane or de-solventization of hexane from the meal is critical in the obtention of a safe meal for animal feed and the nutraceutical industries [14]. MAE is a novel green technique that has gained the considerable attention by industry due to its ease of use, high efficiency, and higher yields [14]. Yet, its application in the oilseed industry was limited due to the associated costs. The new green economy initiative of the Canadian government, however, would facilitate the use of such techniques as energy efficient and improved extraction methods over the conventional solvent extraction methods [1].

The targeted co-extraction of the phenolic compounds, particularly canolol, using the MAE from the meal could facilitate the production of value-added products as sources of nutraceuticals [7]. Canolol formation is closely associated with high temperature processing as temperature-dependent parameters are required to improve its yield [5,15]. The powerful antioxidant and antimutagenic properties of canolol has been reported in many instances[16]. Consequently, the extraction, isolation, and purification of canolol and other thermos-generative compounds would create invaluable co-processing streams for the canola industry.

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Khattab et al. [7] successfully demonstrated the formation of canolol using the microwave. As a solvent extraction technique, however, the microwave has not been evaluated for extracting canolol and other thermo-generative compounds. MAE has many advantages including reducing the surface tension and viscosity of the extracting solvents at higher extraction temperatures This improves the solubility and mass transfer of targeted phenolics including canolol and other thermos-generative compounds [15]. Hence, the targeted extraction of canolol and other thermos-generative phenolic compounds from canola meal should substantially increase its value as a source of nutraceuticals. The present study investigated the efficacy of MAE for enhancing the yield of canolol and other thermos-generative phenolic compounds. The total phenolic content (TPC) and antioxidant activity of the obtained extractants were assessed to determine the effectiveness of the MAE. Milled canola meal with a particle size of 0.75 mm was used for the current study. Two solvent extractants mixtures, methanol:water and ethanol:water were examined at a pre-optimized concentration of 70:30 (v/v) using four different temperatures (140, 150, 160, and 170^{o}C and five different time points (5, 10, 15, 20, and 30 min). The present targeted extractions have implications in the co-processing of the canola meal for producing value-added phenolic compounds.

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2. Materials

Mechanically crushed (double expeller pressed) canola meal containing an oil content of 4-6% (w/w) (*Brassica napus* L.) was used in this study. All the raw materials were obtained from the Viterra group, St. Agathe, Manitoba. Sinapic acid (purity > 98%) were purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada). Sinapine (purity > 97%) was purchased from ChemFaces Biochemical Co., Ltd (Wuhan, Hubei, China) Canolol was synthesized in the lab (purity > 97%) and its purity confirmed via HPLC. Cellulose filter papers were purchased from Thermo Scientific Canada Ltd (Mississauga, ON, Canada). All the extraction solvents were purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada).

3. Methods

3.1 Sample preparation

Canola meal was de-oiled using the Soxtec 2050 (Foss-Tecator, Foss North America, MN, USA) Khattab et al. [6] with few modifications. In brief, 15 g of canola meal sample was put into each extraction thimble and extracted with an optimized cycle of boiling, rinsing, and recovery for 30, 60, and 20 min, respectively. De-oiling was conducted for two consecutive cycles including all five replicates. At the end of the de-oiling process the meal was separated and the remaining oil was decanted. De-oiled meal sample was milled using a ball mill to obtain the particle size of 0.75 mm. Further, the particle size of the obtained meal was confirmed via the Mastersizer 2000 (Malvern Instruments Ltd, Malvern, United Kingdom). The milled samples were stored at -20°C until further analyzed.

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3.2 Determination of Moisture Content

Moisture content of the defatted canola meal samples was conducted using a rapid method by the moisture meter (Denver instrument IR35, Denver, CL, USA). Samples were kept at 130℃ for 4 minutes to determine its moisture content. Ten replicates were analyzed, and the average moisture content was calculated to determine the phenolic content on the dry weight basis.

3.3 Microwave Assisted Solvent Extraction (MAE)

Microwave assisted solvent extraction of the defatted canola meal samples was conducted using the Multiwave^{TM} 5000 (Anton Paar, Montreal, QC, Canada) microwave system containing a rotor (20SVT50) with 20 vessels. Each vessel was filled in with 2.0g of defatted canola meal sample and extracted using 20.0 mL of 70% (v/v) methanol and 70% (v/v) ethanol. Prior to each extraction a magnetic stirrer was added along with heated elements to evenly distribute the heat inside the vessel. The smart vent technology associated with the Multiwave^{TM} 5000 system ensure the proper maintenance of temperature and pressure throughout the experimental process. The power of the microwave system was kept at 1000 W and during each extraction the sample vassals were monitored for the changes in the temperature using an IR temperature probe. The temperature calibration of the equipment was done prior to the extraction with the aid of water. The MAE was carried out at the experimental conditions including the temperatures of 140, 150, 160, and 170℃ for 5, 10, 15, 20 and 30 minutes. After each extraction the phenolic extract was taken out using plastic pasture tube and centrifuged at 7800 g for 15 minutes at 4℃. The supernatant of the centrifuged samples was collected and volumed up for 25.0 mL using the respective solvent (methanol or ethanol) and kept at -20℃ until further analysis.

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3.4 Identification of major sinapate derivates using HPLC-DAD

The changes associated to the phenolic composition of the phenolic extracts obtained by MAE were analyzed using the high-performance liquid chromatography with diode array detection (HPLC-DAD) according to the method described by Nandasiri *et al*. [5]. The HPLC-DAD (Ultimate 3000; Dionex, Sunnyvale, Torrance, CA, USA) analysis was carried out using a reversed phased Kinetex Biphenyl C_{18} 100 Å RP column (2.6 mm, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA) with a flow rate of 0.4 mL/min and 10 µL injection volume. The separation was carried out at 30℃, using a gradient elution of water (0.1% [v/v] formic acid) as solvent A and (0.1% [v/v] formic acid) methanol as solvent B. The gradient system was operated as follows: 25% B (0–3 min), 25% – 40% B (3 – 8min), 40%B (8 – 13min), 40% – 60% B (13 – 25 min), 60% – 70% B (25 – 38 min), 70% – 100% (38 – 41 min), 100% (41 – 44 min), 100% – 25% (44 – 47 min), and 25% B (47 – 57min). The chromatograms were acquired at both 320 nm (sinpaine and sinapic acid) and 270 nm (canolol) using Chromeleon software Version 7.2 SR4 (Dionex Canada Ltd., Oakville, ON, Canada). Major phenolic compound canolol was identified using the authentic standards with a detection limit of 0.001 mg/mL. Calibration curves for each standard were obtained from 1.0 to 100 µg/mL (n=11) concentration range with R^2 = 0.999 for sinapic acid, R^2 = 0.999 for canolol, and R^2 = 0.999 for sinapine.

3.5 Assessment of the total phenolic content and total flavonoid content

3.5.1 Determination of total phenolic content (TPC)

The TPC of the phenolic extracts obtained by MAE were determined using the Folin-Ciocàlteu assay as described by Thiyam *et al*. [17] with few modifications. In brief, samples were diluted with distilled water with 1:100 (v/v) ratio. The diluted extract was mixed with 0.5mL of

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Folin-Ciocalteu's reagent and 1.0mL of 19% (v/v) Na_2CO_3. Distilled water was then added to make the total volume up to 10mL of solution and then vortexed. The reaction was conducted in dark for 60 min with intermittent vortexing (VWR™ Analog Vortex Mixer) at 30 min. Absorbance was measured using the UV-Visible Spectrometer FL6500 (Perkin Elmer Inc., Shelton, Connecticut, U.S.A) at 750nm. Methanol was substituted as blank, and sinapic acid solution (1.0 mM) was used to assemble the standard curve as presented in **Figure S1A** (supplementary data).

3.5.2 Determination of total flavonoid contents (TFC)

The TFC of the phenolic extracts obtained by MAE were measured using $AlCl_3$ colorimetric method described by Zhishen *et al.* [18] with slight modifications. In brief, 0.5 mL of the extract was diluted with distilled water in a ratio of 1:4 (v/v). The diluted sample was then mixed with 0.15 mL of $NaNO_2$, 5% (w/v) and kept at the room temperature for 6 minutes. Afterwards, 0.3mL of $AlCl_3$10% (w/v) was added to the sample mixture and kept for additional 5 minutes. After 5 minutes, 1.0 mL of NaOH (1 M) was mixed with the previous solution by a Vortex mixer (VWR™ Analog Vortex Mixer). The absorbance was measured at 510 nm. Quercetin was used to prepare the standard curve (0.1 to 1mM) **Figure S1B** (supplementary data) and the total flavonoid content of the phenolic extracts were expressed based on equivalent milligrams of quercetin per gram of dry weight of canola meal (QE mg/gDW).

3.6 Antioxidant activity of the phenolic extracts obtained by MAE

3.6.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity of the phenolic extracts obtained by MAE was determined according to the method of Girgih *et al.* [19] with slight modifications using a 96-well

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micro plate reader (Bio-Tek Powerwave XS2, Vermont, USA). A 100 µM DPPH working solution was prepared using 95% methanol. A 100 µL aliquot of extracts obtained at different microwave time/temperature conditions was diluted 1:20 times and the diluted samples were mixed with 140 µL of the DPPH radical solution in a clear 96-well micro plate and incubated in the dark for 30 min at room temperature. A 70% (v/v) methanol solution was used as the blank. Absorbance was measured at 517 nm wavelength, and the percentage DPPH radical scavenging activity was determined using the following equation:

DPPH radical scavenging activity (%) = $(Ab - As/Ab) \times 100$

where Ab and As are the respective absorbance of the blank and sample, respectively.

3.6.2 Metal-ion chelation properties of the extractants

The metal ion chelating ability was evaluated according to the modified method of Xie *et al.* [20]. In brief, a 100 µL aliquot of each phenolic extract obtained by MAE was diluted 10 times (1:10) and then combined with 25 µL $FeCl_2$ and 50 µL of ferrozine reagent and the resultant mixture made up to 1mL with deionized water in a reaction tube. The resultant mixture was allowed to stand for 10 min at room temperature. Thereafter, a 200 µL aliquot from the resultant mixture was pipetted into a clear 96-well micro plate and the absorbance was measured at 562 nm using a microplate reader (Bio-Tek Powerwave XS2). Methanol (70 % v/v) was used as the blank and the results were expressed the % metal ion chelating activity

% Metal ion chelating activity = $(Ab - As/Ab) \times 100$

where Ab and As are the absorbance of the blank and sample, respectively.

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3.7 Statistical analysis

All the experiments were carried out in four replicates. Results were presented as mean ± standard deviation of four replicate analysis. Data points were checked for their normality prior to the statistical analysis and required transformations were carried out to obtain normalized data [21]. To achieve the normalized data for the statistical model square transformations were conducted [21]. For the current statistical analysis different independent factors including solvent (methanol, ethanol), temperature (140, 150, 160, and 170℃), time (5, 10, 15, 20 and 30 minutes) were assessed for the final concentration of the individual phenolic compounds including the major sinaptes and other unknown compounds. In addition, the relationship between the major sinapates and other unknown phenolic derivatives were determined using the response surface model analysis (RSM) analysis.

The model fit statistics was conducted using the RSM analysis. Over the years RSM technique was applied to obtain the best fitting model with the optimal response using minimal number of variables [22]. Further, RSM analysis provides complete information related to interaction effects between individual parameters for determining the stationary point which is the optimal condition [22]. Hence, to validate the proposed mathematical model created by the RSM analysis, analysis of variance (ANOVA) is often required to assess the level of significance and model adequacy [23]. Statistical modeling and analysis were carried out using the R statistical software version 3.6.0 [24].

Similarly, the results of different antioxidant mechanisms were further assessed to determine the optimum extraction time/temperature combinations for the microwave assisted solvent extraction.

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4. Results and Discussion

4.1 Impact of microwave assisted solvent extraction on the major sinapates

The impact of MAE was conducted to determine the compositional changes in sinapine, sinapic acid and thermally generated canolol. These were assessed based on different time/temperature regimes used for both extraction solvents, methanol (70%, v/v) and ethanol (70%, v/v). When subjected to microwave treatment, the major sinapates extracted increased with time and temperature reaching a maximum prior to degradation. The thermally favored reactions involved in the conversion of sinapic acid to canolol and other thermo-generative compounds progressed over time [5,7]. A previous study by Mayengbam *et al.* [25] reported a 60% reduction in the original concentration of sinapine after roasting the canola seeds at 115^{o}C for 5 minutes, while the sinapine concentration further decreased to 90% after extraction for 20 min at 240^{o}C [25]. In a study conducted by Zago *et al*. [14] they reported that application of super heated steam prior to the microwave treatment increased the sinapine content of the meal fraction by 28%. This confirms that additional pre-treatments prior to MAE further facilitated the extractability of sinapic acid derivatives.

The results of the current study showed that both extraction time and temperature significantly affected the extractability of the major sinapates (**Figure 1**). Furthermore, the two solvents produced different yields for the major sinapates with the MAE (**Table 3**). Previous reports found that MAE exhibited better extraction efficiency due to its synergetic effect on mass and heat transfer throughout the extraction process [26]. The yields obtained with the extractants depended on the composition of the extracting material, water content, solvent to substrate ratio, extraction time and the temperature [26]. In addition, the intensity of the microwave also plays an

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important role in the extraction process. The intensity of the sample is also recorded as the power density (W) per gram of sample. In the current study, the intensity was kept constant to minimize the variation throughout the extraction process. The solvent extractions conducted after the microwave-assisted pre-treatment, found ethanol extracted higher amounts of sinapine compared to methanol (**Table 3**). RSM analysis between time and temperature on the concentration of major sinapates established the optimum extraction condition**s** for sinapine, sinapic acid and canolol (**Figure 1a, b**). For both ethanol (adjusted R^2-0.27) and methanol (adjusted R^2-0.89) as extraction solvents, only the main effects (time and temperature) had a significant impact towards the extractability (**Table 1a**). The lower adjusted R^2 value associated with ethanol may be due to the high variability of sinapine extractability at the relatively higher temperatures and prolonged extraction times. Furthermore, the statistical model indicated that there is no stationary point for the extraction of sinapine under the current extraction conditions using the microwave for both extractants. This was attributed to the longer processing times and conversion of sinapine into sinapic acid, canolol, and other sinapate derivatives during thermal processing [12,27,28]. It was further reported that the extractability of sinapine decreases with the increase of the processing temperatures [4]. This was evident from the results of the ratio analysis between sinapine and sinapic acid (**Figure 3**). In addition, our recent studies have shown that the conversion of sinapine to sinapic acid was higher compared to the conversion of sinapine/sinapic acid to canolol [11].

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Table 3: Impact of MAE on the major sinapates

Solvent	Temp (℃)	Time (min)	Wavelength (320 nm)								Wavelength (270 nm)		
			SP (µg/g DW)	SA (µg/g DW)	6.09 RT (µg SAE/g DW)	21.36 RT (µg SAE/g DW)	32.18 RT (µg SAE/g DW)	14.46 RT (µg SAE/g DW)	8.21 RT (µg SAE/g DW)	17.89 RT (µg SAE/g DW)	CL (µg/g DW)	7.53 RT (µg CLE/g DW)	10.10 RT (µg CLE/g DW)	13.66 RT (µg CLE/g DW)
Methanol	140	5	3541.83 ± 155.11	556.97 ± 57.17	181.56 ± 7.58	556.97 ± 57.17	4838.84 ± 118.08	258.23 ± 12.20	83.31 ± 4.11	210.78 ± 7.98	2343.56 ± 92.77	619.01 ± 36.52	287.41 ± 25.28	596.67 ± 15.14
		10	4046.75 ± 484.96	741.69 ± 120.51	222.07 ± 9.93	741.69 ± 120.51	6200.16 ± 191.57	318.07 ± 4.31	71.88 ± 2.54	258.27 ± 1.31	2983.46 ± 146.31	768.88 ± 38.90	356.14 ± 87.22	766.07 ± 12.28
		15	3158.83 ± 181.69	420.51 ± 88.93	243.20 ± 10.66	519.20 ± 88.61	6049.42 ± 111.09	297.49 ± 8.30	83.87 ± 9.45	269.85 ± 1.97	2847.28 ± 86.07	877.13 ± 29.00	349.37 ± 69.97	703.20 ± 25.17
		20	2872.39 ± 341.07	428.91 ± 97.34	231.71 ± 27.78	428.91 ± 97.34	6191.98 ± 401.07	308.62 ± 7.25	72.81 ± 2.78	256.73 ± 14.97	2852.00 ± 200.93	769.80 ± 29.59	302.71 ± 120.35	700.33 ± 53.17
		30	1759.47 ± 254.58	221.23 ± 20.80	314.12 ± 29.55	221.23 ± 20.80	7962.29 ± 253.80	341.19 ± 0.47	81.92 ± 6.38	308.68 ± 4.80	2547.76 ± 35.07	867.89 ± 51.68	760.42 ± 159.77	1208.22 ± 88.33
	150	5	2352.41 ± 84.48	334.21 ± 20.12	223.65 ± 13.72	334.21 ± 20.12	5759.54 ± 30.40	244. 98 ± 5.13	85.62 ± 4.05	232.79 ± 4.59	3006.33 ± 30.21	904.49 ± 30.85	720.48 ± 66.92	860.45 ± 63.07
		10	1744.25 ± 48.95	309.10 ± 32.83	229.32 ± 12.86	309.10 ± 32.83	5956.16 ± 123.09	270.60 ± 7.86	83.12 ± 2.15	248.94 ± 7.99	2965.80 ± 71.13	904.86 ± 34.47	762.96 ± 108.16	911.78 ± 105.42
		15	2585.63 ± 269.01	264.59 ± 34.52	301.83 ± 11.94	264.59 ± 34.52	6761.47 ± 599.85	292.47 ± 29.83	77.91 ± 4.01	287.09 ± 9.12	3158.14 ± 101.36	1123.46 ± 189.18	627.24 ± 18.76	803.68 ± 130.51
		20	1451.95 ± 40.07	190.18 ± 7.64	333.19 ± 16.55	187.49 ± 6.64	7202.72 ± 30.47	289.64 ± 8.31	86.40 ± 1.69	286.69 ± 2.65	3053.81 ± 20.02	1070.19 ± 64.62	1380.24 ± 132.61	1371.80 ± 79.54
		30	904.37 ± 32.75	234.61 ± 9.23	438.06 ± 10.38	237.57 ± 8.68	8257.27 ± 71.75	315.50 ± 2.29	94.77 ± 6.54	335.51 ± 7.92	2346.51 ± 59.78	1357.16 ± 44.19	2273.39 ± 224.10	1837.20 ± 35.43
	160	5	1411.93 ± 161.27	216.39 ± 4.72	315.38 ± 0.72	216.39 ± 4.72	6157.75 ± 1.91	281.13 ± 3.54	102.38 ± 4.50	271.40 ± 2.22	3202.70 ± 39.46	1171.92 ± 70.88	1695.63 ± 144.75	1314.73 ± 49.91

Solvent	Wavelength (320 nm)	Wavelength (270 nm)

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	Temp (℃)	Time (min)	SP (µg/g DW)	SA (µg/g DW)	6.09 RT (µg SAE/g DW)	21.36 RT (µg SAE/g DW)	32.18 RT (µg SAE/g DW)	14.46 RT (µg SAE/g DW)	8.21 RT (µg SAE/g DW)	17.89 RT (µg SAE/g DW)	CL (µg/g DW)	7.53 RT (µg CLE/g DW)	10.10 RT (µg CLE/g DW)	13.66 RT (µg CLE/g DW)
		10	1118.13 ± 147.94	232.74 ± 9.31	365.19 ± 10.23	232.74 ± 9.31	6770.70 ± 15.26	295.06 ± 14.93	112.07 ± 3.52	288.29 ± 4.52	3188.63 ± 29.96	1456.58 ± 67.43	2111.43 ± 238.84	1572.32 ± 84.69
		15	986.23 ± 20.44	237.16 ± 8.00	449.00 ± 25.90	237.16 ± 8.00	7740.05 ± 377.98	326.97 ± 17.39	129.50 ± 17.10	323.20 ± 9.95	3292.28 ± 50.80	1542.32 ± 172,48	2285.47 ± 534.93	1722.32 ± 248.64
		20	585.30 ± 110.11	208.61 ± 7.24	380.91 ± 26.83	208.61± 7.24	6648.04 ± 105.03	276.34 ± 6.83	129.90 ± 7.80	267.83 ± 5.37	2866.28 ± 39.30	1531.88 ± 89.23	2462.21 ± 506.36	1711.62 ± 189.13
		30	387.48 ± 11.25	232.77 ± 15.49	409.36 ± 12.25	232.77± 15.49	7459.99 ± 211.69	251.12 ± 11.77	109.86 ± 1.67	249.58 ± 11.04	1945.17 ± 32.29	1327.85 ± 7.83	3393.27 ± 151.29	2314.18 ± 64.22
	170	5	540.57 ± 68.50	186.63 ± 5.04	194.85 ± 16.41	186.63± 5.04	3455.70 ± 225.08	141.98 ± 9.18	100.59 ± 10.17	151.86 ± 12.34	1658.91 ± 93.44	719.88 ± 82.12	1279.74 ± 185.92	708.36 ± 103.34
		10	582.02 ± 169.18	219.38 ± 0.81	253.12 ± 35.32	219.38± 0.81	4599.25 ± 313.93	172.66 ± 3.20	112.85 ± 3.38	202.37 ± 1.75	1903.08 ± 38.79	855.28 ± 216.67	1931.11 ± 99.46	1065.82 ± 224.87
		15	481.70 ± 9.63	205.11 ± 2.60	319.09 ± 6.58	205.11 ± 2.60	5335.94 ± 139.30	195.41 ± 5.23	115.67 ± 12.72	209.67 ± 5.70	1985.13 ± 16.78	1276.08 ± 29.20	2166.43 ± 205.13	1464.37 ± 98.86
		20	421.36 ± 16.57	238.97 ± 3.86	377.30 ± 11.04	238.97 ± 3.86	6894.05 ± 120.57	258.06 ± 18.95	150.11 ± 1.33	250.05 ± 13.32	2610.34 ± 24.91	1636.74 ± 35.40	3642.72 ± 137.02	2169.27 ± 55.91
		30	ND	231.90 ± 7.32	322.13 ± 20.50	231.90 ± 7.32	8203.42 ± 275.74	221.97 ± 8.30	145.56 ± 1.80	187.77 ± 6.48	2049.56 ± 98.59	1464.48 ± 46.56	5593.86 ± 167.19	3050.06 ± 59.64
Ethanol	140	5	13394.58 ± 394.83	417.42 ± 11.00	629.55 ± 16.18	417.42 ± 11.00	138.50 ± 16.79	276.87 ± 10.45	110.81 ± 5.42	282.35 ± 2.56	4350.52 ± 54.57	1169.91 ± 49.43	1697.94 ± 132.17	1810.11 ± 110.97
		10	14044.18 ± 613.39	652.28 ± 58.64	565.26 ± 83.13	652.28 ± 58.64	109.28 ± 109.09	238.00 ± 18.34	90.48 ± 15.66	265.95 ± 26.55	4169.30 ± 139.70	950.81 ± 179.61	1021.11 ± 272.01	1523.05 ± 170.04
		15	1095.05 ± 95.90	170.76 ± 1.85	324.53 ± 12.22	170.76 ± 1.85	125.16 ±19.93	750.63 ± 21.13	2122.54 ± 68.84	859.48 ± 34.65	538.81 ± 27.04	1525.70 ± 84.35	328.36 ± 22.39	430.76 ± 10.36
		20	731.83 ± 28.05	142.07 ± 1.57	261.80 ± 37.63	142.07 ± 1.57	66.71 ± 1.79	611.53 ± 9.80	2226.05 ± 31.34	514.25 ± 25.58	270.67 ± 33.72	946.23 ± 93.50	nd	377.84 ± 73.87
		30	10327.27 ± 598.03	258.08 ± 33.51	704.34 ± 35.90	258.08 ± 33.51	69.61 ± 0.58	246.42 ± 14.81	125.14 ± 22.66	328.16 ± 24.40	3760.24 ± 23.55	1507.96 ± 154.60	1953.09 ± 351.98	1811.69 ± 243.16
Solvent			Wavelength (320 nm)								Wavelength (270 nm)			

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	Temp (℃)	Time (min)	SP (µg/g DW)	SA (µg/g DW)	6.09 RT (µg SAE/g DW)	21.36 RT (µg SAE/g DW)	32.18 RT (µg SAE/g DW)	14.46 RT (µg SAE/g DW)	8.21 RT (µg SAE/g DW)	17.89 RT (µg SAE/g DW)	CL (µg/g DW)	7.53 RT (µg CLE/g DW)	10.10 RT (µg CLE/g DW)	13.66 RT (µg CLE/g DW)
	150	5	13340.43 ± 466.82	668.37 ± 72.76	336.87 ± 28.19	668.37 ± 72.76	65.79 ± 5.48	202.75 ± 9.94	54.52 ± 4.95	162.75 ± 12.37	3918.48 ± 70.76	512.99 ± 35.21	445.64 ± 68.75	1091.00 ± 163.78
		10	11928.58 ± 587.55	313.74 ± 14.92	825.75 ± 17.45	313.74 ± 14.92	179.26 ± 2.60	297.90 ± 8.31	159.14 ± 6.41	359.33 ± 3.36	5029.25 ± 102.36	1794.05 ± 119.72	2778.22 ± 360.85	2520.22 ± 164.79
		15	878.97 ± 40.82	108.69 ± 34.34	289.28 ± 16.68	108.69 ± 34.34	146.69 ± 1.05	678.77 ± 3.57	1965.64 ± 241.53	855.42 ± 19.14	852.33 ± 70.13	1480.60 ± 504.98	nd	455.58 ± 75.95
		20	939.93 ± 14.51	247.26 ± 8.24	273.74 ± 40.11	247.26 ± 8.24	181.81 ± 6.89	654.99 ± 31.13	1716.51 ± 19.07	515.48 ± 2.17	737.24 ± 23.34	1943.03 ± 133.25	nd	644.68 ± 65.59
		30	6785.0 ± 538.36	265.40 ± 9.83	638.69 ± 26.42	265.40 ± 9.83	224.89 ± 13.76	258.31 ± 14.98	176.61 ± 4.25	336.76 ± 8.87	4282.47 ± 84.33	1908.96 ± 30.18	3764.29 ± 165.32	2834.63 ± 73.84
	160	5	8302.35 ± 860.25	328.12 ± 22.21	631.72 ± 15.45	328.12 ± 22.21	261.06 ± 72.65	272.09 ± 42.43	199.40 ± 12.95	295.88 ± 14.10	5404.57 ± 124.98	2095.57 ± 104.81	4546.45 ± 685.77	2999.54 ± 270.58
		10	8272.26 ± 840.31	302.36 ± 2.55	684.68 ± 49.72	302.36 ± 2.55	243.06 ± 10.69	305.89 ± 9.28	185.33 ± 5.23	291.94 ± 4.67	4734.04 ± 11.84	2081.81 ± 58.34	3909.07 ± 344.63	2857.57 ± 123.19
		15	1094.40 ± 28.28	327.38 ± 12.44	254.10 ± 18.16	327.38 ± 12.44	146.25 ± 0.48	504.83 ± 36.63	1834.48 ± 20.88	581.44 ± 17.46	1560.00 ± 37.14	2821.81 ± 58.34	nd	821.35 ± 38.38
		20	944.80 ± 20.99	294.51 ± 27.21	285.19 ± 12.46	312.22 ± 28.53	189.17 ± 7.11	470.56 ± 12.64	1150.89 ± 66.09	478.56 ± 8.09	894.85 ± 25.89	3747.86 ± 241.71	nd	1034.51 ± 98.33
		30	3854.23 ± 599.14	238.82 ± 9.57	334.72 ± 21.76	238.82 ± 9.57	235.23 ± 14.66	278.73 ± 15.50	184.71 ± 12.07	210.10 ± 8.44	3569.89 ± 7.25	1175.90 ± 52.81	5148.58 ± 679.12	3380.19 ± 294.13
	170	5	5341.16 ± 379.86	301.95 ± 5.04	463.19 ± 8.19	301.95 ± 5.04	470.52 ± 15.76	329.48 ± 44.20	223.39 ± 28.94	258.83 ± 11.57	5003.13 ± 249.93	1831.71 ± 79.53	5920.86 ± 554.38	3539.47 ± 294.13
		10	3245.36 ± 382.16	276.55 ± 42.98	412.01 ± 67.52	276.55 ± 42.98	216.79 ± 29.15	326.69 ± 28.49	202.20 ± 4.00	240.26 ± 22.37	4296.95 ± 126.97	1625.46 ± 153.06	5404.53 ± 499.06	3334.62 ± 315.11
Solvent			Wavelength (320 nm)								Wavelength (270 nm)			

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Temp (℃)	Time (min)	SP (µg/g DW)	SA (µg/g DW)	6.09 RT (µg SAE/g DW)	21.36 RT (µg SAE/g DW)	32.18 RT (µg SAE/g DW)	14.46 RT (µg SAE/g DW)	8.21 RT (µg SAE/g DW)	17.89 RT (µg SAE/g DW)	CL (µg/g DW)	7.53 RT (µg CLE/g DW)	10.10 RT (µg CLE/g DW)	13.66 RT (µg CLE/g DW)
	15	937.75 ± 69.16	394.65 ± 20.76	287.31 ± 4.83	394.65 ± 20.76	146.25 ± 0.48	523.75 ± 26.06	1379.06 ± 188.27	530.22 ± 21.78	1504.42 ± 39.98	3151.65 ± 88.78	nd	923.38 ± 51.81
	20	855.03 ± 49.93	267.87 ± 28.44	282.82 ± 6.53	298.73 ± 34.15	214.01 ± 6.75	406.18 ± 18.74	647.17 ± 198.94	289.21 ± 9.86	1066.19 ± 27.66	4154.29 ± 328.15	nd	1058.08 ± 65.12
	30	1504.51 ± 65.14	231.89 ± 15.70	295.98 ± 15.16	253.84 ± 28.15	239.13 ± 3.88	347.94 ± 19.44	237.64 ± 7.91	210.80 ± 13.28	3478.94 ± 80.29	1088.08 ± 67.76	6447.80 ± 457.07	4502.06 ± 263.64

SP; sinapine, SA; sinapic acid, CL; canolol, temp; temperature, SAE; sinapic acid equivalents, CLE; canolol equivalents, min; minutes, RT; retention time, DW; dry weight, nm; nanometer, µg; microgram, g; gram, nd; not detected

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Table 1a: Response surface analysis of optimized conditions for major sinaptes

	RSM Parameters	Estimate	STD Error	t-value	Level of Significance
Methanol	**Sinapine**				
	Time	-283.68	54.26	-5.23	0.00*
	Temp	-373.24	32.57	-11.46	0.00*
	R^2 - 0.9010				
	Adj R^2 - 0.8886				
	Sinapic Acid				
	Time	21.10	14.45	1.46	0.16
	Temp	60.90	20.52	2.97	0.01*
	Time*Temp	12.26	3.09	3.97	0.00*
	$Temp^2$	7.85	2.38	3.30	0.01*
	R^2 - 0.8273				
	Adj R^2 - 0.7812				
	Canolol				
	Time	-430.74	132.88	-3.24	0.01*
	Temp	-395.77	87.28	-4.53	0.00*
	$Time^2$	-73.92	25.73	-2.87	0.01*
	$Temp^2$	-41.96	11.15	-3.76	0.00*
	R^2 - 0.7043				
	Adj R^2 - 0.6255				
Ethanol	**Sinapine**				
	Time	-1164.29	545.42	-2.14	0.05*
	Temp	-756.26	335.72	-2.25	0.04*
	R^2 - 0.3617				
	Adj R^2 - 0.2866				
	Sinapic Acid				
	Time	-38.58	16.80	-2.30	0.04*
	Temp	-4.92	10.34	-0.48	0.64
	R^2 - 0.2444				
	Adj R^2 - 0.1555				
	Canolol				
	Time	2162.86	435.07	4.97	0.00*
	Temp	64.99	81.65	0.80	0.44
	$Time^2$	505.72	84.24	6.00	0.00*
	R^2 - 0.727				
	Adj R^2 - 0.676				

*significant at the level of 0.05; STD, standard; Temp, Temperature; RSM, response surface methodology analysis; R^2, coefficient of correlation; Adj R^2, adjusted coefficient of correlation

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Table 1b: Analysis of variance (ANOVA) table for the major sinaptes

		DF	Sum Sq	Mean Sq	F value	Level of Significance
Methanol	**Sinapine**					
	FO (Time, Temp)	2	21463937	10731968	72.77	0.00*
	Residuals	16	2359552	147472		
	Lack of fit	16	2359552	147472		
	Pure error	0	0			
	Sinapic Acid					
	FO (Time, Temp)	2	199644	99822	22.60	0.00*
	TWI (Time, Temp)	1	69559	69559	15.75	0.00*
	PQ (Temp)	1	48112	48112	10.89	0.01*
	Residuals	15	66245	4416		
	Lack of fit	15	66245	4416		
	Pure error	0	0			
	Canolol					
	FO (Time, Temp)	2	1294540	647270	6.66	0.01*
	PQ (Time, Temp)	2	2177360	1088680	11.20	0.00*
	Residuals	15	1457565	97171		
	Lack of fit	15	1457565	97171		
	Pure error	0	0			
Ethanol	**Sinapine**					
	FO (Time, Temp)	2	169613846	84806923	4.82	0.02*
	Residuals	17	299383971	17610822		
	Lack of fit	17	299383971	17610822		
	Pure error	0	0			
	Sinapic Acid					
	FO (Time, Temp)	2	91906	45953	2.75	0.09
	Residuals	17	284183	16717		
	Lack of fit	17	284183	16717		
	Pure error	0	0			
	Canolol					
	FO (Time, Temp)	2	6902440	3451220	3.31	0.06
	PQ (Time)	1	37546925	37546925	36.04	0.00*
	Residuals	16	16667785	1041737		
	Lack of fit	16	16667785	1041737		
	Pure error	0	0			

*significant at the level of 0.05; DF, degrees of freedom; Temp, Temperature; Sum Sq, sum of squares; mean sq; mean sum of squares, F-value; , FO; , TWI; , PQ;

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RSM analysis indicated that an extraction temperature of 126^{o}C, for 33.84 minutes resulted in the highest conversion of sinapine to sinapic acid for methanol (adjusted R^2-0.93) while 170^{o}C, for 18.82 minutes (adjusted R^2-0.62) was most effective for ethanol. The ratio analysis confirmed that methanol was a better extractant by facilitating the conversion of sinapine to sinapic acid at a lower temperature and time combination with the added benefit of lower energy costs (**Figure 3**). Similar results were found in our previous studies with methanol and ethanol extractants using accelerated solvent extraction (ASE) [5,11]. In addition, the higher adjusted R^2 values for both extractants indicates that sinapine is the precursor for of sinapic acid. This was previously reported by Khattab *et al*. [6], in which sinapine could be converted in sinapic acid, sinapoyl glucose and canolol. Moreover, the ratio analysis between sinapine and canolol also resulted a higher adjusted R^2 value for both methanol (adjusted R^2-0.92) and ethanol (adjusted R^2-0.75). These higher adjusted R^2 values implies that formation of canolol is dependent on sinapine as one of its precursors (**Figure 3**).

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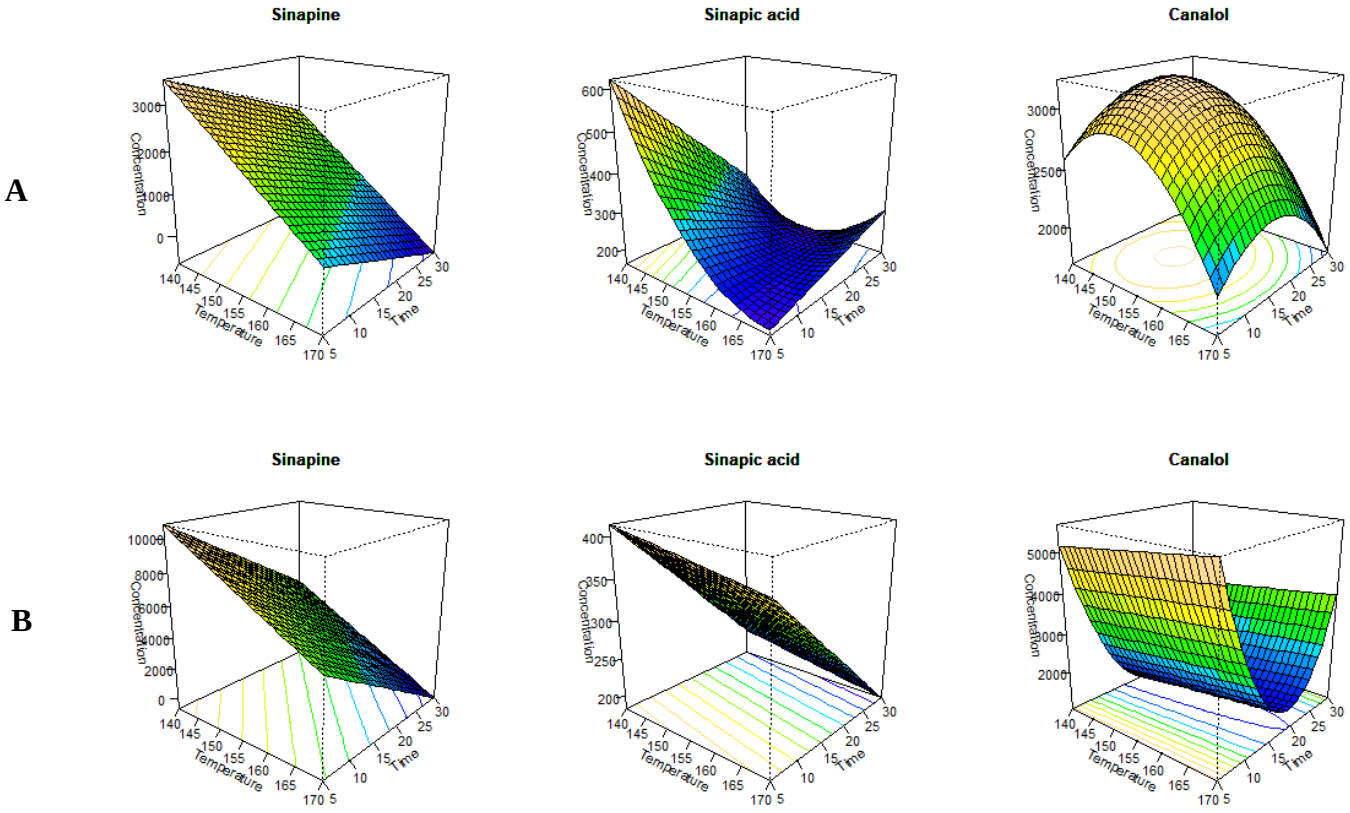

Figure 1: Response surface analysis of the major sinaptes (**A**-methanol, **B**-ethanol)

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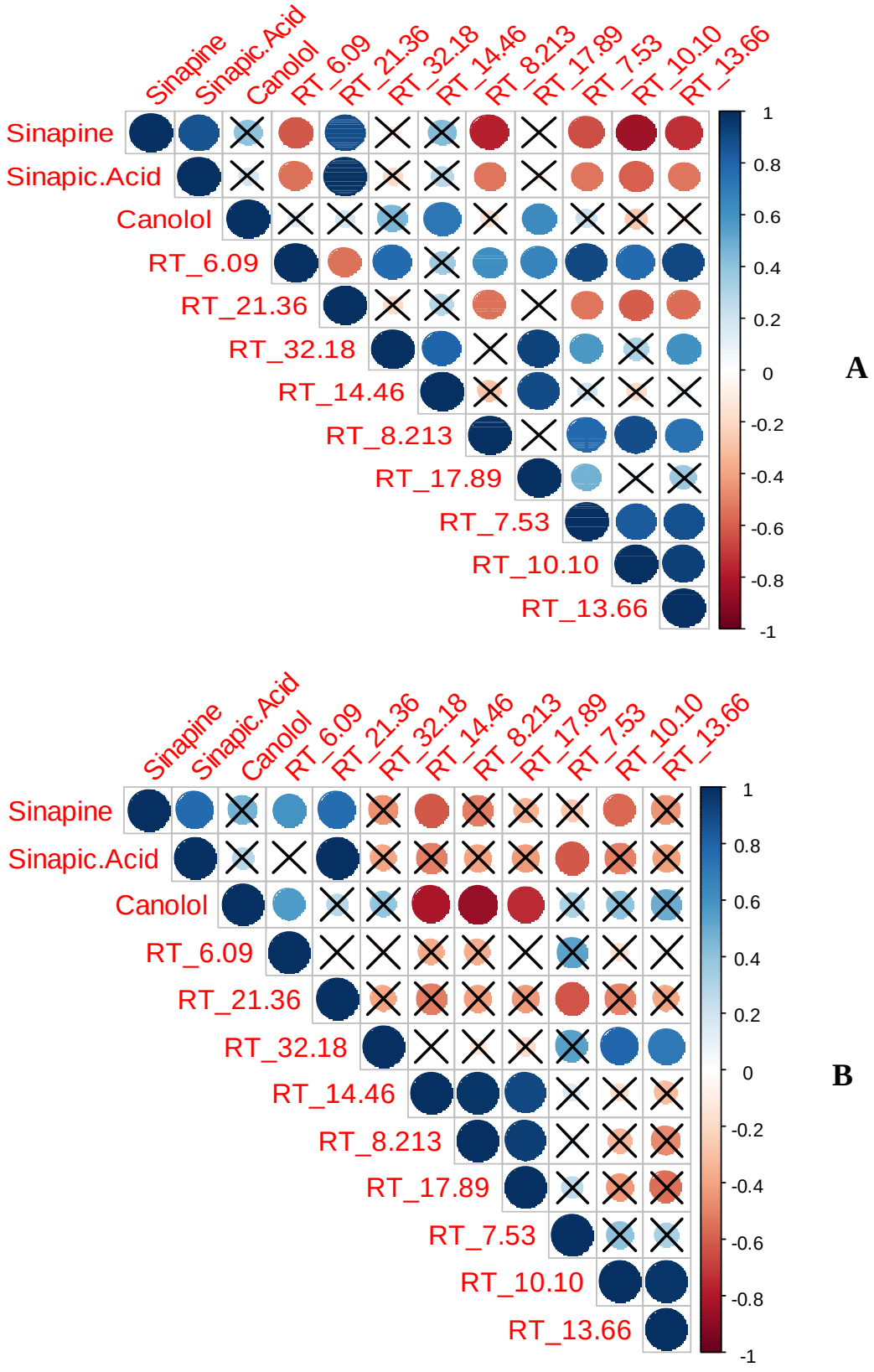

Figure 2: Correlation plot for the phenolic compounds (**A**-methanol, **B**-ethanol)

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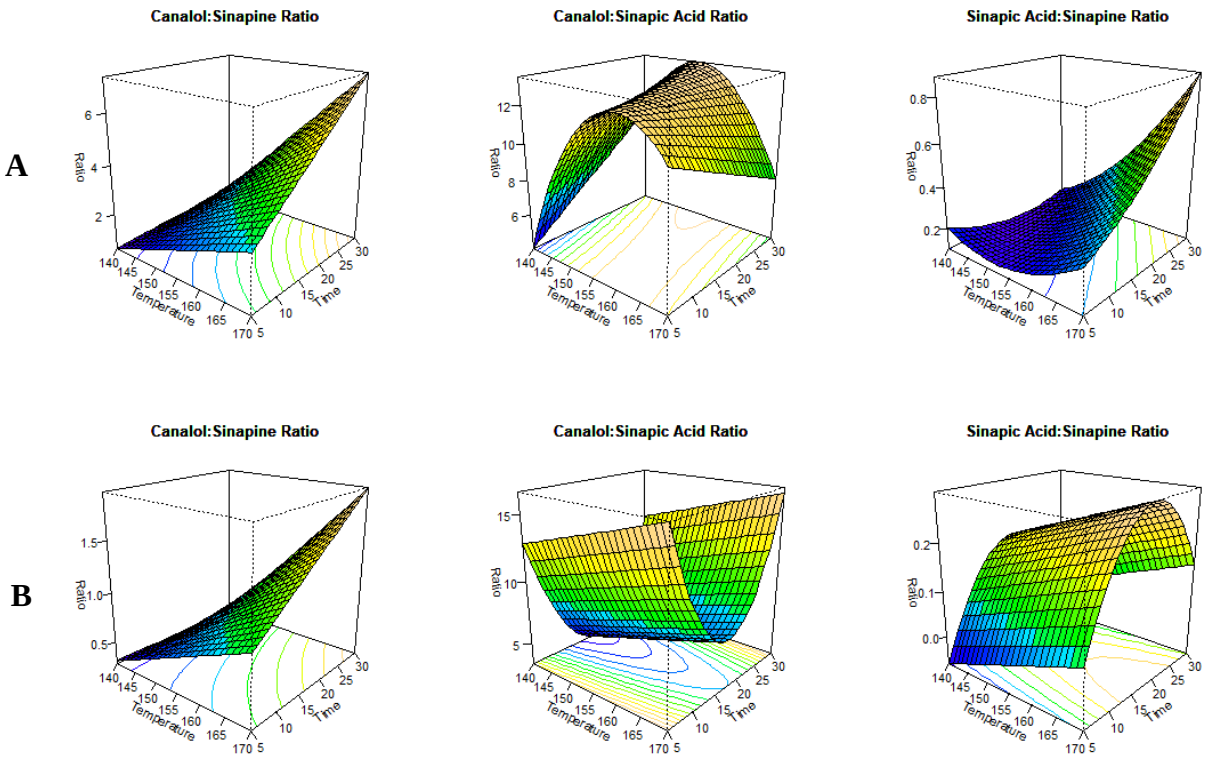

Figure 3: Ratio analysis of the major sinapates (**A**-methanol, **B**-ethanol)

Similarly, for sinapic acid with ethanol as the extractant, the main effects (time and temperature) had a significant impact on its extractability although no stationary point was observed (**Table 1a**). However, a stationary point at 163^{o}C with 16.18 minutes was observed for the extractant methanol indicating that the sinapic acid concentration increased with the processing temperature and time reaching an optimum at 163^{o}C with a processing time of 16.18 minutes (**Table 1a**). Interestingly, the best response surface modeling observed for canolol with both extractants although different stationary phases were recorded. For methanol, the stationary phase of canolol was at 151^{o}C with 15.43 minutes whereas, for ethanol its stationary point was located at 170^{o}C at 19.31 minutes (**Table 1a**). Two different stationary points for each extractant further

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indicates that the extractability of canolol using the microwave can be optimized for each solvent. Based on the current results, methanol appears to be a better extractant compared to ethanol by using a lower processing time/temperature to generate canolol. Similar findings were reported by Khattab *et al.* [7] establishing the superiority of methanol as an extracting solvent for canolol. They also reported that around 95% of the total phenolics in canola meal were converted to sinapic acid with approximately 55% of sinapic acid decarboxylated to canolol using the microwave. Based on the ratio analysis it was evident that the conversion of sinapic acid to canolol had different values for both methanol and ethanol (**Figure 3**). For methanol the stationary point for ratio analysis was at 159^{o}C with 10.89 minutes (adjusted R^2-0.55) whereas for ethanol it was at 170^{o}C with 17.63 minutes (adjusted R^2-0.50). Consequently, methanol was the preferred medium for the conversion of sinapic acid to canolol as it was more energy efficient. The adjusted R^2 value ranging around 0.5, however, indicates that sinapic acid was not the only precursor for the production of canolol.

4.2 Relationship among the sinapates and other phenolic derivatives

Apart from sinapine, sinapic acid, canolol, nine other phenolic derivatives were observed with the microwave aided solvent extraction at the different time/temperature combinations. Two different correlation plots were created for eacht extraction solvent (**Figure 2a & b**). Strong as well as weak and positive as well as negative correlations were evaluated using correlation plots.

Using methanol**,** sinapine and sinapic acid had a significant and very strong positive correlation. A positive relationship was evident between sinapic acid and canolol as well as sinapine and canolol but it was not significant (**Figure 2a**). Interestingly, sinapine had a significant negative relationship with unknown compounds including RT-6.09, RT-8.21, RT-7.53, RT-10.10, and RT-13.66 (**Figure 2a**). Of the unidentified compounds, RT-7.53, RT-10.10, and RT-13.66

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were observed at 270 nm while RT-6.09, RT-8.21 were observed at the wavelength of 320 nm. A similar correlation pattern was also observed for sinapic acid with the above mentioned unidentified phenolic compounds (**Figure 2a**). This confirmed the strong positive correlation among the sinapine and sinapic acid. The significant negative correlation between both sinapine and sinapic acid and the other phenolic compounds indicates the possibility that both sinapine and sinapic acid could be precursors for the generation of unknown phenolic compounds or degradation products of these major sinapates. A strong positive significant relationship was observed between sinapine and sinapic acid with the unknown compound of RT-21.36. Similar to sinapine and sinapic acid this unknown RT-21.36 compound showed a negative relationship with RT-8.21, RT-7.53, RT-10.10, and RT-13.66 (**Figure 2a**). This results further suggest that unknown RT-21.36 compound could be a derivative of sinapine or sinapic acid. Moreover, the unknown compounds including RT-6.09, RT-32.18, RT-8.21, RT-17.89, RT-7.53, RT-10.10, and RT-13.66 exhibited positive correlations among themselves which shows these compounds carries similar extractabilities among them with methanol as the extraction solvent.

The same compounds with ethanol as the extractant demonstrated a quite different extractability for the unknown compounds. Of the identified compounds RT-7.53, RT-10.10, and RT-13.66 were observed at 270 nm while RT-6.09, RT-8.21, RT-14.46, RT-17.89, RT-21.36, and RT-31.18 were observed at the wavelength of 320 nm (**Figure 2b**). A strong and significant negative relationship was found between canolol with the unidentified compounds, RT-8.21, RT-14.46, and RT-17.89 (**Figure 2b**). This indicates that the concentration of canolol was impacted by these unidentified compounds. It was also found that these 3 unidentified compounds had a strong positive correlation among themselves. It appeared that these unidentified compounds may contribute to the formation or degradation of canolol. Similar to methanol both sinapic acid and

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RT-21.36 had significant negative relationship with the unknown RT-7.53 compound (**Figure 2b**). Furthermore, only the compounds RT-10.10 and RT-14.46 showed a negative correlation with the extractability of sinapine (**Figure 2b**). The unidentified compounds RT-10.10 and RT-13.66, both showed a strong positive correlation which further indicated that these two compounds showcase similarly to their extraction with ethanol.

4.3 Impact of MAE on the Antioxidant Activity

To determine the impact of MAE on the antioxidant activity of the phenolic extracts, three different antioxidant assays were used, each targeting a different mechanism. The first measured radical scavenging activity using the DPPH radical scavenging method. The second assay determined the chelating ability of the extracts using the metal ion chelating activity method. Furthermore, both total phenolic (TPC) and total flavonoid contents (TFC) of the samples were determined to assess the efficacy of MAE using different solvent systems with time-temperature regimes using a three-way ANOVA (**Table 2**). The results indicated that for TPC all the major effects including type of solvent, time, and temperature had significant effect ($p>0.05$). Except for time*temperature interaction all other two-way and three-way interaction had a significant impact ($p>0.05$) on the total phenolic content (**Table 2**). The statistical analysis further indicates that total phenolic content is dependent on type of solvent, time, and temperature and can be manipulated using these main effects. Interestingly, for TFC only the main effects of type of solvent and temperature was significant ($p>0.05$). However, similar to TPC except for time*temperature interaction all other two-way and three-way interaction had a significant impact ($p>0.05$) on the extractability of TFC (**Table 2**). The time factor being non-significant indicates that extractability of TFC is independent on the duration of extraction.

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Table 2: Three-way Analysis of variance (ANOVA) table for the Antioxidant Activity

		DF	Sum Sq	Mean Sq	F value	Level of Significance
TPC	Solvent	1	42092	42092	416.67	0.00*
	Time	1	2402	2402	23.78	0.00*
	Temp	1	52303	52303	517.75	0.00*
	Solvent:Temp	1	3299	3299	32.66	0.00*
	Solvent:Time	1	1015	1015	10.05	0.00*
	Time:Temp	1	95	95	0.95	0.33
	Solvent:Time:Temp	1	5306	5306	52.53	0.00*
	Residuals	228	23033	101		
TFC	Solvent	1	158372	158372	193.82	0.00*
	Time	1	1242	1242	72.77	0.22
	Temp	1	207308	207308	253.72	0.00*
	Solvent:Temp	1	13562	13562	16.60	0.00*
	Solvent:Time	1	91400	91400	111.86	0.00*
	Time:Temp	1	1	1	0.00	0.97
	Solvent:Time:Temp	1	15322	15322	18.75	0.00*
	Residuals	139	113575	817		
DPPH	Solvent	1	13.14	13.14	1.03	0.31
	Time	1	33.64	33.64	2.64	0.11
	Temp	1	772.06	772.06	60.55	0.00*
	Solvent:Time	1	413.09	413.09	32.40	0.00*
	Solvent:Temp	1	2000.75	2000.75	156.91	0.00*
	Time:Temp	1	4.43	4.43	0.347	0.56
	Solvent:Time:Temp	1	6.28	6.28	0.49	0.48
	Residuals	103	1313.39	12.75		
MIC	Solvent	1	4.41	4.41	0.43	0.52
	Time	1	9.65	9.65	0.93	0.34
	Temp	1	123.76	123.76	11.96	0.00*
	Solvent:Time	1	1.03	1.03	0.10	0.75
	Solvent:Temp	1	138.92	138.92	13.42	0.00*
	Time:Temp	1	41.51	41.51	4.01	0.05*
	Solvent:Time:Temp	1	0.87	0.87	0.08	0.77
	Residuals	76	786.73	10.35		

*Significant at the level of 0.05; DF, degrees of freedom; Temp, Temperature; Sum Sq, sum of squares; Mean sq; mean sum of squares, F-value; TPC, total phenolic content; TFC, total flavonoid content; MIC, metal ion chelation activity; DPPH, 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity

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For both DPPH and MIC, it was observed that in addition to extraction time, the type of solvent extractant was also not significant. For both antioxidant assays, their activity was primarily dependent on the extraction temperature. This was similar to both TPC and TFC, except for time*temperature interaction all other two-way interactions had a significant impact ($p>0.05$) on the DPPH radical scavenging activity (**Table 2**). However, the three-way interaction of solvent*time*temperature had no significant impact on its antioxidant activity. Interestingly, for the MIC only solvent*temp and time*temp interactions were significant ($p>0.05$) except the solvent*time two-way interaction. Recent studies indicated that both sinapic acid and canolol had higher radical scavenging activity targeting. Higher radical scavenging activities are often closely associated with a reduction in cell oxidative stress [29]. Statistical analysis further indicated that, similar to DPPH, the 3-way interaction of solvent*time*temperature was insignificant for the metal ion chelating activity of the extracts. The chelating power of the metal ions can be impacted by many factors including the geometry of the metal complexes, ionic radii of the metal cations, valency of the metal, and hard-soft acid-base considerations [30]. Hence, in the current experiment the statistical results indicated that extraction temperature was the most important factor affecting the chelation power of the metals and its radical scavenging activity. Yet both type of solvent and extraction temperature are the crucial factors for TPC and TFC.

The co-relation analysis between TPC, TFC and antioxidant activity provided very interesting results. It was found that there was a strong positive and significant co-relation among both TPC and TFC (**Figure 4**). This further indicated with MAE, both TPC and TFC levels increased significantly. An increase in both TPC and TFC levels could be associated with the formation of novel phenolic compounds while thermal processing including dimers, trimers and other oligomers of sinapate derivatives and other flavor active kaempferol derivatives [11].

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Interestingly, no significant correlation was observed between MIC and DPPH (**Figure 4**) which further confirms the two different mechanisms of actions between the two antioxidant activities. Both DPPH and MIC showed a negative correlation with TPC. However, the correlation was not significantly different (**Figure 4**). Furthermore, DPPH radical scavenging activity showed a strong significant negative relationship with TFC. One of the limitations of the Folin-Ciocalteu assay is that *it is* based on colorimetry and often the reaction could be reversible and facilitated by the presence of NH-groups of the protein compounds [31]. Therefore, when it shows relatively higher TPC values it could be due to the presence of other compounds. TPC also measures the reducing power of the extracts and is often recorded there is a positive correlation between the TPC and the antioxidant activity. Hence, it is recommended to use different assays to measure the antioxidant activity of the samples [32].

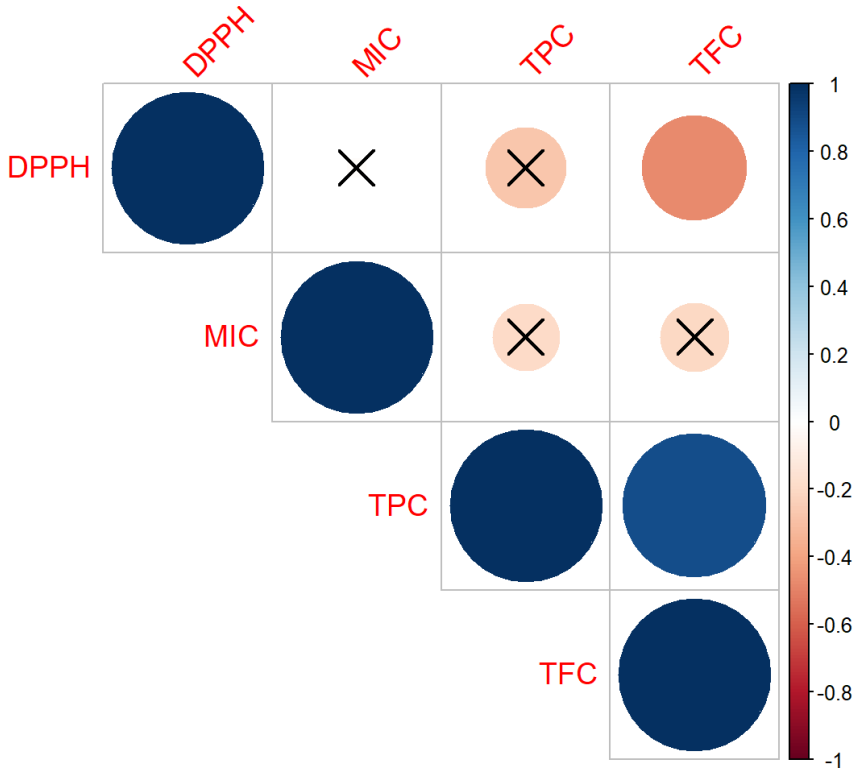

Figure 4: Correlation plot for the antioxidant activity

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TFC formation of chromogen could also be impacted by several factors. These flavonoids consist of many different classes including anthocyanin, catechins, flavanone glycosides, flavanone, flavons, flavonol glycosides, flavonols and isoflavons and synthesized from the precursor phenyl alanine [33]. TPC, like TFC is also measured using a colorimetric assay based on the formation of a complex between the aluminum ion and the carbonyl and hydroxyl groups of flavonoids to produce a yellow colored complex [33]. Some complexed flavonoid compounds show little or no antioxidant activity which could explain the strong negative correlation between the TFC and the DPPH radical activity. In addition, the antioxidant activity of DPPH is dependent on the formation of radicals. With the more complexed and larger flavonoid molecules, the antioxidative radical scavenging activity could be limited to its structure-function relationship. Further analysis of the more structure-based activity of antioxidants is required for confirmation of the above correlations.

Conclusion

MAE is a novel and innovative green technique which requires less solvents and a shorter time. Multiwave^{TM} 5000 has been shown to be an effective method for extracting valuable phenolic compounds from the canola meal. Using the response surface analysis, extraction conditions for major sinapates were optimized for the MAE. The results confirmed that conversion of sinapine to sinapic acid and canolol is not only dependent on time and temperature but other intrinsic and extrinsic factors. Also, the correlation analysis between the phenolic compounds indicated that extractability of sinapates can be impacted by the type of solvent extractants which can be manipulated to improve the extractability of the phenolic compounds including canolol. The

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results from the antioxidant activity indicate that the extraction temperature in the most important factor for the antioxidant activity while the type of solvent can have a significant impact on its TPC and TFC levels. This study further confirms that MAE can be applied in the canola industry as a novel method to efficiently extract valuable phenolic antioxidants from the meal by-product

5. Author Contribution

RN designed and conceptualized the study, performed the experiments, and interpreted the results, and drafted the original manuscript. OF and TN both helped with the extractions and antioxidant assays. MA helped with the statistical modeling and analysis. ME, OF, and EZ were involved in proof reading and writing the manuscript.

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Supplementary Data

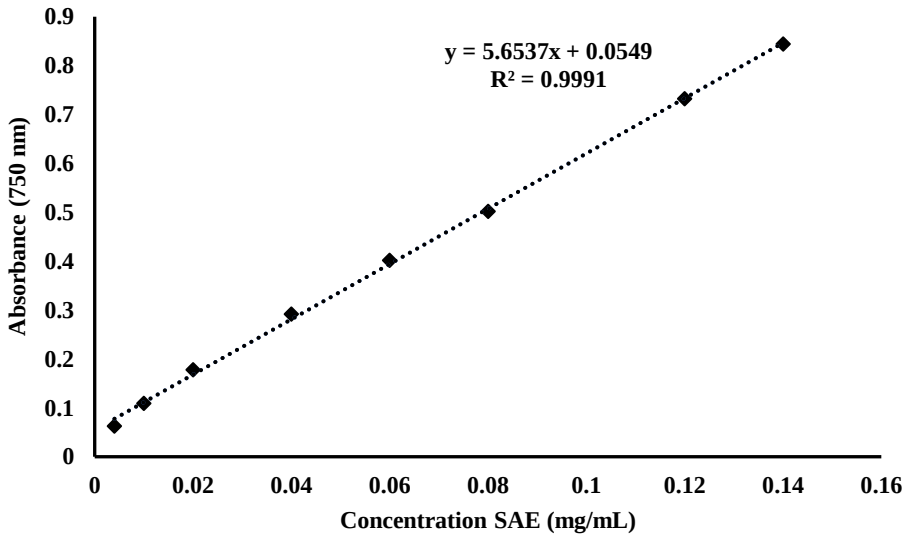

Figure S1A: Total Phenolic Content Standard Curve uisng Sinapic Acid Solution (1mM) as Standard (SAE - sinapic acid equivalents, mg - miligram, mL - mililiter, nm – nanometer, R^2 - coefficient of variance)

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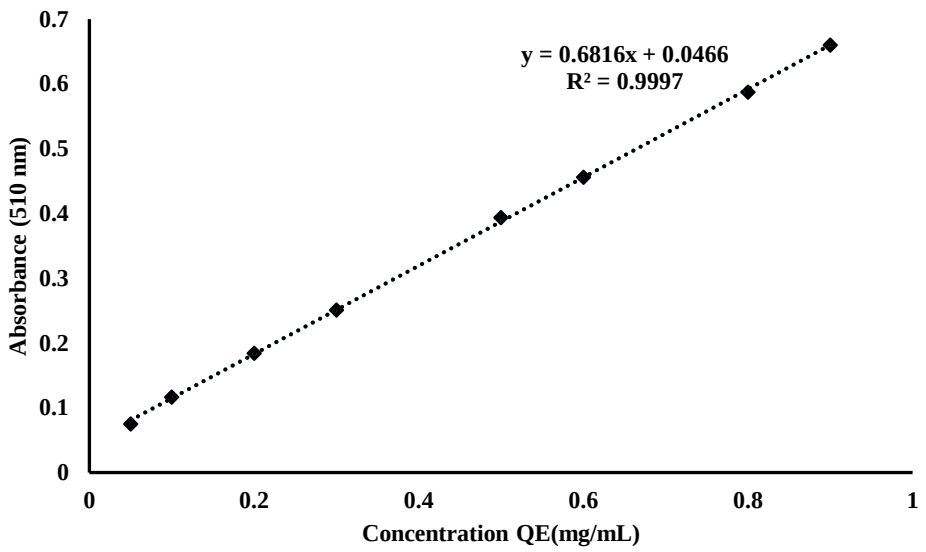

Figure S1B: Total Flavonoid Content Standard Curve uisng Quercetin solution (1mM) as Standard (QE - qercetin equivalents, nm - nanometer, mg - miligram, mL - mililiter, R^2 - coefficient of variance)

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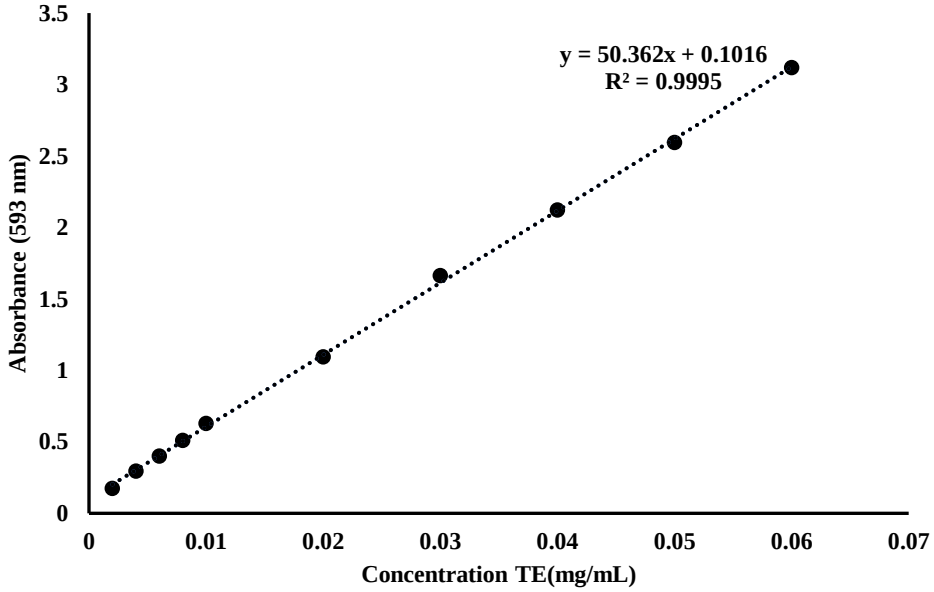

Figure S1C: Trolox as standard for the FRAP assay (TE - trolox equivalents, nm - nanometer, mg - miligram, mL - mililiter, R^2 - coefficient of variance)

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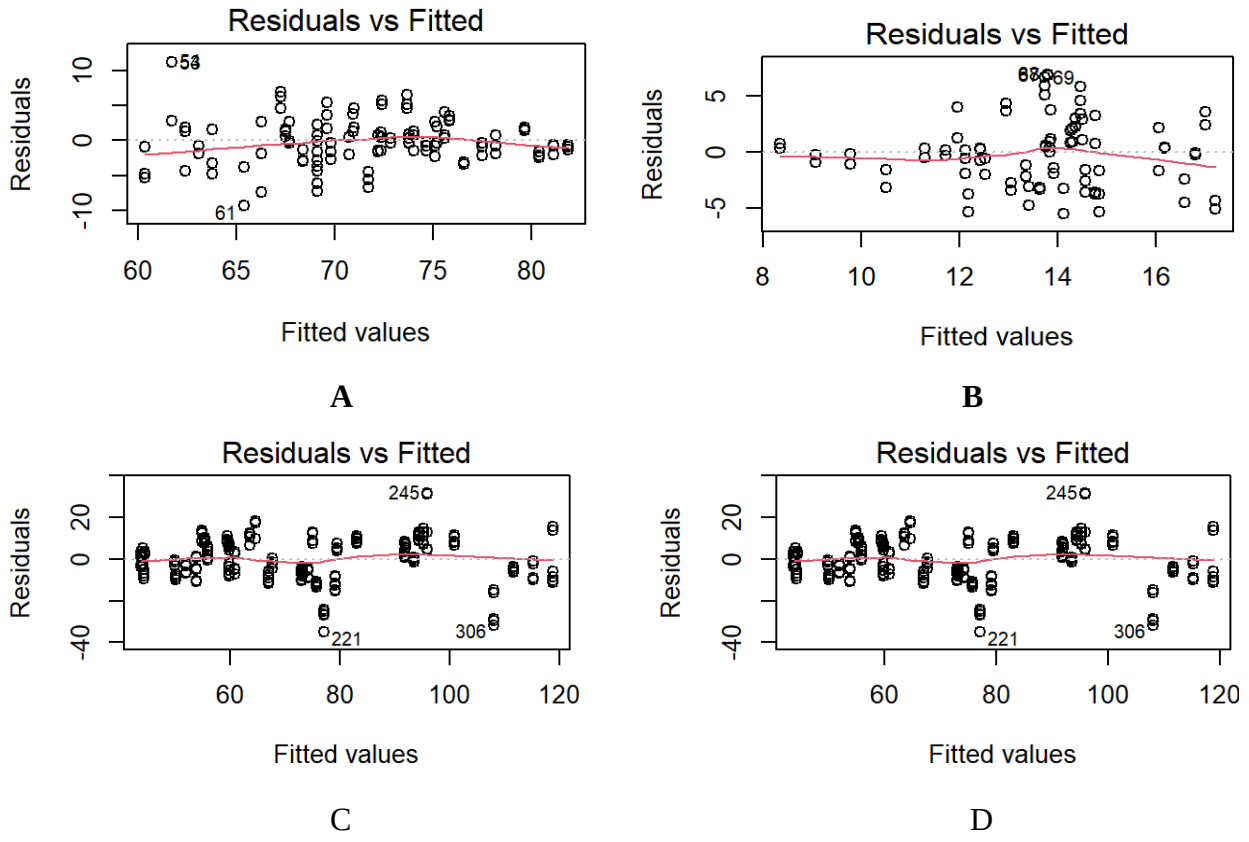

Figure S2: Residual Plots for Antioxidant activity (Residuals Vs Fitted Plots) A – DPPH activity, B- Metal Ion Chelation activity, C- Total phenolic content (TPC), D- Total flavonoids content (TFC)

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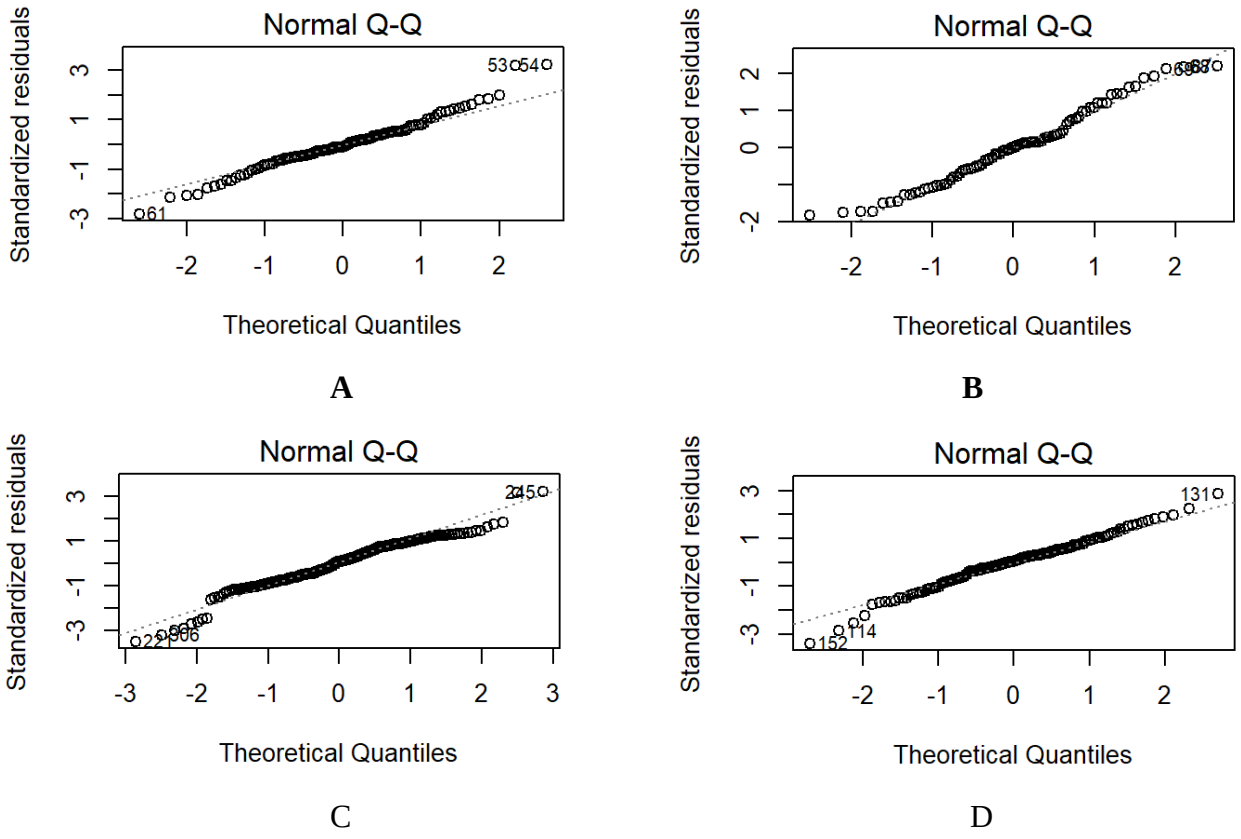

Figure S3: Normal Probability Plots for Antioxidant activity A – DPPH activity, B- Metal Ion Chelation activity, C- Total phenolic content (TPC), D- Total flavonoids content (TFC)