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FUNCTIONAL CHARACTERISTICS OF AVOCADO, KIWIFRUIT, PINEAPPLE AND POMEGRANATE BY-PRODUCTS

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ABSTRACT

Fruits by-products have significant source of fibers, vitamins and minerals and nutraceutical compounds. Therefore, the use of such by-products is expected to enhance produced products nutritional value. The effects of partial replacement of wheat flour using flours made of avocado peel and seeds, kiwifruit peels, pineapple peels and pomegranate skin and seeds on treatment functional characteristics were investigated. Fruit by-products inconsistently impacted treatments functional properties. Pomegranate water holding capacity (WHC) held at 35°C was significantly increased from 109.3% to 119.9 with the increase in substitution from 5 to 20%. The use of fruits by-products negatively impacted treatments freeze-thaw stability and significantly ($P<0.05$) reduced treatments pasting viscosities. Results of consumer studied indicated an acceptable application of fruit by-products in a cookie model system having an overall liking score of more than 6/9. Fruits by-products impacted treatments WHC, syneresis and pasting viscosities due to changes in treatment's chemical composition; especially its fiber content.

KEYWORDS:
Fruits by-products, avocado, kiwifruit, pineapple, pomegranate.

INTRODUCTION

There are increasing demands for fruits production due to the increasing markets for processed fruits including juice, pulps, nectars and jams. Fruit by-products of such fruits include its peels; seeds and pomace are thus increasing the pressure to discard them to the environment [1]. Several agricultural by-products from various sources are usually discarded by the food and pharmaceutical industry or used as animal feeds or fertilizers regardless of its nutritional value [2].

Agricultural and fruits by-products were reported to be a significant source for fibers, vitamins, minerals, phenolic compounds and antioxidants [3]. For example, avocado (*Persea Americana)* seeds (i.e., representing 26% of the fruit) contain more than 70% of the amino acids of the fruit and possess significant amounts of antioxidant activity [4]. Raw pineapple wastes are usually high in crude fibers (16-25%) soluble sugars (40-75%) and pectin [5]. Kiwifruit (*Actinidia deliciosa*) and pomegranate (*Punica granatum* L., Peel) skins are also considered a significant source of antioxidants [4]. Moreover, pomegranate was reported to contain significant amounts of tannin having antibacterial and anti-inflammatory qualities [6].

The use of fruits by-products thus can provide natural sources of valuable nutrients. For instance, El-Baroty [7] evaluated the total phenolic compounds and antioxidant activities in mango (*Mangifera indica* L.) seeds pulp and kernel, pomegranate (*Punica ranatum* L.) and peanut (*Arachis hypogaea* L., Giza 6, shell). Mango kernel peel and pomegranates were reported to have a radical scavenging activity comparable to that of Butylated Hydroxyanisol (BHA). Sah [8] on the same manner studied the effect of pineapple waste powder as probiotic growth, antioxidant and ant mutagenic activities in yogurt. The authors reported a 0.3 - 1.4 log cycle increases in probiotic populations as a result of pineapple supplementation and a significant increase in antioxidant activities. De Camargo [9] added peanut skins in ratio of 1.3, 1.8 and 2.5% to cookies aiming to increase its poly-phenol content. An addition of 2.5% was resulted in 52% increase in insoluble fiber content and 30% poly-phenols. Antioxidant activity was also increased with the increased peanut skin addition.

Therefore, the use of fruit by-products is critical to increase biological value, enhance functional properties and reduce fruit wastes (i.e., has an impact on the environment). It becomes important to investigate the use of fruit by-products for human consumption. Therefore, the objective of this work was to study the effects of partially

TABLE 1
Ingredients and quantities (grams) used for cookies preparation

	Wheat flour	Fruit by- product	Butter	Sugar	Eggs	Milk	Vanilla	Baking powder
Control	250.0	0.0	240	112.5	30	07. Mai	Jan 75	Jan 35
TRT 5%	237.5	12. Mai	240	112.5	30	07. Mai	Jan 75	Jan 35
TRT 10%	225.0	25.0	240	112.5	30	07. Mai	Jan 75	Jan 35
TRT 15%	212.5	37.5	240	112.5	30	07. Mai	Jan 75	Jan 35
TRT 20%	200.0	50.0	240	112.5	30	07. Mai	Jan 75	Jan 35

replacing wheat flour by flours made with selected fruit by-product flours namely avocado peel and seeds, kiwifruit peels, pineapple peels and pomegranate skin and seeds on flour mixtures functional characteristics. The study also aims at investigating the potential utilization of prepared flour treatments in the production of cookies.

MATERIALS AND METHODS

Materials: Avocado (*Persea Americana*) (i.e., seed and skin), kiwifruit (*Actinidia deliciosa*) (i.e., skin), pineapple (*Ananas comosus*) (i.e., peel) and pomegranate (*Punica granatum* L.) (i.e., skin and seeds) were used in this study. Fruit by-products were acquired from a local fruit and salad manufacturer in Amman, Jordan and kept frozen (-20ºC) until use. Fruit by-products were sliced into 2x2 cm pieces to increase its surface area, and blanched (90ºC for 2 min.) before cooling to room temperature (25.2ºC). Blanched fruits by-products were then separately dried in a conventional oven at 55°C for 48 hours before grinding to flour to pass through a 100 µm sieve. Fruit by-product flours were then used to prepare wheat flour-fruit treatments.

Design of the experiment: For treatment formulation, fruit by-product flours were used to replace whole purpose wheat flour in ratio of 5, 10, 15 and 20%. Flour treatments were then mixed thoroughly using household mixer at speed of 6 for 15 minutes before used in physiochemical measurements and cookies preparation. 100% whole purpose wheat flour was used as control.

Proximate composition: Ash, protein, lipids and fiber contents of avocado, kiwifruit, pineapple and pomegranate by-products were determined according to AOAC [10]. Carbohydrate content was determined by difference. The analyses were performed in duplicates.

Water holding capacity (WHC): A 5% suspension of homogenized (x 120, Igenieurbuero CAT, Stufen, Germany) of fruits by-products treatments were used to measure treatments WHC as described by Saleh [11]. Treatments were held separately at 25, 35, 45 and 55ºC for one hour before WHC measurements. After one hour holding at the required temperature; the dispersed samples were centrifuged (Eppendorf, 5810R, Hamburg, Germany) at 3800 RPM for 30 min. Sediments were weighted and WHC (%) was calculated using the following formula. Duplicate measurements were performed, and averages were reported.

WHC (%) = (Weight of sediment/weight of dry solids) * 100%

Freeze thaw stability: Aqueous dispersions of fruit by-products treatments (5% wt/vol) were prepared and used for freeze thaw stability measurements. In summary, treatments were gelatinized at 95°C with continuous shaking for 30 min. After cooling to 25°C; the gelatinized treatments were subjected to three consecutive freeze–thaw cycles. In summary, gelatinized treatments were frozen at -22°C for 24 h followed by thawing at 30°C for 2.0 h and then centrifuged at 3800 rpm for 30 min. The degree of syneresis per freezing cycle was expressed as the freeze thaw stability of treatments. Each freeze thaw stability cycle was performed in triplicate and results were expressed as the average for each cycle.

Rheological measurements: Flow behavior index and consistency coefficient of 5% homogenized (x 120, Igenieurbuero CAT, Stufen, Germany) suspensions of fruit by-products treatments were measured using a rotational viscometer (SNB-AI Digital Viscometer, Shandong China). Treatments were held at 25ºC for one hour before viscosity parameters being measured during shear rate of 6-60 s^{-1} at 25.0°C.

Pasting measurements: Peak, trough, setback, breakdown, and final viscosities and pasting temperature of treatments were measured using a Rapid Visco Analyzer (RVA Model 4, Newport Scientific Inc, Warriewood, Australia). RVA protocol was followed the AACCI approved method 76-21 AACC [12] method as described by Saleh [13]. Thermocline version 1.2 software (Newport Scientific Inc., Warriewood, Australia) was used to record and process data obtained from the RVA. All samples were measured in duplicate.

TABLE 2
Chemical composition (dry matter) of avocado, kiwifruit, pineapple and pomegranate fruit by-product flours.

Treatments	Macro Nutrients (%)			Fiber (%)		Ash (%)
	Protein	Lipids	Carbohydrates	Insoluble	Soluble	
Wheat Flour	11.9 ± 1.1 [b]	0.9 ± 0.1[d]	79.7 ± 1.1[b]	1.68 ± 0.01[d]	1.52 ± 0.03[c]	4.3 ± 0.7[d]
Avocado	15.4 ± 1.5 [a]	1.9 ± 0.2[b]	73.5 ± 0.1[c]	1.99 ± 0.02[b]	2.41 ± 0.01[b]	4.8 ± 0.5[d]
Kiwifruit	1.0 ± 1.2 [e]	2.8 ± 1.2[a]	82.0 ± 0.1[a]	2.34 ± 0.04[a]	2.56 ± 0.04[a]	9.3 ± 0.5[b]
Pineapple	6.5 ± 0.7 [c]	2.0 ± 0.01[c]	82.3 ± 1.5[a]	1.54 ± 0.01[e]	1.26 ± 0.02[e]	6.4 ± 0.1[c]
Pomegranate	2.7 ± 0.4 [d]	0.2 ± 0.01[e]	83.4 ± 1.4[a]	1.84 ± 0.01[c]	1.36 ± 0.01[d]	10.5 ± 0.2[a]

[1] Protein, lipids, carbohydrate, insoluble and soluble fiber and ash of fruit by-products and wheat flour control having different letter(s) are significantly (P<0.05) different according to the LSD.

Cookies preparation: Table 1 presents the ingredients and quantities used for cookies preparation. For cookies making, sugar and butter were mixed thoroughly before adding the dry ingredients and then the wet ingredients. The formed dough was then mixed to form cohesive dough. Formed dough balls were then baked in household oven at 130°C for 15 min.

Consumer evaluation of cookies made using wheat - fruit by-product treatments: Sensory attributes of cookies made using wheat flour containing fractions of fruit by-products were assessed in a sensory evaluation laboratory, Department of Nutrition and Food Technology, Faculty of Agriculture, The University of Jordan. Cookies were prepared 24 hours before performing consumer liking testing. A total of 50 consumers were performed the consumer evaluation testing. For the consumer testing, three digit identified samples were randomly presented to consumers in Styrofoam cups and consumers were instructed to complete a 9-point hedonic scale (1=dislike extremely, 9=liked extremely) according to Meilgaard [14]. Consumers were asked to wash their palate between samples. Overall liking, firmness, stickiness, flavors and color of each sample was evaluated.

Statistical analysis: Analysis of variance (ANOVA) was carried out on physical treatments data using JMP release 10.0 (SAS institute, Cary, NC). Least significant differences (LSD), at a 5% level of probability, were determined between treatments.

RESULTS AND DISCUSSION

Table 2 presents the chemical composition of avocado, kiwifruit, pineapple and pomegranate by-product flours. Protein, lipids and carbohydrate contents of fruit by-products ranged from 1.0 to 15.4, 0.2 to 2.8 and from 73.5 to 83.4%, respectively. Ash contents of fruit by-product flour were significantly (P<0.05) greater than wheat flour and ranged from 4.8 to 10.5%.

Table 3 presents the WHC of wheat flour substituted with fractions (0, 5, 10, 15 and 20%) of avocado, kiwifruit, pineapple and pomegranate flours over various temperature (25, 35, 45 and 55°C) range. WHC ranged from 76.4% for the control wheat flour to 133.7% for 20% pomegranate treatment. Results clearly demonstrated an increase in WHC with the increase in the degree of substitution regardless to holding temperature. For example, with 5, 10, 15 and 20% avocado treatments held at 25°C for 1 hour, WHC were 81.1, 91.5, 95.5 and 112.0%, respectively. WHC of pomegranate treatments held at 35°C increased from 109.3% to 114.1, 117.3 and 119.9 with the increase in substitution from 5 to 10, 15 and 20, respectively. Similar trends were reported for fruit by-product treatments.

Comparing WHC of various fruit by-products treatments within the same substitution fractions and holding temperature indicated that pomegranate treatments showed the greatest water holding capacity ranging from 98.2 to 133.7%. The increased WHC with the increase of fruit by-products treatments were attributed to the increases fraction of cellulose, lignin and soluble and insoluble fibers contents with the added fruits by-products. These results are in accordance with Hunter [15] who reported a significant increase in water uptake of with the increase in fiber content of flax fiber. More specifically, Boulos [16] reported a significant increase in WHC with the increased content of whole locust and insoluble locust bean gum. Cellulose, lignin and pectin were also reported to increase WHC by 32, 67 and 84 %, respectively. Ngoc [17] positively correlated (r = 0.82) soluble non-cellulosic polysaccharides to WHC.

Table 4 presents the freeze thaw stability of wheat flour substituted with fractions of fruit by-products. Three syneresis (%) cycles are presented for avocado, kiwifruit, pineapple and pomegranate treatments (i.e., 5, 10, 15 and 20% substitutions). Freeze thaw cycle 1 ranged from 6.0 to 14.6, from 5.7 to 12.9, from 5.1 to 14.2 and from 6.9 to 12.2% for avocado, kiwifruit, pineapple and pomegranate treatments, respectively. Freeze thaw cycle 2 and 3 had similar trends with the increase in syneresis (%)

TABLE 3
Water holding capacity (WHC) of treatments containing 0, 5, 10, 15 or 20% of avocado, kiwifruit, pineapple and pomegranate by-product flours [1]

Substitution ratio	Temperature			
	25 ºC	35 ºC	45 ºC	55 ºC
	Avocado treatments			
0%	76.4 ^{e} ± 1.1	90.6 ^{c} ± 0.1	101.2 ^{d} ± 0.6	120.8 ^{e} ± 1.1
5%	81.1 ^{d} ± 0.2	83.1 ^{d} ± 0.8	89.1 ^{e} ± 0.7	126.8 ^{d} ± 1.3
10%	91.5 ^{c} ± 0.1	93.1 ^{bc} ± 0.2	108.3 ^{c} ± 0.5	126.3 ^{c} ± 0.6
15%	95.5 ^{b} ± 0.1	94.1 ^{b} ± 2.3	116.4 ^{b} ± 0.8	130.1 ^{b} ± .7
20%	112.0 ^{a} ± 0.2	98.3 ^{a} ± 0.8	119.6 ^{a} ± 1.3	132.5 ^{a} ±2.9
	Kiwifruit treatments			
0%	76.4 ^{d} ± 1.1	90.6 ^{d} ± 0.6	101.2 ^{d} ± 0.7	120.8 ^{a} ± 2.4
5%	88.2 ^{c} ± 2.0	99.9 ^{b} ± 0.9	111.6 ^{c} ± 0.6	109.6 ^{b} ± 2.6
10%	90.8 ^{c} ± 0.4	98.2 ^{c} ± 0.3	114.0 ^{b} ± 0.4	123.8 ^{a} ± 2.5
15%	107.0 ^{b} ± 0.2	115.5 ^{a} ± 0.1	112.3 ^{c} ± 0.8	119.2 ^{a} ± 3.8
20%	122.6 ^{a} ± 2.3	115.5 ^{a} ± 0.4	120.1 ^{a} ± 0.6	122.7 ^{a} ± 1.1
	Pineapple treatments			
0%	76.4 ^{c} ± 1.1	90.6 ^{d} ± 0.1	101.2 ^{b} ± 0.6	120.8 ^{b} ±1.1
5%	64.7 ^{d} ± 2.4	90.9 ^{d} ± 0.8	69.5 ^{e} ± 1.1	86.3 ^{e} ± 0.8
10%	81.6 ^{b} ± 0.5	105.3 ^{b} ± 0.5	91.0 ^{c} ± 0.4	111.7 ^{d} ± 0.2
15%	110.7 ^{a} ± 0.9	97.5 ^{c} ± 1.1	87.7 ^{d} ± 1.1	116.0 ^{c} ± 0.8
20%	112.6 ^{a} ± 0.1	115.7 ^{a} ± 0.5	119.9 ^{a} ± 1.1	124.9 ^{a} ± 1.2
	Pomegranate treatments			
0%	76.4 ^{d} ± 1.1	90.6 ^{e} ± 0.1	101.2 ^{c} ± 0.6	120.8 ^{b} ± 1.1
5%	107.5 ^{b} ± 1.4	109.3 ^{d} ± 0.8	114.4 ^{b} ± 1.2	120.5 ^{b} ± 0.1
10%	98.2 ^{c} ± 1.4	114.1 ^{c} ± 0.2	116.6 ^{b} ± 1.0	122.3 ^{b} ± 4.2
15%	119.1 ^{a} ± 1.5	117.3 ^{b} ± 0.4	124.5 ^{a} ± 2.5	122.2 ^{b} ± 0.6
20%	120.5 ^{a} ± 1.1	119.9 ^{a} ± 1.0	123.2 ^{a} ± 0.5	133.7 ^{a} ± 2.4

[1] Water holding capacity (%) ± standard deviation within the same fruit by-product, temperature and various fraction used (i.e., same column within the same fruit by-product) having different letter(s) are significantly ($P<0.05$) different according to the LSD.

TABLE 4
Freeze–thaw stability, flow behavior index (n) and consistency coefficient (m) of treatments containing 0, 5, 10, 15 or 20% of avocado, kiwifruit, pineapple and pomegranate by-product flours [1]

Substitution ratio	Syneresis (g/100 g)			Viscosity Parameters	
	Cycle 1	Cycle 2	Cycle 3	n	m
	Avocado				
0%	5.1 ^{d} ± 0.02	6.8 ^{e} ± 0.04	9.7 ^{c} ± 0.03	0.26 ^{d}	15.4 ^{b}
5%	6.0 ^{c} ± 0.17	7.8 ^{d} ± 0.02	10.1 ^{bc} ± 0.26	0.39 ^{c}	14.7 ^{b}
10%	6.4 ^{c} ± 0.05	8.8 ^{c} ± 0.09	9.6 ^{c} ± 0.03	0.56 ^{b}	13.4 ^{c}
15%	6.7 ^{b} ± 0.04	9.8 ^{b} ± 0.22	10.4 ^{b} ± 0.16	0.58 ^{b}	42.1 ^{a}
20%	14.6 ^{a} ± 0.12	15.2 ^{a} ± 0.04	15.4 ^{a} ± 0.19	0.98 ^{a}	2.4 ^{d}
	Kiwifruit				
0%	5.1 ^{e} ± 0.02	6.8 ^{d} ± 0.04	9.7 ^{d} ± 0.03	0.26 ^{e}	15.4 ^{d}
5%	5.7 ^{d} ± 0.03	8.3 ^{c} ± 0.01	10.5 ^{c} ± 0.37	0.55 ^{c}	18.3 ^{b}
10%	5.9 ^{c} ± 0.04	8.8 ^{c} ± 0.02	10.8 ^{d} ± 0.01	0.62 ^{b}	20.8 ^{a}
15%	6.9 ^{b} ± 0.02	9.8 ^{b} ± 0.03	11.3 ^{b} ± 0.04	0.43 ^{d}	17.2 ^{bc}
20%	12.9 ^{a} ± 0.01	13.3 ^{a} ± 0.06	14.3 ^{a} ± 0.01	0.66 ^{a}	16.2 ^{d}
	Pineapple				
0%	5.1 ^{c} ± 0.02	6.8 ^{e} ± 0.04	9.7 ^{d} ± 0.03	0.26 ^{d}	15.4 ^{b}
5%	5.1 ^{c} ± 0.06	7.6 ^{d} ± 0.01	10.2 ^{b} ± 0.02	0.51 ^{c}	16.4 ^{b}
10%	5.8 ^{b} ± 0.02	8.2 ^{c} ± 0.05	9.6 ^{c} ± .01	0.49 ^{c}	17.9 ^{ab}
15%	5.6 ^{b} ± 0.04	9.2 ^{b} ± 0.02	9.8 ^{c} ± 0.04	0.72 ^{a}	19.8 ^{a}
20%	14.2 ^{a} ± 0.05	14.6 ^{a} ± 0.03	14.6 ^{a} ± 0.03	0.67 ^{b}	20.0 ^{a}
	Pomegranate				
0%	5.1 ^{d} ± 0.02	6.8 ^{d} ± 0.04	9.7 ^{b} ± 0.03	0.26 ^{c}	15.4 ^{c}
5%	6.9 ^{c} ± 0.03	8.0 ^{c} ±0.01	8.5 ^{c} ± 0.19	0.30 ^{c}	17.8 ^{c}
10%	6.7 ^{c} ± 0.03	7.9 ^{c} ± 0.13	8.8 ^{c} ± 0.15	0.33 ^{b}	34.4 ^{a}
15%	7.5 ^{b} ± 0.02	9.3 ^{b} ± 0.03	9.5 ^{b} ± 0.10	0.34 ^{b}	21.8 ^{b}
20%	12.2 ^{a} ± 0.02	13.2 ^{a} ± 0.09	13.8 ^{a} ± 0.06	0.54 ^{a}	17.0 ^{c}

[1] Freeze thaw stability cycles ± standard deviation and flow behavior index (n) and consistency coefficient (m) within the same fruit by-product and various substitution (0, 5, 10, 15 and 20%) fractions (i.e., same column within the same fruit by-product) having different letter(s) are significantly ($P<0.05$) different according to the LSD.

TABLE 5
Pasting viscosities (peak, trough, breakdown, final and setback (cP) and pasting temperature (°C) of treatments containing 0, 5, 10, 15 or 20% fractions of avocado, kiwifruit, pineapple and pomegranate by-product flours [1]

Substitution ratio	Peak Viscosity	Trough Viscosity	Breakdown Viscosity	Final Viscosity	Setback Viscosity	Pasting Temp.
			Avocado			
0%	2390 ^{a} ± 8	1140 ^{a} ± 2	1250 ^{a} ± 10	2549 ^{a} ± 6	1408 ^{a} ± 8	67 ^{b} ± 0.0
5%	2046 ^{b} ± 0	943 ^{bc} ± 1	1102 ^{b} ± 1	2180 ^{b} ± 13	1237 ^{b} ± 13	67 ^{b} ± 0.0
10%	1980 ^{b} ± 15	961 ^{b} ± 54	1019 ^{b} ± 39	2183 ^{b} ± 66	1222 ^{b} ± 12	67 ^{b} ± 0.6
15%	1754 ^{c} ± 18	884 ^{cd} ± 14	870 ^{c} ± 4	1966 ^{c} ± 29	1082 ^{c} ± 15	76 ^{ab} ± 11.5
20%	1527 ^{d} ± 91	868 ^{d} ± 23	659 ^{d} ± 68	1864 ^{d} ± 43	996 ^{d} ± 21	84 ^{a} ± 0.4
			Kiwifruit			
0%	2390 ^{a} ± 8	1140 ^{b} ± 2	1250 ^{a} ± 10	2549 ^{a} ± 6	1408 ^{a} ± 8	67 ^{a} ± 0.0
5%	2171 ^{b} ± 50	1055 ^{b} ± 47	1116 ^{b} ± 3	2270 ^{b} ± 46	1215 ^{b} ± 1	66 ^{a} ± 0.0
10%	2247 ^{b} ± 13	1114 ^{b} ± 10	1133 ^{ab} ± 4	2300 ^{b} ± 4	1186 ^{b} ± 6	66 ^{a} ± 1.2
15%	2209 ^{b} ± 72	1102 ^{b} ± 28	1107 ^{b} ± 45	2121 ^{c} ± 67	1019 ^{c} ± 39	66 ^{a} ± 1.3
20%	2461 ^{a} ± 38	1296 ^{a} ± 65	1165 ^{ab} ± 103	2522 ^{a} ± 89	1226 ^{b} ± 24	66 ^{a} ± 0.0
			Pineapple			
0%	2390 ^{a} ± 8	1140 ^{a} ± 2	1250 ^{a} ± 10	2549 ^{a} ± 6	1408 ^{a} ± 8	67 ^{b} ± 0.0
5%	2042 ^{bc} ± 20	982 ^{b} ± 3	1060 ^{cd} ± 17	2117 ^{b} ± 29	1135 ^{bc} ± 26	66 ^{c} ± 0.0
10%	2089 ^{b} ± 42	975 ^{bc} ± 24	1114 ^{b} ± 18	2119 ^{b} ± 24	1144 ^{b} ± 0	66 ^{c} ± 0.0
15%	2005 ^{c} ± 13	937 ^{c} ± 11	1068 ^{c} ± 1	2035 ^{c} ± 6	1098 ^{c} ± 5	66 ^{c} ± 0.0
20%	2009 ^{c} ± 27	973 ^{bc} ± 21	1036 ^{d} ± 6	1935 ^{d} ± 47	962 ^{d} ± 26	68 ^{a} ± 1.0
			Pomegranate			
0%	2390 ^{b} ± 8	1140 ^{c} ± 2	1250 ^{c} ± 10	2549 ^{c} ± 6	1408 ^{a} ± 8	67 ^{a} ± 0.0
5%	2194 ^{d} ± 12	1028 ^{d} ± 13	1166 ^{d} ± 1	2284 ^{e} ± 13	1255 ^{c} ± 1	66 ^{b} ± 0.1
10%	2902 ^{a} ± 9	1505 ^{a} ± 1	1397 ^{a} ± 10	2842 ^{a} ± 3	1336 ^{b} ± 4	66 ^{b} ± 0.2
15%	2868 ^{a} ± 17	1503 ^{a} ± 8	1364 ^{b} ± 9	2730 ^{b} ± 14	1226 ^{d} ± 6	66 ^{ab} ± 0.6
20%	2310 ^{c} ± 28	1321 ^{b} ± 21	989 ^{e} ± 7	2503 ^{d} ± 23	1181 ^{e} ± 2	67 ^{a} ± 0.0

[1] Pasting viscosities (peak, trough, breakdown, final and setback (RAVU) and pasting temperature (°C) within the same fruit with various substitution (0, 5, 10, 15 and 20%) fractions (same column within the same fruit by-product) having different letter(s) are significantly (P<0.05) different according to the LSD.

with the increase in fruit by-product fraction in treatments. These results indicate a limited ability of fruit by-products flour to forming a matrix network during gelatinization that was related to its chemical composition. Although fruit treatments results in weak freeze thaw stability; these results can be manipulated using hydrocolloids. In this regard, several authors have reported an improvement in freeze thaw stability using hydrocolloids [13]. Saleh [13] for example, reported a significant reduction in syneresis of acorn starch when combined with carboxymethylcellulose, carrageenan and xanthan gum. Table 4 also presents flow behavior index (n) and consistency coefficient (m) of wheat flour substituted with 5, 10, 15 and 20% of avocado, kiwifruit, pineapple and pomegranate by-product flours respectively. Flow behavior index ranged from 0.30 to 0.98 regardless to fruit by-product and fractions used compared to 0.26 for control wheat flour. Pomegranate treatment had the lowest flow behavior index value compared to avocado, kiwifruit, pineapple but greater than the control. Results further indicated an increase in flow behavior index with the increase in fraction of fruits by-product flour used. Consistency coefficients of treatments ranged from 2.4 to 42.1 irrespective of wheat-fruit by-product fraction used. Results suggests that the viscoelastic behavior of fruit by-product flour significantly (P<0.05) influenced by the change in treatments chemical composition. The availability of proteins along with some naturally occurring water-soluble gums may be responsible for the increase in flow behavior index of fruit by-products treatments. Pectin was indicated to form significant proportion of the peel of citrus fruit and is expected in the presence of some fractions monosaccharides and various organic acids (mainly citric acid) to enhance treatment's viscosity [5].

Pasting viscosities of wheat treatments are presented in Table 5. Peak, trough, breakdown and final viscosities ranged from 1527, 868, 659 and 1864 cP to 2902, 1505, 1397 and 2842 cP for to 20% avocado and 10% pomegranate, respectively compared to 2390, 1140, 1250 and 2549 for the 100% wheat flour control sample. Setback viscosity ranged from 962 cP for pineapple 20% to 1336 cP for the 10% pomegranate compared to 1408cP for the control sample.

Results indicated a significant ($P<0.05$) reduction in peak viscosity with the addition of fruit by-products, with some exceptions. These results were attributed to the increased fraction of fibers with the increase proportions of fruit by-products in treatments; a resulted alteration in treatment's chemical composition as well as chemical structure. Protein-starch-lipids interactions were long reported to form flours pasting profile with fiber being detrimental to pasting viscosities [18]. Additionally, proteins and starch were reported to play a key role in pasting formation [19]. The ability of proteins-starch-lipid matrix formation is the principal factor in protecting starch granules during swelling from lyses due to the formation of such insoluble rigidity polymeric matrix [18]. Although the use of avocado in treatments formation increased treatment's protein content; it is believed that protein structure rather than content affects treatments pasting viscosities.

Consumer attributes (i.e., overall, appearance, texture, flavor, aftertaste, color and graininess liking) of cookies made using wheat flour substituted with fractions avocado, kiwifruit, raw pineapple and pomegranate by-product flours are presented in Table 6. Overall liking scores ranged from 6.3 for pomegranate (i.e., 15%) to 8.1 for the control. Although the addition of fruit by-products lowered overall liking scores of treatments compared to the control, results indicated an acceptable final product (>6.0). Cookies supplemented with fractions of fruit by-products had slight reduction in texture liking scores compared to the control that was attributed to the increased fiber content of treatments.

Appearance, flavor, aftertaste, color and graininess liking scores varied (i.e., although lower than the control) but with no significant trends. For example, treatment containing 5% pineapple was comparable to the control having hedonic scores ranging from 7.5 to 8.1 for overall, appearance, texture, flavor, aftertaste, color and graininess liking scores that are very close to the classification "liked very much".

TABLE 6
Consumer overall, appearance, texture, flavor, aftertaste, color and graininess liking of cookies made using wheat flour substituted with fractions of avocado, kiwifruit, pineapple and pomegranate by-product flours [1]

Substitution ratio	Overall	Appearance	Texture	Flavor	Aftertaste	Color	Graininess
	Avocado						
0%	8.1 ^{a}	7.8 ^{a}	7.9 ^{a}	8.1 ^{a}	8.2 ^{a}	7.9 ^{a}	8.0 ^{a}
5%	7.4 ^{b}	7.2 ^{bc}	7.2 ^{bc}	7.2 ^{b}	7.3 ^{b}	7.5 ^{ab}	7.5 ^{b}
10%	7.4 ^{b}	7.4 ^{ab}	7.4 ^{ab}	7.1 ^{b}	7.1 ^{b}	7.7 ^{ab}	7.5 ^{b}
15%	7.3 ^{b}	6.8 ^{cd}	7.2 ^{bc}	7.2 ^{b}	7.1 ^{b}	7.1 ^{b}	7.0 ^{bc}
20%	6.6 ^{c}	6.5 ^{d}	6.8 ^{c}	6.3 ^{c}	6.2 ^{c}	6.2 ^{c}	6.9 ^{c}
	Kiwifruit						
0%	8.1 ^{a}	7.8 ^{a}	7.9 ^{a}	8.1 ^{a}	8.2 ^{a}	7.9 ^{a}	8.0 ^{a}
5%	7.5 ^{b}	7.1 ^{b}	7.5 ^{ab}	7.6 ^{a}	7.3 ^{b}	7.3 ^{b}	7.4 ^{bc}
10%	7.1 ^{bc}	7.0 ^{b}	7.5 ^{ab}	7.0 ^{b}	6.7 ^{c}	6.7 ^{c}	7.1 ^{c}
15%	6.7 ^{c}	6.7 ^{b}	7.1 ^{b}	6.6 ^{b}	6.5 ^{c}	6.8 ^{bc}	7.2 ^{c}
20%	7.6 ^{b}	7.6 ^{a}	7.6 ^{ab}	7.9 ^{a}	7.6 ^{b}	7.9 ^{a}	7.8 ^{ab}
	Pineapple						
0%	8.1 ^{a}	7.8 ^{a}	7.9 ^{ab}	8.1 ^{a}	8.2 ^{a}	7.9 ^{a}	8.0 ^{a}
5%	7.8 ^{ab}	8.0 ^{a}	8.1 ^{a}	8.1 ^{a}	7.9 ^{ab}	7.7 ^{ab}	7.5 ^{ab}
10%	7.4 ^{b}	6.6 ^{b}	6.9 ^{cd}	7.4 ^{b}	7.4 ^{bc}	7.2 ^{c}	7.3 ^{b}
15%	6.7 ^{c}	6.5 ^{b}	6.5 ^{d}	6.7 ^{c}	6.6 ^{d}	7.0 ^{c}	6.7 ^{c}
20%	7.6 ^{b}	6.7 ^{b}	7.3 ^{bc}	7.5 ^{b}	7.1 ^{cd}	7.3 ^{bc}	7.3 ^{bc}
	Pomegranate						
0%	8.1 ^{a}	7.8 ^{a}	7.9 ^{a}	8.1 ^{a}	8.2 ^{a}	7.9 ^{a}	8.0 ^{a}
5%	7.9 ^{a}	7.6 ^{a}	7.7 ^{a}	7.7 ^{ab}	7.7 ^{ab}	7.6 ^{a}	7.9 ^{a}
10%	7.3 ^{b}	7.5 ^{a}	7.4 ^{a}	7.3 ^{b}	7.5 ^{b}	7.7 ^{a}	7.3 ^{b}
15%	6.3 ^{c}	6.5 ^{b}	6.7 ^{b}	6.3 ^{c}	6.1 ^{c}	6.5 ^{b}	6.6 ^{c}
20%	6.8 ^{b}	6.7 ^{b}	6.6 ^{b}	6.6 ^{c}	6.6 ^{c}	6.8 ^{b}	6.9 ^{bc}

[1] Overall, appearance, texture, flavor, aftertaste, color and graininess liking scores within the same fruit with various substitution (0, 5, 10, 15 and 20%) fractions (same column within the same fruit by-product) having different letter(s) are significantly ($P<0.05$) different according to the LSD.

The addition of fruit by-products, having a dark brownish color negatively impacted cookies color scores. Furthermore, the presence of slight aftertaste of some fruit by-products, especially kiwifruit and pomegranate may have impacted cookies sensory characteristics. The greater the fruit by-product contribution in treatments, the lower the color liking score. For example, color liking scores significantly ($P<0.05$) decreased from 7.5 to 6.2 and from 7.6 to 6.8 when avocado and pomegranate treatments increased from 5 to 20%. The dark brownish color of cookies made using various fractions of fruit by-products was attributed to fruit by-product's chemical composition as well as its low pH (i.e., 4.2 to 5.4). Turksoy and Ozkaya [20] reported a significant reduction in color liking of cookies supplemented with pumpkin peel powder and carrot pomace powder; rich in β-carotene.

In the same manner, the use of 15% kiwifruit and pomegranate treatment resulted in the lowest aftertaste liking score of 6.5 and 6.1, respectively while the use of 20% avocado resulted in an aftertaste liking score of 6.2. Avocado peels were reported to have significant amounts of phenolic compounds (10,848.27 ± 162.34 mg GAE kg-1) and flavonoids (1,360.34 ± 188.65 mg EQ kg-1) that is believed to impart peels aftertaste [21]. Pomegranate content of Tannins and Alkaloids was also related to the lower aftertaste liking of produced cookies [22].

CONCLUSION

The use of avocado, kiwifruit, pineapple and pomegranate treatments produced exceptionally successful acceptable cookies characteristics and is expected to influence cookies nutritional aspects, especially their fiber content. Although the use of fruit by-products resulted in darker cookies color, this apparently had no impacts on cookies sensorial attributes. Treatments that included fractions of fruit by-product's flours resulted in increased WHC, syneresis and pasting viscosities; results that was attributed to the increase fiber content. Improvement in freeze thaw stability, however, can be improved through the use of hydrocolloids that are known to provide stability in food applications. Nutritional value and anti-nutritional factors of fruit by-products are not studied and are believed to be a fruitful research area.

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THE USE OF NEW HYPER IMMUNE SERA AND REAL-TIME PCR ASSAY FOR THE DETECTION OF MEAT SPECIES

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ABSTRACT

The increase in the population and in costs of production cause a competitive platform in the supplying, selling and marketing of the food. Adulteration in meat products has become a significant issue to be addressed for public health and also for economic issues. In this context, it is important to apply the most reliable, accurate and practical meat species detection methods. In this study, three assays including agar gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA) and newly developed real-time PCR kits were compared in order to determine which method is the most successful in detecting twelve different meat species. Also, hyper immune sera of beef, pork, chicken and horse were produced in rabbits and used in AGID assays. It was found that real-time PCR kits could detect meat species in the meat mixtures up to 0.1%, ELISA detected up to 1% and AGID to up to 5%. The results showed that while real-time PCR method was more sensitive, reliable and less expensive than ELISA, its low sensitivity might be a problem in discrimination of adulteration in meat products. Although hyper immune sera based AGID is less expensive and less practical than other assays, cross-reactions between the species of the same class (chicken-turkey; beef-sheep-goat-deer; and horse-donkey) found to be the main limitation of the method. Determination of meat species in meat products is of great importance in terms of adulteration, food legislation, religious limitations, consumer health and rights. The findings of this study could provide information in choosing a reliable, easily applicable, accurate and affordable method.

KEYWORDS:
AGID, ELISA, real-time PCR, hyper immune serum, meat species identification

INTRODUCTION

Meat and meat products play an important role for individuals to live their lives in a healthy way. The increase in the population and in costs of production cause a competitive platform in the supplying, selling and marketing of the food. Consequently, as the prices escalate, on behalf of reducing the costs, producers can sometimes resort to tricky ways. Adulteration in meat products has become a very common issue worldwide, and has been frequently seen in both developed and developing countries [1,2]. In this context, the most common adulteration is mixing different type and/or species of meats into products. Following the horsemeat crisis in Europe in 2013, it was stated that adulteration and fraud in meat products is one of the most important problem to be addressed for public health [3].

Determination of meat species used in the production of meat products and controlling their compliance with the legislation are of great importance in terms of both food legislation and consumer rights. Adding cheap meat types into meat products provides unfair economic gain to the producers, while causing health problems, inducing allergic reactions in people who are sensitive to some meat types, and deceiving people who do not consume some meat species due to religious beliefs [1,4,5]. In addition, because of the emergence of some hazardous conditions, likely in BSE (Bovine Spongiform Encephalopathy) disease, the identification of meat species has gained even more importance [6].

Mixing meats into products obtained from illegally slaughtered animal species by ignoring the public health causes zoonotic infections such as rabies, ruam, tuberculosis, anthrax, trichinellosis etc. in the food producers, handlers and consumers [7]. In Turkey, because of religious rules breeding and consumption of pork is very limited. However, it is thought-provoking that one of the biggest trichinellosis outbreak in the world with 542 cases was recorded in January 2004 in Turkey, due to the consumption of raw meatballs (a traditional ready-to-eat food with raw meat) prepared with illegally hunted pork [8].

There are many methods for determining the meat species such as immunochemical (e.g. agar gel immunodiffusion – AGID), protein (e.g. enzyme-linked immunosorbent assay - ELISA) and DNA-based (e.g. polymerase chain reaction - PCR) [9,10]. PCR-based methods come to the fore since protein

denaturation takes place especially in meat products. Also, PCR provides opportunity to distinguish many different types at the same time with multiplex option and is used easily in many routine laboratories. Within these PCR techniques, due to its high sensitivity, specificity and quantitative results, real-time PCR can be considered as one of the most effective methods [9,11,12]. In real-time PCR, nucleic acids are quantified by increased signals after each amplifying cycle with the help of fluorescent dyes in proportion to the number of PCR amplicons produced [13]. Frequently, TaqMan® probes have been preferred in real-time PCR analysis [9,14,15]. With the use of these probes, specific fluorescent signals can be obtained for target species, and hereby the meat of different animals in a meat product can be detected in a single reaction [16].

In this study, it was aimed to *i)* produce hyper immune sera for beef, pork, chicken and horse; *ii)* determine sensitivity, specificity and titers of the sera *iii)* perform sensitivity, specificity and accuracy tests of newly developed commercial real-time PCR kits for the detection of meat species for the first time; and i*v)* compare agar gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA) and real-time PCR in order to determine which method is the most effective in detecting meat species.

MATERIALS AND METHODS

Meat samples. Meats from the following animals were used in this study; beef (*Bos taurus domesticus*), sheep (*Ovis ovis*), goat (*Capra hircus*), roe deer (*Capreolus capreolus*), camel (*Camelus dromedarius*), chicken (*Gallus gallus*), turkey (*Melagris gallopavo*), rabbit (*Lepus europaeus*), horse (*Equus caballus*), donkey (*Equus asinus*), pork (*Sus scrofa*) and common carp (*Cyprinus carpio*).

Hyper immune serum production. Specific hyper immune sera for beef, pork, chicken and horse were produced in rabbits. For this purpose, 10-12 weeks old New Zealand breed rabbits were used. All rabbits were clinically examined and one week adaptation was achieved with the ad libitum feeding protocol prior to the experiment. The Ethics Committee Decision about the study was taken from the Kirikkale University Animal Experiments Local Ethics Committee (Date: 26/07/2018, No:38)

Albumins that specific to bovine, pig and horse (Sigma Aldrich, USA) and blood serum of chicken were suspended as 1% in PSS, supplemented with adjuvant (Complete Freund's Adjuvant) and then injected to the rabbits subcutaneously. Five rabbits were used for each animal species. Immunizations were continued for five weeks (one dose per week) in order to increase the serum titer. In the sixth week, the blood of the rabbits was taken through the ear vein into sterile tubes and kept overnight at 4°C. After the incubation, hyper immune (precipitate) sera were obtained by centrifuging the tubes at 1200 g for 2-4 minutes [17,18].

Determination of hyperimmune sera titer. Blood sera specific to animal species were used to determine the titer of the sera. Blood sera were diluted with PSS in the ratio of 1/10, 1/100, 1/1000, 1/5000, 1/7000, 1/10,000 and 1/50,000. Then the tubes were observed for precipitation. The dilution rate with the last precipitation was determined as the titer of the serum.

Determination of the sensitivity and specificity of hyperimmune sera. The sensitivity and specificity of hyper immune sera were examined with agar gel immunodiffusion (AGID) test. Initially, macerated sera of each animal species (cattle, chicken, pig and horse) were prepared. For this purpose, 5 grams of meat was macerated with 45 ml PSS, and incubated at 37°C overnight. Mixtures were filtered through Whatman no. 4 filter paper. Then, decreasing concentrations (100%, 50%, 30%, 10%, 5%, 1%) of the both macerated sera and hyper immune sera were prepared with PSS, and after the sera were dripped into the wells on the 1% Noble agar, plates were incubated at 37°C for 24 h. The hyper immune sera that gave reaction with macerated fluid were considered as positive. Also, cross-reactions and the lowest detectable meat ratio in raw meat mixtures was determined.

Enzyme-linked immunosorbent assay. Five grams of the diced meat samples of each animal species (cattle, camel, sheep, goat, roe deer, chicken, turkey, rabbit, horse, donkey, pig and common carp) were mixed with 100 ml PSS and thoroughly mixed in the stomacher for 60 seconds. Then the mixture was heated in a water bath at 100°C for 15 minutes, prior to filtering through Whatman no. 4 filter paper. ELISA kits (ELISA-TEK, Microwells Cooked Meat USDA Kit: Beef, poultry, pork, horse, turkey - Gainesville, FL) were used for the detection of meat species according to the manufacturer's instructions. Wells with distinct green color interpreted as positive.

Real-time PCR meat species detection assays. Newly developed real-time PCR kits (DIAGEN Real Time PCR Meat Detection Kit: Beef, sheep, goat, chicken, turkey, pork, horse, donkey, beef/pork, chicken/turkey and horse/donkey - Ankara, TURKEY) were used for determining meat species. The sensitivity, specificity and accuracy of the kits were performed for the first time in this study.

Preparation of meat samples and DNA extraction. Meat mixtures of animal species were prepared as listed in Table 1. Tissue DNA extraction kit

(Vivantis GF-1 Tissue DNA Extraction Kit) was used according to the manufacturer's instruction. The purity of DNAs, which were planned to be used as template DNA, were determined using NanoDrop Spectrophotometer (NanoDrop ND-100, Delaware, USA). For this purpose, values of about 1.8 and above at 260/280 wavelength were accepted. Also, DNA of single animal species were standardized to 50 ng and diluted up to 10^{-4} (5 ng, 0.5 ng, 0.05 ng, and 0.005 ng) with serial dilutions for testing sensitivity, specificity and accuracy of the kit.

Real-time PCR analysis. Real-time PCR was carried out with Bio-Rad CFX96 Touch Real Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). Analysis were performed by DIAGEN Real Time PCR Meat Detection Kits prepared by species-specific primers and TaqMan® probes. Reactions were carried out in a single/multiplex format. Each kit specific for meat species were consist of Mix A, Mix B (Primers and TaqMan® probes specific to the relevant regions of animal-specific mitochondrial DNA), molecular-grade water, DNA Taq polymerase and control DNA. real-time PCR analyses were performed in a total volume of 20 µl containing 10 µl Mix A, 3.5 µl Mix B, 3.3 µl molecular-grade water, 0.2 µl Taq DNA polymerase, and 3 µl template DNA (20-100 ng). Each run was carried out with the initial denaturation at 95 °C for 5 min, and then 30 cycles of denaturation at 95 °C for 10 s, annealing at 59 °C for 30 sec, and extension at 72 °C for 5 sec, with cooling for 1 min at 25°C. All runs were analyzed with CFX Manager 3.1 Software (Bio-Rad, Hercules, CA) and samples with Ct value for the tested meat species were accepted as positive. All samples were analyzed three times.

RESULTS AND DISCUSSION

In the study, hyper immune sera for cattle, chicken, pig and horse were produced in New Zealand rabbits. The titers of horse and pig sera were determined as 1/50,000 and the titer of chicken and cattle sera were found 1/10,000. Since the desired titers of the sera were higher than 1/5,000, the immunization period (five week) of the serum production step was not extended.

The sensitivity and specificity of hyper immune sera were examined with AGID test. Although the AGID test is an easy, inexpensive and common used method for determining meat species, the inevitable cross reactions between the sera of animals belong to the same class can cause misinterpretations. According to our analysis, cattle hyper immune serum gave positive reaction with cattle, sheep and goat meat; chicken hyper immune serum gave positive reaction with chicken and turkey meat; pig hyper immune serum gave positive reaction only with pork; and horse hyper immune serum gave positive reaction with horse and donkey meat. These multiple positive reactions indicated that AGID cannot be able to discriminate beef from sheep, goat and deer; chicken from turkey; and horse from donkey. Munir and ur Rahman [10] compared horse and donkey hyper immune sera using AGID and Countercurrent Immunoelectrophoresis (CCIE), and stated that due to the false positive cross reactions it was not possible to discriminate these species meat with AGID. On the other hand, our hyper immune sera did not cross-react with any animal blood sera and meat maceration fluids that belong to other animal species (Table 2). Based on these results, it has been determined that AGID test can be used in ruminant, poultry, pork and equidae meat differentiations.

ELISA tests for beef, poultry, pork, horse and turkey were performed on cattle, camel, sheep, goat, roe deer, chicken, turkey, rabbit, horse, donkey, pig and common carp in the study. ELISA showed 100% specificity with the related animal species tested (beef, horse, pork and turkey) while the poultry test gave positive reaction for both chicken and turkey as expected. No positive/false positive reactions were observed for camel, sheep, goat, roe deer, rabbit, donkey and common carb. As the same, in our study real-time PCR showed 100% specificity with DNAs of beef, sheep, goat, chicken, turkey, pork, horse and donkey. The DNAs of meat species were analyzed three times as individually and as mixture. It was determined that the real-time PCR kits did not cross-react with non-specific DNA of meat species and the DNA of the other animals tested. In a study that compared the same ELISA kit and a real-time PCR kit for beef and pork, the specificity was indicated as 100% same as in our study [9].

Sensitivity of the real-time PCR single and multiplex meat species detection kits (beef, sheep, goat, chicken, turkey, pork, horse, donkey, beef/pork, chicken/turkey and horse/donkey) were performed with meat mixtures as given in Table 1. The sensitivity (or minimum detection limit) was determined experimentally with three replicate tests. According to the analysis, the sensitivity of the real-time PCR kits was found 0.1% in all meat mixtures. On the other hand, ELISA was able to detect animal species in meat mixture up to 1%, and when hyper immune sera were tested with the decreasing concentrations (100%, 50%, 10%, 5%, 1%) of meat mixtures, the lowest detectable meat rate in the raw meat mixtures was found 5% for AGID (Table 2). The sensitivity of real-time PCR was found quite high compared to ELISA and AGID. Some studies detecting pork and beef species were reported the same sensitivity percentage for real-time PCR used with TaqMan™ probes [9,19). However, Perestam et al. [9] were found 5% specificity for pork and 10% for beef/pork mixture using ELISA-TEK kit. USDA [20] was also tested

TABLE 1
Meat mixtures used for the sensitivity, selectivity and accuracy tests of the meat species detection methods.

Cattle	Sheep	Goat	Deer	Chicken	Turkey	Horse	Donkey	Pork	Fish	Rabbit
100 %	-	-	-	-	-	-	-	-	-	-
-	100 %	-	-	-	-	-	-	-	-	-
-	-	100 %	-	-	-	-	-	-	-	-
-	-	-	100 %	-	-	-	-	-	-	-
-	-	-	-	100 %	-	-	-	-	-	-
-	-	-	-	-	100 %	-	-	-	-	-
-	-	-	-	-	-	100 %	-	-	-	-
-	-	-	-	-	-	-	100 %	-	-	-
-	-	-	-	-	-	-	-	100 %	-	-
-	-	-	-	-	-	-	-	-	100 %	-
-	-	-	-	-	-	-	-	-	-	100 %
33%	-	-	-	33%	-	33%	-	1%	-	-
33%	-	-	-	33%	-	1%	-	33%	-	-
33%	-	-	-	1%	-	33%	-	33%	-	-
1	-	-	-	33%	-	33%	-	33%	-	-
33%	33%	-	-	-	1%	33%	-	-	-	-
33%	1%	-	-	33%	33%		33%	-	-	-
-	10%	10%	10%	10%	10%	10%	10%	10%	10%	10%
10%	-	10%	10%	10%	10%	10%	10%	10%	10%	10%
10%	10%	-	10%	10%	10%	10%	10%	10%	10%	10%
10%	10%	10%	-	10%	10%	10%	10%	10%	10%	10%
10%	10%	10%	10%	-	10%	10%	10%	10%	10%	10%
10%	10%	10%	10%	10%	-	10%	10%	10%	10%	10%
10%	10%	10%	10%	10%	10%	-	10%	10%	10%	10%
10%	10%	10%	10%	10%	10%	10%	-	10%	10%	10%
10%	10%	10%	10%	10%	10%	10%	10%	-	10%	10%
10%	10%	10%	10%	10%	10%	10%	10%	10%	-	10%
10%	10%	10%	10%	10%	10%	10%	10%	10%	10%	-
5%	-	-	-	95%	-	-	-	-	-	-
-	5%	-	-	-	95%	-	-	-	-	-
-	-	5%	-	-	-	-	-	-	-	-
95%	-	-	-	5%	-	-	-	-	-	-
-	95%	-	-	-	5%	-	-	-	-	-
-	-	-	-	95%	-	5%	-	-	-	-
-	-	-	-	95%	-	-	5%	-	-	-
-	-	-	-	-	-	95%	-	5%	-	-
-	-	-	-	-	-	-	-	-	-	-
0.5%	-	-	-	95.5%	-	-	-	-	-	-
-	0.5%	-	-	95.5%	-	-	-	-	-	-
95.5%	-	-	-	0.5%	-	-	-	-	-	-
-	-	-	-	95.5%	0.5%	-	-	-	-	-
95.5%	-	-	-	-	-	0.5%	-	-	-	-
-	-	-	-	95.5%	-	-	0.5%	-	-	-
-	-	-	-	95.5%	-	-	-	0.5%	-	-
0.1%	-	-	-	99.9%	-	-	-	-	-	-
-	0.1%	-	-	99.9%	-	-	-	-	-	-
99.9%	-	-	-	0.1%	-	-	-	-	-	-
-	-	-	-	99.9%	0.1%	-	-	-	-	-
99.9%	-	-	-	-	-	0.1%	-	-	-	-
-	-	-	-	99.9%	-	-	0.1%	-	-	-
-	-	-	-	99.9%	-	-	-	0.1%	-	-

TABLE 2
Comparison findings of real-time PCR, ELISA and AGID test for the prepared meat mixtures.

Meat mixtures	Number of analysis	Real Time	ELISA	AGID
100% of related species	3	3/3	3/3	3/3
33% of related and 67% other species	3	3/3	3/3	3/3
10% of related and 90% other species	3	3/3	3/3	3/3
5% of related and 95% other species	3	3/3	3/3	3/3
1% of related and 99% other species	3	3/3	3/3	-
0.5% of related and 99.5% other species	3	3/3	-	-
0.1% of related and 99.9% other species	3	3/3	-	-
0% of related and 100% other species	3	3/3	0/3	0/3

-: not detected.

ELISA-TEK kits and reported the detection limits 1% for beef and deer, 4% for pork, poultry, sheep and horse. The reason for having different results despite using the same kit may be related to the amount of meat species in the mixtures tested and/or efficiency of extraction protocol. Considering the sensitivity of the tests, it may be thought that ELISA can also provide effective results in determining the types of meat usage for fraud, considering that different meats are added with more than 5-10 % in order to gain economically unfair profits.

The reliability of the real-time PCR kits was achieved with analyzing the samples obtained from the meat mixtures prepared (Table 1) and the samples of the pure meat DNA diluted up to 10^{-4} with serial dilutions in three replicates. With this we can determine the limit of detection (LOD) of each species-specific primer/probe in RT-PCR analysis. In none of the trials, false positive or false negative results were observed (Table 2). Also, it was found that real-time PCR protocol could detect the pure DNAs' of animal species as low as 0.005 ng (10^{-4} dilution).

In terms of time and ease of use, ELISA was found the easiest to perform and needed shorter time compared to AGID and real-time PCR. Depending on the hands skill of the researcher/technician, ELISA test could be finished in 3.5 - 5 h. For real-time PCR, time of analysis vary from 3 to 6 hours depending on the increase in the number of samples. The longest analysis period belongs to AGID with almost 48 h because of the incubations. However, ELISA was found the most expensive assay, followed by real-time PCR and AGID, respectively.

CONCLUSION

Overall, the sensitivity, specificity and accuracy of AGID, ELISA and real-time PCR assays used in meat species determination were compared. The results showed that the real-time PCR method performed with newly developed real-time PCR kits were more sensitive, reliable and less expensive than ELISA. However this low sensitivity may be a problem in the discrimination of adulteration in meat products. Although hyper immune sera based AGID is less expensive and practical, as well as the meat to be analyzed must be raw, cross reactions between chicken with turkey; beef with sheep, goat or deer; and horse with donkey is the main limitations of the method.

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Conflict Of Interest. The authors declare no conflict of interest.

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IMPACT OF DIFFERENTIAL SIEVING ON NUTRITIONAL PROPERTIES OF BUCKWHEAT FLOUR FRACTIONS

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ABSTRACT

The nutritional and functional properties of differentially sieved buckwheat flour fractions were determined in the present study. Sieving, a low-cost technique to pre-process fours. Milled buckwheat flour was differentially sieved to get coarse and fine flour fractions. The flour factions and whole flour were analysed for nutritional composition, antinutrients, fatty acids, amino acids, *in vitro* protein digestibility and mineral bioaccessibility following standard techniques. The results showed highest protein content (14.2%) with better digestibility and amino acid composition in fine flour fraction in comparison to whole and coarse flour. Among essential amino acids, leucine and lysine content were higher, and overall glutamic acid constituted a major proportion of amino acids. Coarse flour had a high content of dietary fibre (74.6%). The minerals largely present in coarse flour were Ca, Mn, Mg, K, and Cu, inversely fine flour had higher Fe and Zn content. Oleic and linoleic acid were the major fatty acids present in flour samples ranging from 39.3-43.9% and 30.8-34.3% respectively. In conclusion, differential sieving of buckwheat flour influenced the composition of flour fractions significantly. The separated fractions can be potentially utilized for the product formulation with desired properties.

KEYWORDS:
Nutritional composition, antinutrients, mineral bioaccessibility, fatty acids, amino acids, functional properties

INTRODUCTION

Buckwheat is a pseudocereal grain, belonging to the family '*Polygonaceae*' and genus '*Fagopyrum*', which is a "smartweed" family having broad-leafed herbaceous annual plant [1]. Structurally, the seeds are dicotyledon and brown in color with an irregular shape, having four triangular surfaces. Around the world, two types of buckwheat are used namely common buckwheat (*Fagopyrum esculentum*) and Tartary buckwheat (*Fagopyrum tataricum*). Buckwheat is usually consumed as whole grain and thus contributes to higher nutrients and dietary fibre content in the diet in comparison with common cereals [2].

In recent times, consumption of buckwheat is increasing, as consumers are switching over to nutritious foods, especially gluten-free products. Buckwheat is identified as a good source of nutrients such as protein, lipid, dietary fibre, minerals, and phenolic compounds. Particularly, buckwheat protein has a well-balanced amino acid composition with high biological value and associated with many health benefits such as plasma cholesterol level reduction, anticancer, antidiabetic, anti-hypertension, anti-inflammatory, and neuroprotective effects. Additionally, buckwheat has prebiotic and antioxidant activities [3]. Dietary treatment for chronic and metabolic diseases, such as diabetes, hypertension, and celiac disease could consider buckwheat as an alternative food, leading to a daily diet rich in nutrient content. Ultimately, buckwheat serves as a functional food to improve human health and also treat diseases [4].

Primary processing like milling, sieving, grinding of the flour are essential steps for the utilization of grains [5]. Studies on common buckwheat processed into flour using different milling methods i.e. high-speed universal grinder, wet-milling and a stone mill for its physicochemical properties reported lower average particle size and damaged starch content with intact granular structures in wet-milled flour. And this flour showed high water absorption index, swelling power, and low water solubility index and water-binding capacity [6]. The trend of fractionation or enrichment and recombination techniques is increasing in the food industry to improve the quality of foods. Other fractionation techniques such as pearling/grinding, sieving, and air classification could result in grain fractions with added value and higher concentration of nutrients in each fraction by the process of separation or enrichment [7].

Previous studies on buckwheat milled fractions separated into various grades of flour reported different nutrient composition as the light flour present in the central portion consists of starchy endosperm and embryo and the aleurone layer contains most of the protein, lipids, and minerals [8]. Practically applying such milling processes in commercial setup would increase the cost of production and also the wholesomeness of the nutrients present in the grain

won't be utilized. Alternatively, differential sieving would separate the major fractions of the flour, resulting in the flour containing all the nutrients of the grain in different proportions. Taking this into account, the purpose of this research was to compare the nutrient composition and functional properties of fractions separated from whole buckwheat flour by differential sieving.

MATERIALS AND METHODS

Materials. Materials and differential sieving. The common buckwheat (*Fagopyrum esculentum*) was obtained from a local certified organic shop in Mysuru, India in a single batch. The grains were sorted, cleaned, dried at 40°C for 4 hr in the hot-air oven, and milled into flour in plate mill. The milled whole flour was differentially sieved by passing through a standard 100 mesh sieve with 149 µm pore size, allowing small particle size to pass through which was labelled as fine flour (FF) and the residual flour in the sieve was coarse flour (CF). The coarse flour was further pulverized into finer particle size flour to facilitate analysis. A portion of whole flour (WF) was retained as such which served as the control. The FF accounted for 70% and the rest 30% was CF of the whole buckwheat flour. All the experimental flours were stored in air-tight zip-lock pouches at a low temperature of 4°C until further analysis.

Reagents and enzymes. The enzymes used in the study were pepsin (GRM9155), pancreatin (RM3867), and bile salt procured from Himedia, and α- amylase from *Bacillus amyloliquefaciens* (Lot#SLBF7325V) procured from Sigma-Aldrich chemicals Pvt Ltd. The dialysis tube (LA395) from Himedia used for *in-vitro* studies had the following specification 31.71mm width, 21.5mm diameter, 2.4nm pore size, and molecular cut off between 12,000 to 14,000. Other chemicals, solvents, and acids were procured from Sisco Research Laboratories Pvt Ltd (SRL), Sd Fine-chem Ltd and Merck Life Science Pvt Ltd, Mumbai, India. All the chemicals used in the study were of analytical grade. All analysis was performed in triplicates and triple distilled water was used throughout the study.

Methods. The WF and differentially sieved flours of buckwheat, FF and CF were analysed for nutritional composition, amino acid, and fatty acid profile, bioaccessible iron, zinc and calcium, *in-vitro* protein digestibility and functional properties.

Nutrient composition. The nutrient content of the flour samples was evaluated by following standard AOAC methods [9]. The moisture content was determined gravimetrically by removing the moisture of the flour samples in the hot-air oven and the total ash was determined by recording the weight of ash after charring the flours followed by carbonizing in muffle at 550°C. Soxhlet method of solvent extraction for fat content and the Kjeldahl method for protein were used. The percent protein was obtained by multiplying the nitrogen value with the factor 6.25. Dietary fibre content was analysed as per the method of Asp et a., [10] by rapid enzymatic method.

Antinutrients. Phytic acid was estimated in flours following Thompson and Erdman's [11] method. A conversion factor of 3.55 was used to convert phosphorous into phytic acid. Condensed tannins were determined colorimetrically by vanillin-HCl method, wherein the flour samples were extracted with methanol. A known aliquot of methanol extract was mixed with freshly prepared vanillin-HCl reagent, set aside for 20 min and read at 500 nm. The results were expressed as milligrams of (+)-catechin equivalents per 100g [12].

In vitro protein digestibility. *In vitro* digestible protein was estimated in the flour samples by digesting with pepsin and pancreatin enzymes with appropriate pH adjustment. Soluble protein was separated and estimated by the Kjeldahl method [13].

Mineral content and bioaccessible iron, zinc and calcium. The total ash obtained through incineration (as given earlier) was heated with concentrated HCl over hot water bath and solubilized with triple distilled water. This solution was filtered through ashless filter paper, made-up to a known volume and estimated for Fe, Zn, Ca, Mg, Mn, K, and Cu in an Inductively coupled plasma optical emission spectrometer (ICP-OES) (Perkin Elmer Optima^{TM} 8300, MA, USA). The flour samples were also estimated for bioaccessible Fe, Zn, and Ca by dialysis method after treating with digestive enzymes [14]. The dialysates were estimated for dialyzable mineral using ICP-OES. Percent bioaccessibility of the minerals was calculated by dividing dialyzable mineral over total mineral present in the analysed flour.

Fatty acid profile. The fatty acid profile was measured in gas chromatography (GC) fitted with a capillary column and flame ionization detector into which fatty acid methyl esters were fed. The GC model used was GC-2010plus, Shimadzu Corporation, Tokyo, Japan, and the column was 30m x 0.25mm ID, DF – 0.25µm thickness. The injector and detector temperature were maintained at 260 - 280°C. The fatty acids were identified and quantified with reference to the retention time of standard fatty acid methyl esters under similar conditions and expressed as a percentage of relative area [15].

Amino acid Profile. The amino acid profile was determined by ion chromatography in an Amino acid analyzer released by hydrolysis on boiling with

semi-concentrated hydrochloric acid. The instrument used was the Biochrom 30+ series of Amino acid analyzer from Biochrom Ltd. Cambridge, UK with two-channel A/D converter card on a PC using Software for peak integration. The aminograms were detected at 570 nm and 440 nm [16].

Functional properties. For measurement of functional properties, flour samples were dried in an oven at 40°C for 24hrs to attain equilibrium moisture and analysed using standard procedures. The methodologies used were as follows, water absorption capacity (WAC) reported as water absorbed in g/100g of flour by the method of Elhardallou and Walker [17]; water solubility index (WSI) expressed as the weight ratio of dissolved solids in the supernatant and dry sample as described by Anderson et al., [18]; oil absorption capacity (OAC) as the measure of oil taken up by the known amount of flour and reported as g of oil absorbed/100g of dry flour as given by Sosulski et al., [19]; and foaming capacity and stability expressed as volume of foam per 100 ml according to Coffmann and Garcia [20].

Statistical analysis. The values are presented as means ± standard deviation (SD) of three consecutive measurements in each experiment. The comparison between the sample flours to test the significant difference was done by one-way analysis of variance (ANOVA), followed by the T-test in SPSS 16.0 [21], and the statistical significance was defined as $P<0.05$.

RESULTS AND DISCUSSION

Nutritional composition. The nutritional composition of flour samples as analysed and on dry weight basis (dwt) is presented in Table 1. The moisture content of analysed flour varied from 7.2 to 8.5%. The FF fraction had significantly higher protein (14.2% *dwt*) and fat contents (3.07% *dwt*), followed by WF. Inversely, the CF had a higher content of minerals (2.80 g/100g). CF also had significantly higher total and insoluble dietary fibre content of 14.6 and 74.6 g/100g respectively in comparison to FF, which had a very low content. Buckwheat has a relatively small amount of soluble dietary fiber (1.4%), most of which was retained in FF. These differences in composition of FF and CF of buckwheat in comparison to WF can be attributed to the grain structure, wherein most of the endosperm, embryo and a part of bran contributed towards higher protein and fat contents of FF. Sine the CF retained most of the cellulosic bran portion, the fiber content

TABLE 1
Chemical composition of buckwheat flour fractions (per 100g

		Whole flour	Fine flour	Coarse flour
Proximate composition (g)				
Moisture		7.2±0.04^{c}	7.7±0.05^{b}	8.5±0.09^{a}
Protein		11.6±0.07^{b} (12.9)	12.7±0.14^{a} (14.2)	8.89±0.24^{c} (9.9)
Fat		2.72±0.02^{b} (2.93)	2.83±0.03^{a} (3.07)	1.66±0.01^{c} (1.82)
Total ash		2.06±0.02^{b} (2.29)	1.95±0.01^{c} (2.18)	2.52±0.02^{a} (2.80)
Dietary fibre	Total	22.6±0.46^{b} (25.1)	5.08±0.18^{c} (5.7)	67.02±2.01^{a} (74.6)
	Insoluble	21.3±0.51^{b} (23.7)	4.13±0.17^{c} (4.6)	66.55±2.01^{a} (74.1)
	Soluble	1.23±0.06^{a} (1.4)	0.95±0.05^{b} (1.1)	0.47±0.06^{c} (0.52)
Minerals (mg)				
Iron		7.51±0.08^{b} (8.33)	8.40±0.12^{a} (9.39)	5.82±0.11^{c} (6.47)
Zinc		2.58±0.06^{b} (2.86)	2.84±0.05^{a} (3.18)	2.46±0.13^{bc} (2.74)
Calcium		71.00±2.5^{b} (79.0)	31.00±1.8^{c} (35.0)	195.0±0.8^{a} (217)
Manganese		2.58±0.02^{b} (2.86)	1.66±0.02^{c} (1.86)	4.75±0.02^{a} (5.28)
Magnesium		225±4.5^{b} (250)	224±1.9^{bc} (250)	257±5.4^{a} (286)
Potassium		495±5.0^{b} (550)	446±6.8^{c} (499)	660±3.3^{a} (735)
Phosphorus		355±6.4^{b} (394)	405±3.6^{a} (453)	215±1.1^{c} (240)
Copper		1.09±0.02^{bc} (1.21)	1.13±0.01^{b} (1.26)	1.35±0.07^{a} (1.51)
Sodium		3.14±0.85^{abc} (3.48)	3.57±0.04^{b} (3.99)	4.11±0.01^{a} (4.57)
Antinutrients				
Phytic acid (g)		1.16±0.12^{ab} (1.29)	1.27±0.06^{a} (1.42)	0.89±0.08^{c} (0.99)
Condensed Tannins (mg)		206.2±3.6^{c} (228.8)	265.2±7.0^{b} (296.6)	311.9±4.4^{a} (347.0)

The values are mean ± SD from three replicates. Mean values followed by different superscripts in each row are significantly different at $P<0.05$ and the values in parenthesis are on dry weight basis.

TABLE 2
***In vitro* digestible protein and bioaccessible minerals in buckwheat flour fractions**

		Whole flour	Fine flour	Coarse flour
***In vitro* digestible protein (g/100g)**				
Content		5.04±0.34^{b} (5.59)	6.83±0.19^{a} (7.64)	4.14±0.19^{c} (4.61)
Percent		39.2^{bc} (43.5)	48.3^{a} (53.9)	41.9^{b} (46.6)
Bioaccessible minerals (mg/100g)				
Iron	Content	1.33±0.01^{b} (1.48)	1.04±0.02^{c} (1.16)	1.37±0.01^{a} (1.52)
	Percent	17. Jul	48.7	33.3
Zinc	Content	1.25±0.0^{a} (1.39)	0.5±0.0^{c} (0.56)	1.08±0.0^{b} (1.20)
	Percent	12. Apr	17. Mai	66.2
Calcium	Content	23.6±0.02^{b} (26.2)	20.4±0.01^{c} (22.9)	24.1±0.02^{a} (26.8)
	Percent	23. Mai	43.9	12. Mrz

Mean values with different alphabetical superscripts were significantly different within a row at $p<0.05$ and figures in the parenthesis represent values on dry weight basis.

was exceptionally high. A study by Sytar et al., [22] reported the presence of 55% protein in buckwheat embryo followed by 35% in endosperm and rest in the hull in contrast to cereals having 10-20% of the protein in the embryo and a major portion of around 80-90% in the endosperm of the grain. Bonafaccia et al., [23] reported a protein content of around 21% in the bran and 10% in the flour of common buckwheat. The presence of higher dietary fibre content in buckwheat is said to reduce high blood pressure, lower cholesterol, control blood sugar, and prevent cancer [24].

The mineral content of buckwheat showed compositional variations among the two fractions of flour. The FF had higher content of Fe, Zn, and P, while CF was higher in Ca, Mn, Mg, K, Cu, and Na. The CF was exceptionally high in Ca with 217 mg/100g in comparison to WF and FF. The higher ash content in CF reflected the higher mineral content. Overall buckwheat flour samples were rich in magnesium and potassium. Potassium rich foods are specifically recommended for cardiovascular and kidney diseases [25]. Additionally, buckwheat contained the highest amounts of zinc, copper, and manganese in comparison to the major cereals such as rice, wheat, and cornflour as mentioned by Przybylski and Gruczyńska [26] in their review. An increase in mineral content (particularly Fe, K, Mg, Mn, and Zn) of wheat bread incorporated with buckwheat ranging from 10-20% or 10-40% was reported as compared to control wheat bread [27, 28].

Phytic acid complexes with minerals and influences their absorption. Phytic acid content was highest in FF (1.42%) and lowest in CF (0.99%). Condensed tannins, which also have the ability to bind minerals, though function as antioxidants too, were highest in CF. Condensed tannins are known to bind to fibre fraction of the grain and are released during the digestion process upon action by enzymes [29]. Since the CF had high dietary fibre content, the condensed tannins were also higher. Bilgiçli and İbanoğlu [27] reported a phytic acid content of 1335 mg/100g in buckwheat flour. Accordingly, buckwheat incorporated bread had a higher phytic acid content.

In vitro protein digestibility and bioaccessible minerals . Digestibility of protein is an index of its quality. A good quality protein should also be digestible apart from having a desirable amino acid balance. Similarly, bioaccessibility of a nutrient is a measure of the potential absorbable amount of that nutrient in the body. At the physiological level, the nutrients which can function in the body should be absorbed through the digestive tract. The extent of absorption depends on multitude of factors related to the host, efficiency of digestion, as well as the structure and composition of food being consumed. Specifically, mineral absorption is influenced by many inhibitors and promotors present in the diet [30]. Hence, determination of bioaccessibility gives an idea about the probable extent of the absorption of a nutrient. The content and percent *in vitro* digestibility of protein was highest in FF (53.9%), as this fraction also had highest concentration of protein (Table 2). The WF and CF had lower digestibility with no significant difference between them (43.5% and 46.6% respectively).

There were significant differences in bioaccessible Fe, Zn, and Ca between the differentially milled and whole flours. CF showed a better bioaccessibility of Fe, though it had least Fe content and FF showed the least bioaccessibility despite having highest Fe content. However, in terms of percent bioaccessibility, 48.7% of Fe was available from FF, while it was much lower for CF (33.3%) and WF (17.7%). Zn bioaccessibility was highest in whole flour, (though as percent of total it was least), followed by CF. CF also showed better percent bioaccessibility. FF had least bioaccessible Zn and Ca. Ca bioaccessibility was almost similar in WF and CF, though when considered as percent, there were differences. CF had highest Ca content but a small percentage was bioaccessible. It may be noted that CF also had highest condensed tannins, which tend to

bind Ca. A lower bioaccessibility of minerals in FF can be attributed to higher phytic acid content.

Fatty acid profile. The fatty acid profile of whole and fractionated flours of buckwheat are presented in Table 3. Buckwheat fat constituted a higher proportion of oleic acid ranging from 39.3 – 43.9% as the major monounsaturated fatty acid with highest concentration in the FF. Alvarez-Jubete et al., [31] reported that linoleic acid was the major fatty acid present in pseudocereals and buckwheat had a higher content of oleic acid compared to amaranth, quinoa, and wheat. Whereas, in our study, linoleic acid was the second predominant fatty acid present in WF (34.3%) followed by FF (33.19%) and CF (30.86%). In support of our results, Golijan et al., [32] found oleic acid as a dominant fatty acid in organic and conventional common buckwheat grains. The saturated fatty acid identified in our study were myristic, palmitic, stearic, and arachidic with highest concentration in CF. Palmitic acid was the major saturated fatty acid present in the buckwheat samples (range, 17.43 - 19.30%) and it was the third most abundant fatty acid present in CF. Sinkovič et al., [33] reported similar results of a higher content of palmitic acid being present in hulls (19.7 – 26.4%) than in bran (14.4-15.0%) or light flour (13.9-15.9%) of buckwheat. These slight changes in the fatty acid composition could be because of cultivar, seasonal or production variables. Other fatty acids present in a lower amount were stearic, linolenic, and behenic acid. EPA and DHA were also reported in a smaller amount of about 0.2% and 1.9% respectively. Overall, higher content of saturated fatty acid was observed in CF (27.2%), MUFA in FF (44.2%) and PUFA in WF (36.9%).

Amino acid profile. The amino acid composition of buckwheat flour samples are presented in Table 4. Comparison of the overall amino acid content of differentially sieved buckwheat flours shows significantly higher content in FF followed by WF and CF. The predominant essential amino acid reported were leucine and lysine ranging from 0.905 – 0.531% and 0.755 – 0.422% respectively in all the three flours samples. The other essential amino acids present in notable amounts were valine and phenylalanine. These values were comparable to earlier studies by Mota et al., [34] and Sytar et al., [35]. According to the former study, buckwheat contained five time more lysine than rice indicating that it could supplement a rice based diet with lysine effectively. Methionine, tryptophan, and histidine were the limiting essential amino acids present in the analysed flours. Threonine and methionine were identified as first and second limiting amino acids in buckwheat by Giménez-Bastida et al., [36] in their review on buckwheat. Thus, in present study, the total essential amino acid accounted for 4.073% in WF, 4.859% in FF, and 2.817% in CF.

Glutamic acid (2.413%) was the major amino acid present all amino acids followed by aspartic acid (1.275%) in FF. Arginine and glycine were present in higher amount amongst the conditionally essential amino acids in the analyzed flour. The presence of higher amides content (glutamine, acid-glutamine, aspartic acid-asparagine, and arginine) in buckwheat confirms its role as a storage protein [37]. Overall, non-essential amino acids could be estimated in higher proportion than the conditionally essential or essential amino acids in buckwheat flours. The biological value buckwheat was reported to be very high (90%) on account of well-balanced amino acids [22]. Thus, the use of FF as a functional ingredient in food formulations would provide all the essential amino acids in adequate amount, which would impart multiple health benefits.

TABLE 3
Fatty acid profile of buckwheat flour fractions (%)

	Whole flour	Fine flour	Coarse flour
Myristic acid (C14:0)	0.23 ± 0.01^{ab} (0.26)	0.19 ± 0.02^{c} (0.21)	0.25 ± 0.01^{a} (0.28)
Palmitic Acid (C16:0)	15.7 ± 0.03^{c} (17.43)	16.0 ± 0.02^{b} (17.94)	17.4 ± 0.02^{a} (19.30)
Palmitoleic Acid (C16:1)	0.31 ± 0.04^{b} (0.35)	0.3 ± 0.01^{c} (0.33)	0.43 ± 0.02^{a} (0.48)
Stearic Acid (C18:0)	3.31 ± 0.02^{b} (3.68)	2.86 ± 0.03^{c} (3.20)	4.29 ± 0.04^{a} (4.77)
Oleic Acid (C18:1)	37.4 ± 0.01^{b} (41.5)	39.2 ± 0.03^{a} (43.88)	35.3 ± 0.02^{c} (39.28)
Linoleic Acid (C18:2)	30.9 ± 0.06^{a} (34.3)	29.7 ± 0.34^{b} (33.19)	27.7 ± 0.02^{c} (30.86)
Linolenic Acid (C18:3)	2.35 ± 0.01^{a} (2.61)	1.34 ± 0.04^{c} (1.49)	2.3 ± 0.01^{b} (2.56)
Arachidic Acid (C20:0)	0.73 ± 0.02^{c} (0.81)	0.88 ± 0.01^{b} (0.99)	2.59 ± 0.02^{a} (2.88)
Behenic Acid (C22:0)	1.91 ± 0.03^{c} (2.12)	2.1 ± 0.04^{ab} (2.35)	2.17 ± 0.02^{a} (2.41)
EPA (C20:5)	0.2 ± 0.01^{a} (0.23)	0.18 ± 0.01^{c} (0.20)	0.2 ± 0.01^{ab} (0.22)
DHA (C22:6)	1.2 ± 0.02^{b} (1.33)	1.02 ± 0.01^{c} (1.14)	1.79 ± 0.01^{a} (1.99)
Others	5.64 ± 0.02^{b} (6.26)	5.94 ± 0.02^{a} (6.64)	5.52 ± 0.02^{c} (6.14)

Mean values with different alphabetical superscripts were significantly different within a row at $p<0.05$ and figures in the parenthesis represent values on dry weight basis.

TABLE 4
Amino acid profile of buckwheat flour fractions (%)

	Whole flour	Fine flour	Coarse flour
Essential amino acid			
Methionine	0.181±0^{b} (0.201)	0.214±0^{a} (0.239)	0.118±0^{c} (0.131)
Lysine	0.563±0^{b} (0.625)	0.675±0^{a} (0.755)	0.379±0^{c} (0.422)
Threonine	0.401±0^{b} (0.445)	0.467±0^{a} (0.522)	0.297±0^{c} (0.330)
Tryptophan	0.16±0^{b} (0.178)	0.194±0^{a} (0.217)	0.099±0^{c} (0.110)
Isoleucine	0.397±0^{b} (0.441)	0.469±0^{a} (0.525)	0.273±0^{c} (0.304)
Leucine	0.685±0^{b} (0.760)	0.809±0^{a} (0.905)	0.477±0^{c} (0.531)
Valine	0.524±0^{b} (0.582)	0.618±0^{a} (0.691)	0.361±0^{c} (0.402)
Histidine	0.255±0^{b} (0.283)	0.296±0^{a} (0.331)	0.187±0^{c} (0.208)
Phenylalanine	0.503±0^{b} (0.558)	0.602±0^{a} (0.674)	0.34±0^{c} (0.379)
Conditionally Essential amino acid			
Arginine	0.944±0^{b} (1.048)	1.173±0^{a} (1.312)	0.584±0^{c} (0.650)
Cystine	0.238±0^{b} (0.264)	0.295±0^{a} (0.330)	0.146±0^{c} (0.162)
Glycine	0.633±0^{b} (0.703)	0.746±0^{a} (0.835)	0.45±0^{c} (0.501)
Proline	0.443±0^{b} (0.492)	0.495±0^{a} (0.553)	0.349±0^{c} (0.388)
Non-essential amino acid			
Serine	0.509±0^{b} (0.564)	0.598±0^{a} (0.669)	0.368±0^{c} (0.409)
Alanine	0.47±0^{b} (0.522)	0.549±0^{a} (0.614)	0.332±0^{c} (0.370)
Aspartic acid	0.951±0^{b} (1.055)	1.14±0^{a} (1.275)	0.66±0^{c} (0.735)
Glutamic acid	1.789±0^{b} (1.985)	2.157±0^{a} (2.413)	1.135±0^{c} (1.263)
Methionine+cystine	0.419±0^{b} (0.465)	0.509±0^{a} (0.569)	0.264±0^{c} (0.294)

Mean values with different alphabetical superscripts were significantly different within a row at $p<0.05$ and figures in the parenthesis represent values on dry weight basis.

TABLE 5
Functional properties of buckwheat flour fractions (%)

		Whole flour	Fine flour	Coarse flour
Water absorption capacity (g/100g)		201.6±1.9^{b}	180.3±4.2^{c}	281.2±1.2^{a}
Water solubility index (g/100g)		8.1±0.06^{b}	2.63±0.06^{c}	14±0.25^{a}
Oil absorption capacity (g/100g)		97.9±0.4^{a}	75.2±0.7^{c}	96.5±0.4^{b}
Foaming capacity (ml/100ml)		6.89±0.75^{bc}	13.22±0.38^{a}	7.43±1.05^{b}
Foaming stability (ml/100ml)	30 min	6.75±0.5^{b}	12.72±0.5^{a}	6.75±0.13^{bc}
	60 min	6.03±0.75^{bc}	11.2±0.63^{a}	7.06±0.39^{b}

Functional properties. The composition of food determines its end use in product formulation. Food macromolecules define and influence the quality parameters of food systems. These can be measured as functional properties such as water absorption, swelling, foaming capacity, emulsifying properties, etc. The starch content of food is responsible for gelatinization, whereas fibre is linked to high water absorption. Similarly, a high protein content aids in emulsification. The functional properties of buckwheat flour samples showed higher water absorption capacity and water solubility index in CF of 281.2±1.2 and 14±0.25 % respectively with a significant difference between different flours (Table 5). This could be possibly explained due to the presence of higher content of dietary fiber which has a greater capacity to hold water. The WF showed higher oil absorption capacity followed by CF, contradictorily FF showed greater foaming capacity. Buckwheat flour also shows good foam stability on setting aside the foam for half an hour to one hour. Overall, it can be observed that buckwheat flours possessed good functional properties and thus can be used for value addition to traditional prepared dishes or for novel food formulations.

CONCLUSION

This research shows the effect of differential sieving on the nutrient distribution in the obtained flour fractions of buckwheat flour. Largely, a significant effect of differential sieving on the composition of buckwheat flour was observed. The FF had the highest protein content and *in vitro* digestible protein as well as fat content while the CF showed least amount. CF was rich in minerals especially Ca and K, and dietary fibre, both total and insoluble. The major fatty acids in flour fractions were oleic, linoleic, and palmitic, each observed in a higher proportion in FF, WF, and CF respectively. The FF was

comparatively a good source of leucine and lysine among essential amino acids. Buckwheat flour fractions also demonstrated high water and oil absorption capacities which could be useful in product formulations. Thus, these data on the compositional profile and functional properties of buckwheat flour fractions would facilitate it's utilization in food formulations.

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Conflict of interests. The authors declare that there is no conflict of interest regarding the publication of this paper.

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ANTIOXIDANT AND ANTICANCER EFFECTS OF PUMPKIN AND LETTUCE SEED OILS ON CHEMICALLY-INDUCED COLON AND LIVER CANCERS IN RATS

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ABSTRACT

Pumpkin (*Cucurbita pepo*) and lettuce (*Lactuca sativa*) seed oilswere prepared previouslyused in the present study.The present study aims to investigate the antioxidant and anticancer activities of pumpkin (P) and lettuce (L) seed oils against chemically-induced colon and liver carcinogenesis in male albino rats using 1,2 dimethyl hydrazine (DMH) and and diethylnitrosamin (DENA) as well establish carcinogenic substances. Administration of DMH and DENA to rats showed significant increased in alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in sera of experimental rats. Significant decreases in sera albumin and tissues antioxidant as glutathione transferase (GSH-T), glutathione peroxidase (GSH-P), glutathione reductase (GSH-R) and superoxide dismutase (SOD) activities were observed in DMH and DENA-induced rat groups. Administration of P and L seed oils with DMH and DENA induced rats exhibited improve in biochemical changes of liver function enzymes. A marked reduction were observed in the levels of ALP, ALT and γ-GT in sera of rat groups firstly received P and L seed oils respectively compared to DMH and DENA control rats groups, indicating protective effects of both seed oils. Significant decreases in the levels of lipid peroxidase (LP) in sera of rats administered P and L seed oils compared to those DMH and DENA control rats. Higher significant decrease in the level of LP was observed in sera of rats administered P seed oil more than those given L seed oil. Anticancer activity was evaluation through determination of CEA and C19.9 in DMH and DENA-induced colon and liver carcinogenic rat groups treated with either P and L seed oils compared versus carcinogenic control rat groups. P and L seed oils showed more effective for inhibiting DMH-induced colon cáncer than that of DENA-induced liver cancer. The present results showed the activity of GSH-R was increased in liver and kidney of rat groups treated with P and L seed oils respectively as compared to those of DMH and DENA control rats. GSH-P activity was also increased in liver and kidney. P and L seed oils modify GSH-T activity in all treated rat groups. P and L seed oils exhibited higher increase of SOD activity in liver and kidney as compared to those of DMH and DENA control rats. The most significant findings of the present study is that the P and L seed oils have shown beneficial effect not only on colon and liver cancer but also on antioxidant defense enzyme activities in DMH and DENA- induced colon and liver carcinogenesis in rats as well as protect cell against DMH and DENA oxidative stress by antagonizing DMH and DENA toxicitys. Results of the present study suggest that both seed oils showed a significant beneficial effects against chemically-induced colon and liver carcinogenesis in rats. These finding suggested that the seed oils (200 mg/kg b.w.) could be apotential compounds used in protective and treatments of colon and liver tumors. According to these observations, the use of P and L seed oils can be recommended as antioxidant and anticancer agents for production of many types of inexpensive seed oils have shown beneficial effects in treatments and combating oxidative damages of colon and liver carcinogenesis.

KEYWORDS:
Pumpkin, Lettuce, Seed oils, Anticancer, Antioxidant, Rat.

INTRODUCTION

Cancer is the major health problem for researchers and medical professionals in most regións of the world and is the leading cause of death in both economically developed and developing countries. Different types of cancer diseases infect and inherent human but the lung, breast, liver and colorectal cancer are being the most common forms [1,2]. Malignant cancers consider the second leading cause of death over the world and the numbers of cases are continuously arising[2]. Colon cancer is the most malignant tumor and the third most common cause of death in many regions of the world with very high morbidity and mortality rates due to unsuccessful treatments using traditional therapy [3,4]. Liver cancer represents a great public health problem as the most common cancer in developed countries [5,6]. Hepatocellular carcinoma is the most malignant tumour of the liver caused by oxidative stress and inflammation and considered the second prominent

cause of cancer-related mortalities [7,8] Diets, nutrition and oxidative stress were found to be the major factors affects in many chronic diseases and human carcinogénesis [9,10,11] through reduction of antioxidant defenses against cancer cells which consider the main factor in development of most cancer types.

Diets included high fruits and vegetables containing some phytochemicals provide cancer chemoprevention[12,13,14]. Consumption of vegetables rich in phytochemicals compounds has become a useful method to reduce the risk in developing of many types of cancer by interfering with cell cycle and inducing apoptosis [15]. Diets rich in vegetables are associated with a lower risk of chronic diseases including cancer [16,17] reported some functional foods containing olive, turnip, flax and cabbage exhibited higher inhibitory activity against the growth of human cancer cell lines[18,19]. Plant derived diets containing phytochemicals used to reduce the risk and inhibit or retard the development of colon cancer [13,15]. Different parts of plant have been common among people and pharmaceutical industry from ancient times, were found to be used in food and medical industries for production of inexpensive drugs with lesser side effects for treatment of most diseases [20,21] they reported the antioxidant are scavengers of free radicals and are modulated during carcinogenesis or after tumor formation [22,23]. Plant-derived extracts can be used as apotential treatment for cancers and was found to induce apoptotic cell death of human colon and hepatoma cells in vitro and in vivo [24,25].Various plant products as polysaccharides, alkaloids, saponins, triterpenes, polyphenols and flavonoids have shown antioxidant and anticancer properties in vitro and in vivo [23,26,27]. Moreover, different plant deivedsubstances are used in structure of new anticancer drugs development[28,29]reported the anticancer drugs from natural products have low cost and exhibited several effective actions of chemotherapy against resistant cancer cells. Many plants and plant-derived compounds possess anticancer properties and have wide applications in cancer therapeutics [14,20,30]. Numerous studies suggest that certain plant materials might be useful as anticancer and chemopreventive agents [31,32].Plant derived anticancer drugs such as paclitaxel, vinblastine, vincristine and colchicine have been aproved effective anticancer drugs and are widely used in clinical practice against most of the cancer types [21,28]. Most of the modern chemotherapeutic and radiotherapeutic agents have been reported to exhibit severe toxicity to normal tissues and resistance of cancer cell, accompanied by undesirable side effect [3]. The continuing need for effective anticancer agents, several researchs concentrates for production of novel anticancer drugs from natural products with potential antitumour activity [9,20,29].Current research concentrates heavily on novel anticancer drug development from natural products particularly the bioactive compounds with potential antitumour and chemopreventive activities [13,31,33].Natural compounds, extracted from plants or animals as natural sources were used in developing novel chemopreventive compounds for cancer therapeutic strategies that could overcome limitations of conventional therapies[21,30]. Natural products are a rich resource of cancer chemotherapy drugs shown antiinflammatory activity [30] and anticancer potential in a variety of bioassay systems and animal models [33]. Many anticancer drugs used in medicine are derived from natural sources of fruits and vegetables involving phenolic, flavonoides and polyphenols as different kinds of antioxidants and free radical scavenging agents [22,24,29].Recently, many natural compounds such as terpenoids, phenolic, flavonoids and lignans were discovered from plant sources as antioxidant substances capable of scavenging free superoxide radicals, protecting biological systems against harmful effects of oxidative processes and play an important role in cancer treatment [33, 34,35].The antioxidant compounds have anti-inflammatory, antitumor and anticarcinogenic activities.[16,36] established the anticancer effect of antioxidants as inhibit cancer cell proliferation, differentiation, induce apoptosis, and interfere in angiogenesis, inflammation and inhibit metastasis [23,34].However, natural compounds with antioxidant activity can target tumor cells after disease occurrence, directly inhibit cell proliferation and prevent tumor recurrence or metástasis [12,24,37]. Plant seeds research increases during the last decade, especially focusing in the field of diet and disease due to its contents of the many chemical ingredients used by ancient peoples as food, feed or medicine (17,37,38). Plant seeds have rich nutritional and nutraceutical components used for protection against some diseases [19,39, 40] reported the consumption of pumpkin andflax seed was associated with potential health benefits such as reduction of cáncer risk and atherosclerosis.Several investigators [12,21,24,41]reported the seeds extract from seeds of different plant family exhibited anticancer and pharmacological effects in vitro, in vivo and in medical trials. Many investigators provided evidence that the consumption of some constituents of plant seed results in treated and protection against chemically induced colon and hepatocellular carcinoma [5,8,42,43]. Among natural products, oils extracted from plant seeds, have antimicrobial, antifungal, antioxidant, antitumor and cytotoxic activities [35,36,44].

Oils are biological mixtures of plant origin consisting of mixtures from glycerol and chain of fatty acids [45] and suitable edible among people used as a food ingredient in variousfood items and consumedindifferent amounts in most diets [17,19]. Plant seed oils can be considered as bioactive molecules in medicine have been demonstrated to have antitumor [36] and chemopreventive effects [14,31].

Many investigators [8,17,33,44] evidence the different seed oils have anticancer properties, nutritional quality and health benifits. Plant seed oils were found to be used in treated and protection against chemically induced colon and hepatocellular carcinoma using rats [8,46,47] reported some plant seed oils containing phytochemicals and antioxidant compounds were beneficial to protect the mucosa against chemical carcinogénesis induced by 1, 2-dimethylhydrazine [42,46]and protect the liver against lipid peroxidation impairment in antioxidant status induced by CCl4 [5,7]. Phytochemicals exhibit antitumor activities through improvement the defences of antioxidant enzymes, remove oxidative stress, followed inhibition of cacinogenesis and direct absorb the reactive oxygen species [15,26,48].Dietary phytochemicals include phenolic compounds, essential oils and polyunsaturated fatty acids are widely distributed in fruits and vegetables (38,49], may contribute to health-promoting effects through powerful antioxidant properties, decrease metastasis, induce apoptosis, and inhibit cell proliferation [24,34,50].Seed oils have antioxidant [23,35] and cytotoxic activities against different diseases [51,52].Seed oils have higher anticancer ingredients, including fatty acids, phenolic and flavonoid as antioxidant compounds [50,53] referred to as prebiotics properties being associated with improved human health with their physiological effects and reduced risk of colon cancer [31]. Plant seed oils are nontoxic and biodegradable that consequently suitable for different pharmaceutical and biomedical uses and play important roles in several physiological and pathological conditions [17,19].Pumpkin (*Cucurbita pepo*), lettuce (*Lactuca sativa*), flaxseed and coriander seed oils have different biological activities in medicine and treatment of prostatic hyperplasia and colorectal carcinogenesis in human[53,54]. Oils were found to be used as antiviral, antibacterial and anticancer [35,55] they found the pumpkin and lettuce seed oils posses antioxidant, antibacterial, antiviral, antimicrobial and cytotoxic activities. Seed oils with their constituents of fatty acids and phytochemicals contents posses various bioactivities including cytotoxicity [51,52],anticancer [8,48]and antidiabetic [53,56]. Seed oils of different plants have been shown the potential health impacts in preventing some diseases and have antiinflammatory [57,58], antiproliferative[23,25], anti-angiogenic [34] and antigenotoxic activities[36]. Recent study has shown that some oil intake cause improve in some biochemical parameters of oxidative stress and exhibited reduce the risk of some diseases [7,42,59].Pumpkin and lettuce seeds are commonly consumed as functional food or in medicine in many regions of the world and consider a good sources of diet health-promoting compounds, contains phytochemical possess biological activities, including antioxidant and anticancer activities [12,40,44]. Pumpkin and lettuceseed oils contains polunsaturated fatty acids, phenol, flavonoids, phytosterols,tocopherols, β-carotene and vitamins that are considered nutraceutical and pharmaceutical properties with different health benefits and appreciated as natural products[17,56,60].In previous studies, we have extracted pumpkin and lettuce seed oils (P and L) with a 34.4% and 30.2 % yields respectively using cold pressed extraction [44].Fatty acid contents of both seed oils (P and L) were analysis and identified using Gas Liquid Chromatography. Analysis of P and L seed oils revealed the presence of higher polyunsaturated fatty acids (92.2% and 90.4% respectively) thanthat of saturated fatty acids (7.8% and 9.6%). Phenolic and flavonoid contents of P seed oil (42.4mg GAE/g and 30.4 mg CE/g) were highest than that of L seed oil (1.8 mg GAE/g and 1.2mg CE/g).Interestingly, the pumpkin and lettuce seed oils have anticancer and cytotoxic activities on various cancer cell lines in vitro [44]. However, the properties of many plants and plant drived compounds particularly it's cytotoxic, anticancer and antioxidant activities have not yet been fully investigated. Thus, the objective of the present study was to evaluate the anticancer and antioxidant effects of pumpkin and lettuce seed oils on chemically induced colon and liver carcinogenesis in vivo using experimental male albino rats.The present results of preclinical trials may be helpful to develop a novel inexpensive anticancer and antioxidant drugs containing bioactive substances as well as some functional foods supplemented with pumpkin and lettuce seeds oil as antioxidant and treatment agents effectively used in the chemotherapeutic treatment of different cáncer types particularly colon and liver cáncers.

MATERIALS AND METHODS

Carcinogenic materials. Diethylnitrosamine (DENA) and 1, 2 dimethylhydrazine dihydrochloride 99+% (DMH) were purchased from Sigma-Aldrich® chemie, Gmbh, Riedstr. 2, D-89555 Steinheim, Germany. All other chemicals used in the present study were of the highest purity and analytical grade were obtained from Sigma Chemical Company (Sigma-Aldrich), Steinheim, Germany.

Pumpkin (*Cucurbita pepo*) and Lettuce (*Lactuca sativa*) seed oils were previously prepared and their different contents particularly, fatty acids, phenol and flavonoid were estimated [44].

Cancer induction. Induction of colon cancer experimentally in rats was done using 1, 2 dimethyl hydrazine (DMH) according to methodsdescribed previously [46,61]. Induction of liver cancer (hepatocellular carcinoma) experimentally in rats was done using diethylnitroseamine (DENA)according to methodsdescribed previously[8, 47].

Animals and experimental design. Seventy male albino rats, 8 weeks of age, weighing about 160±1.1g were purchased from the National Research Center for biological products. The rats had free access to fed commercial diet and tap water. The animal room was controlled (25±1ºC) and had a 12-hour light-dark cycle and humidity at 60±5%. The rats were acclimatized for a period of two week before the experiments began. The rats were randomly divided into ten groups (7rats/group) and distributed into first five groups and second five groups were housed in a wire screen cage. Three groups of rats from the first five groups were administrated for 5 weeks (twice /week) subcutaneous injections of 1,2-dimethyl-hydrazine (DMH) at a dose of 40 mg/kg body weight [46,62]. The first group (C1) was maintained without any treatment over experimental period (16 weeks) and used as colon carcinogenic control group (C1). The other 2 groups of these three groups of rats administrated DMH for 5 weeks (twice/week) were then treated with oral dose (200mg/kg body weight) of Pumpkin (P) and Lettuce (L) seed oils (C1/P group and C1/L group respectively) from week 6 till the end of experimental period (16weeks). Two groups of rat remaining from the first five groups were administrated, from the first week, with oral dose (200mg/kg body wt) for 5 weeks of P and L seed oils (P/C1 group and L/C1 group respectively) and then they were administrated for 5 weeks (twice/week) subcutaneous injections of DMH at a dose of 40 mg/kg body weight and treated with oral dose (200mg/kg body weight) of P and L seed oils (C1/P group and C1/L group respectively) from week 6 till the end of experimental period (16weeks). Three groups of the second five groups of rats were administrated for 6 weeks (five/week) subcutaneous injections of diethylnitrosamine (DENA) at a dose of 20 mg/kg body weight [8,47]. The first group (C2) was maintained without any treatment over experimental period (16 weeks) and used as liver carcinogenic control group (C2). The others 2 groups of these three groups of rats administrated DENA for 6 weeks (five/week) were then treated with daily oral dose (200mg/kg body weight) of P and L seed oils (C2/P group and C2/L group respectively) from week 7 till the end of experimental period (16weeks). Two groups of rat remaining from the second five groups were administrated with daily oral dose (200mg/kg b. w.) for 6 weeks, from the first week, of P and L seed oils (P/C2 group and L/C2 group respectively) and then they were administrated for 6 weeks (five/week) subcutaneous injections of DENA at a dose of 20 mg/kg body weight and treated with daily oral dose (200mg/kg body weight) of P and L seed oils (C2/P group and C2/L group respectively) from week 7 till the end of experimental period (16 weeks).The experimental protocol was done according to [43]. All animals' procedures were performed in accordance with the ethical guidlines and policies approved by the Ethics Committee of NRC (2013).

Samples preparation. At the end of experimental period (16 weeks), blood samples were drawn from 7 rats per each group separately using capillary tubes, centrifuged at 4000xg for 10 min. Separated sera or plasma were stored at-60ºC till used. Liver, kidney and Heart tissues were removed immediately, weighed, washed (using saline 0.9%), minced and homogenized (10% w/v) separately with cold sodium potassium phosphate buffer (0.01 M, pH 7.4) using homogenizer (Mechanika precyzyjna warszawa model MPW-309, Poland). The homogenates were centrifuged at 10,000g for 20 min at 4ºC and the resultant supernatants were stored at -70ºC till used. Stored sera or plasma and tissues homogenates were used for estimation of biochemical parameters and antioxidant enzymes. Colon and liver tissues were removed and used for pathological examinations.

Biochemical parameters. Alkaline phosphatase (ALP) level was carried out referring the DGKC indications, Germany [63]. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured [64] using kits of QCA, Spain. Gamma glutamyl transferase (γ-GT) was carried out according to the kinetic colorimetric method [65] using Biodignostic kits, Egypt.Total protein was estimated [66] using Biodignostic kits, Egypt. Serum albumin level was measured according to the method described previously [67]. Globulin was calculated by subtracting albumin form the total protein [68[. Glutathione transferase (EC 2.5.1.18) activity (GSH-T), Glutathione peroxidase (EC1.11.1.9) activity (GSH-P) and Glutatione reductase (EC1.6.4.2) activities (GSH-R) in plasma and homogenates of liver, kidney, and heart tissues were assessed [69,70,71]. Superoxide dismutase (EC 1.15.1.1) activity (SOD) was measured by the NADH oxidation procedure method[72]. Lipid peroxidase (LP) was also estimated [73]. Determination of CEA was performed with commercially available Enzyme Immunoassay Kit[74]. CA 19.9 was performed with commercially available Enzyme Immunoassay Kit [75].

Histopathology. Histpathological assessments of colon and liver tissues were carried out using Hematoxyline and Eosin (H&E) staining technique[76].

Statistical Analysis. Data from the biochemical analysis was statistically analyzed [77] using student T-test.

RESULTS AND DISCUSSION

Pumpkin (P) and lettuce (L) seeds associated with people from ancient time in food and generally consumed for its nutritive values and medicinal therapeutic properties.P and L seed oils are mainly composed of polyunsaturated fatty acids, phenolic and flavonoids [44].Other investigators [17,39,50]reported the P and L seed oils exhibited higher anticancer activity due to their antioxidants and polyunsaturated fatty acids contents. The health benefits of these oils have been reported in the last decade and their prebiotic effects demonstrate the content of these oils depends on their constituents of polyunsaturated fatty acid and phytochemicals [17,40,49,60]. In vitro cytotoxicity test revealed that the P and L seed oils exhibited anticancer activity against different cancer cell lines due to different percentages of phytochemical and polyunsaturated fatty acid contents [44,49,50,78] they found the phytochemical, phenolic and flavonoid containing oils reduce the risk and inhibit or retard the development of carcinogenic activities. P and L seed oils inhibits cell proliferation of colon (HCT-116) and liver (HEPG2) human cancer cell lines that could arrest the cell cycle and generate apoptosis, which explains the in vitro anti-proliferative effects [44,79,80]. Moreover, anti-proliferative effects of differemt seed oils against different human cáncer cells were reported by several investigators [16,34,62].

Cancer induction. Antioxidant and anticanceractivities of P and L seed oils used in the present study were done on chemically induced colon and liver cáncer in vivo using male albino rats. The chemical carcinogenic commonly used is 1, 2-dimethyl-hydrazine (DMH) which is well-established for inducing colon cáncer in vivo through initiation, promotion and progression [32,46,62].1,2-Dimethyl hydrazine (DMH) was used as a potent and complete carcinogen for the colon, since it has been reliably used to induce the initiation and promotion steps of colon carcinogenesis after five doses over 5 successive weeks (twice/week). Rats is expected to aberrant crypt foci formation in DMH induced colon carcinogénesis [81] reported the increased number of preneoplastic neoplasm formation may be attributed to the metabolic dysfunction and prominent cell proliferation [62].The colon cancer in the present study was induced by intraperitoneally injection of 1, 2-dimethyl-hydrazine (DMH) at a dose of 40 mg/kg body weight twice a week for 5 weeks[43]. DMH was used as a potent and complete carcinogen for the colon, since it has been reliably used to induce the initiation and promotion steps of colon carcinogenesis after five doses (twice/week) over 5 successive weeks [43,46,62] they reported the DMH has been reliably used to induce the initiation, promotion and progression of colon carcinogenesis. Many investigators [24,4282] found pre-neoplastic lesions, oxidative stress, biotransforming enzymes and histopathological alterations in DMH-induced rat colon carcinogenesis. Other researchers [32,83] reported intraperitoneally DMH administered rats showed aberrant crypt foci initiation and adenoma development indicated the progression of colon cancer[62].DMH was specifically targets the colon of rats[81,84], where it induces DNA damage, preneoplastic lesions and is detected as aberrant crypts and tumours. Previous studies indicated that the adminstration periods for 8 or 12 weeks (twice/week) intraperitoneally injection of DMH treatment leads to the development of colon carcinoma [46,81].Adminstration periods for 16 weeks (twice/week) intraperitoneally injection of DMH treatment led to the development of colon carcinoma[31,84]. Moreover, intraperitoneally injection of DMH weekly once for 20 weeks (20mg/kg) leads to the development of colon carcinoma[62].

Diethylnitrosamine (DENA) as carcinogenic substance commonly used for inducing hepatocarcinogenic in vivo [85,86].Intraperitoneally injection with DENA (20 mg/kg) five times weekly for 6 consecutive weeks induce liver cancer [47].Rats are widely used as experimental models to study DENA-induced hepatocarcinogenesis [47,86,87].The liver cáncer (hepatocarcinogenic cáncer) in the present study was induced by intraperitoneally injection of DENA at a dose of 20 mg/kg body weight five a week for 6 weeks. DENA was used as carcinogen for the hepatocarcinogenic, since it has been reliably used to induce hepatocarcinogenic after 6 doses (five/week) over 6 successive weeks [8,47]. DENA administered intraperitoneally injection in rats will metabolized by liver to generate reactive oxygen species causing oxidative stress and liver injury [81]. DENA dose (20 ml/kg body weight) five a week for 6 weeks administered to rats was found an optimal for inducing toxicity,free radicals and hepatocellular carcinoma [47,88].P oil isolated from pumpkin seed has appreciable anticancer activity greater than that of L oil isolated from lettuce seed against HCT-116 and HEPG2 carcinoma cell lines [44]. Oral administration of P andL seed oils at a dose of 200 mg/kg did not produce any signs of toxicity and no animals were ill or died, indicate the P andL seed oils were nontoxic in rat up to an oral dose of 200 mg/kg. Therefore, investigation of antioxidant andanticancer activities were carried out using daily dose levels of 200 mg/kg body weight for a period of 16 weeks. However, administration of pumpkin and lettuce products to human is simple, since they are used as common dietary constituents in many regions of the world.

Biochemical parameters. Biochemical parameters were altered in DMH and DENA-induced colon and liver cáncer rats groups (C1and C2). Results represented the potential effects of P andL seed

oils on the levels of liver marker enzymes (ALP, ALT, AST and γ-GT) and total protein, albumin and globulin levels in sera of carcinogenic (DMH and DENA) and treated rat groups (Tables 1, 2).Reductions in serum total protein, albumin and globulin levels were observed in DMHand DENA-induced colon and liver cáncer rats (C1and C2) as shown in Tables 1, 2. However, the increases in ALP, ALT, AST and γGT levels in rat sera were reported in cancer due to liver dysfunction [43,82,89].

Serum transaminases (ALP, ALT, AST) are considered to be sensitive indicators of liver injury in DMH and DENA-induced cancer rats where the liver was necrotized [43,47,90]. Results showed the ALP, ALT and AST levels were elevated significantly accompanied with significant decrease in albumin concentration in DMH carcinogenic rats (C1 group) as shown in Table 1. These findings attributed to DMH induced colon cáncer that leading to malfunction of the liver [62,91] obtained significant elevations in the levels of ALP, ALT and AST in será of DMH-induced rats. Similar results obtained by other investigators [86,92] found significant elevations in the levels of serum ALP, ALT, AST and γGT in liver diseases and disorders in hepatocellular damage caused by a number of agents including cáncer [89,93].Result also revealed increases in the levels of sera ALP, ALT, AST and γGTof DENA rats (C2) as shown in Table 2. These results are in agreement with those reported by many investigators [82,94] reported significant elevations in the levels of sera ALP, ALT and AST in rat liver diseases. Data in the present study showed that the DENA administration to rats increased the levels of sera liver function enzymes that considered most sensitive markers in diagnosis of toxicity and hepatocellular damage [85,95]. ALP has been markedly increased by action of DENA administration causing hepatic dysfunction and reflect hepatocellular injury [85,88]. ALP levels were elevated due to defective hepatic excretion or increased production of ALP by hepatic parenchymal cells [43,95]. An increase in the ALT and AST levels in plasma might be mainly due to the leakage of these enzymes from the liver into the blood stream which gives an indication of the hepatotoxic effects [86,96]. These results are in consistentwith other previous studies [84,95,97] reported the hepatic damage was indicated by marked elevation of ALP, ALT and AST levels. Liverdamage induced by chronic treatment leads to liver cell necrosis and consequentlyelevated levels of serum transaminases [86,90,97].Treatment of DMH carcinogenic rats with P orL seed oils reduced the activities of ALP and ALT in plasma and consequently alleviated liver damage caused by DMH-induced colon cáncer.The value of ALT and AST activities in sera of rats received P and L seed oils reflected their improvement of liver function enzymes.Results in Table (1) showed significant decreases in the levels of ALP

TABLE 1
Biochemical parameters in sera of experimental rats (first fiverat groups).

Parameters	C1	C1/P	C1/L	P/C1	L/C1
Total protein (g/dl)	5.06±0.10	8.40±0.38	6.96±0.10	8.10 ±0.24	7.26±0.20
Albumin (g/dl)	4.14±0.10	6.08±0.20	5.28±0.10	5.90±0.20	5.44±0.40
Globulin g/dl	0.92±0.08	2.32±0.20	1.68±0.10	2.20±0.20	1.82±0.10
Alkaline phosphatase (IU/L)	240.1±4.14	126.45±2.97	152.04±3.30	118.20±3.9	132.10±3.80
ALT(U/ml)	40.4±1.20	20.3±1.40	32.40±1.80	16.3±0.92	20.4±0.80
AST(U/ml)	46.40±2.90	40.20±2.40	42.94±2.80	28.80±2.10	36.04±2.60
γ-GT (U/L)	140.4±2.86	54.40±1.90	82.02±1.68	44.8±0.44	66.2±0.62
Lipid peroxide (nmol/ml)	4.10±0.60	3.46±0.40	2.66±0.30	3.02±0.10	2.24±0.2

Data was presented as mean value ± SE of 7 rats / group. P<0.05 versus control

TABLE 2
Biochemical parameters in sera of experimental rat (second five rat groups).

Parameters	C2	C2/P	C2/L	P/C2	L/C2
Total protein (g/dl)	3.82±0.10	6.56±0.16	5.20±0.10	6.98 ±0.14	5.82±0.10
Albumin (g/dl)	2.04±0.06	4.18±0.08	3.48±0.04	4.62±0.10	3.94±0.08
Globulin g/dl	1.78±0.08	2.38±0.08	1.72±0.06	2.36±0.06	1.88±0.04
Alkaline phosphatase (IU/L)	270.1±4.64	146.44±2.97	186.04±3.30	112.10±2.95	142.2±3.40
ALT(U/ml)	66.4±1.97	28.3±1.20	42.52±1.90	22.40±0.90	30.40±0.44
AST(U/ml)	54.62±2.80	38.60±1.90	40.64±1.02	24.94±0.94	32.04±1.02
γ-GT (U/L)	180.40±3.20	58.22±1.64	96.14±1.84	52.8±0.92	76.4±1.04
Lipid peroxide (nmol/ml)	6.20±0.36	3.60±0.20	4.80±0.26	3.02±0.16	3.90±0.22

Data was presented as mean value ± SE of 7 rats / group. P<0.05 versus control

(47 % and 37%), ALT (50% and 20%) and AST (18% and 12%) in sera of rat groups (C1/P and C1/L respectively) treated with P and L seed oils as compared to those of rat group C1. A marked reduction were observed in the levels of ALP (51% and 45%) and ALT (60% and 50%), in sera of rat groups (P/C1 and L/C1) respectively compared to rat group C1. These results are in accordance with those reported by other investigators [27,92]. Administration of P and L seed oils (200 mg/kg) showed different changes in biochemical parameters of DENA-induced hepatic cáncer rats (C2) as shown in Table (2). Results showed significant decreases in the levels of ALP (46 % and 31%), ALT (58% and 52%) and AST (29% and 26%) in sera of treated rat groups (C2/P and C2/L) with P and L seed oils respectively as compared to those of DENA rats (C2). The administration of P and L seed oils showed significant decreases in serum AST and ALT activities as compared to the DENA hepatotoxic rats (C2). These findings are closely related to the evidence of Jayakumar et al. [88]. A marked reduction were observed in the levels of ALP (58% and 47%), ALT (66% and 54%) and AST (54% and 41%), in sera of rat groups (P/C2 and L/C2) given P and L seed oils respectively as compared to DENA rats (C2). These results are in accordance with those reported by other investigators [86,98] reported the seed oils exhibited hepatoprotective effect against DENA carcinogenesis. A significant decreases were observed in the levels of serum ALP and ALT by action of P and L seed oils compared to hepatotoxic DENA rats (C2) revealed the improving and protective effects of P and L seed oils on rat liver.These results are in agreement with those repoted by other investigators used different seed oils [87,89,97]. The reduction in the levels of these parameters were observed in rat groups received P and L seed oils indication of the stabilities of plasma membranes and repair of hepatic tissue damage caused by DMH and DENA carcinogens.

Results also showed the hepatic marker enzyme γGT was significant elevated in será of rats group adminstered DMH and DENA rat groups (C1 and C2), indicating damage of the liver cell membrane and other changes as a result of DMH and DENA carcinogenesis [43,86,89,97] reported the elevation in the levels of γGT in sera of rats cause damage liver cell membrane followed liberation of γGT from plasma membrane into the circulation of the hepatic cells as a result of carcinogenesis [87]. However, the levels of γGT and ALT in será of rats have been used in diagnosis of primary liver cancer [95,96].The present results showed higher reduction in the levels of γ-GT (62% and42%) in sera of rat groups (C1/P and C1/L) given P and L seed oils respectively as compared to DMH rat group C1. Higher reduction in the levels of γ-GT (68% and 52%) was observed in sera of rat groups given P seed oil (P/C1) and L seed oil (L/C1) respectively as compared to C1 rat group (Table 1). These results are in agreement to those obtained by other investigators [84,95,96] reported the ALP, ALT, AST and γ-GT are reliable markers of liver function. The results also showed higher reduction in the levels of γ-GT (67% and47%) in sera of rat groups treated with P and L seed oils (C2/P and C2/L) respectively as compared to DENA rats (C2). Highest reduction in the levels of γ-GT (71% and 58%) was observed in sera of rat groups given P (P/C2) and L (L/C2) seed oil respectively as compared to DENA rats (C2) as shown in Table (2).These results are in accordance to those obtained by other studies [88,99] suggesting that the seed oils have potential protective effect against DENA-induced liver cancer and may be improvement and repair the liver damege of DENA injury. P and L seed oils could be prevent the increase of hepatic enzymes, especially in rat groups received P and L seed oils before DENA adminstration (P/C2 and L/C2 groups). Other investigators [8,78,91] studied the hepatoprotective effects of induced liver damage in rats. However, the degree of protection was evaluated by determining the marker enzymes (ALP, ALT and AST) and total proteins.

In the present study, higher decreases in the levels of ALP, ALT and AST activities accompanied with significant increases in albumin concentration were observed in rat groups received P and L seed oils before administered DMH and DENA than those of rats treated with P and L seed oilsafter DMH and DENA.Administration of P and L seed oils (200 mg/kg) showed higher significant increases in the levels of total protein, albumin and globulin in sera of rats group administered P seed oil (C1/P and P/C1) and L seed oil (C1/L and L/C1) compared to DMH control group C1 (Table 1).These results are consistent to other studies [17,39,86] reported the seed oils administred to DMH-induced rats, showed significant reductions in serum protein, albumin and globulin due to inhibition of protein degradation [80]. Significant reductions in serum total protein, albumin and globulin levels were observed in DENA-induced liver cancer rats group (C2) as shown in Table (2).These results are in agreement to those reported by other workers [32,80] reported reduction in albumin level resulting from liver disorders and decrease in albumin synthesis due to the highly toxic effect of carcinogens leads to formation of free radicals damaging proteins [95]. Higher significant increases were observed in the levels of total protein, albumin and globulin in sera of rats group administered P seed oil (P/C2 and C2/P groups) and L seed oil (L/C2 and C2/L groups) compared to C2 control rats group (Table 2).These results are similar to those reported by other investigators [17,39,80]. Other researchers [32,62] showed the reduction in albumin level resulting from liver disorder and decrease in albumin synthesis due to the highly toxic effect of carcinogens leads to formation of free radicals damaging proteins [43,95].Results showed

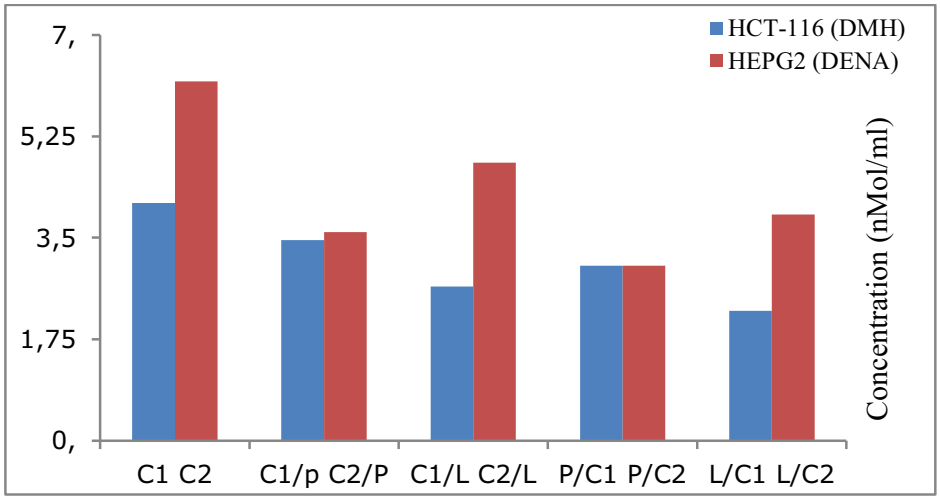


FIGURE 1
Lipid peroxide (LP) levels in sera of experimenta rats.

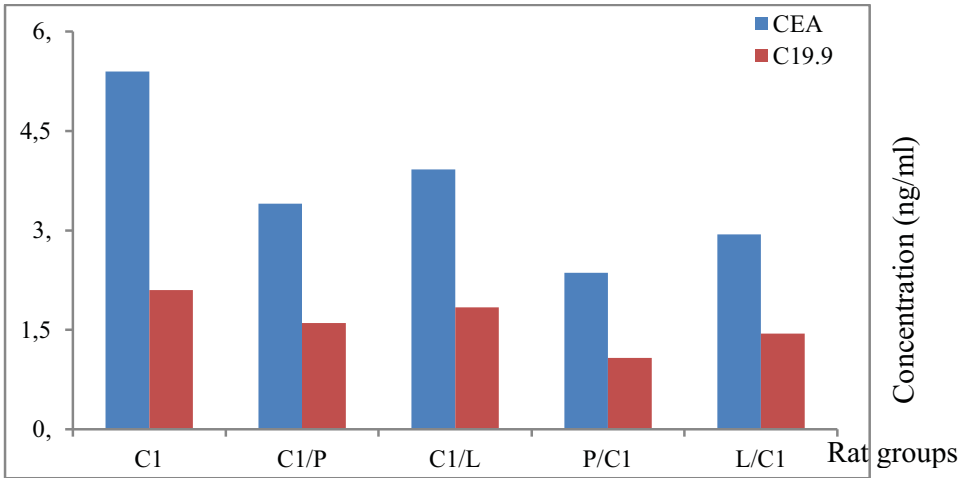


FIGURE 2
CEA and C19.9 levels of experimental rat groups.

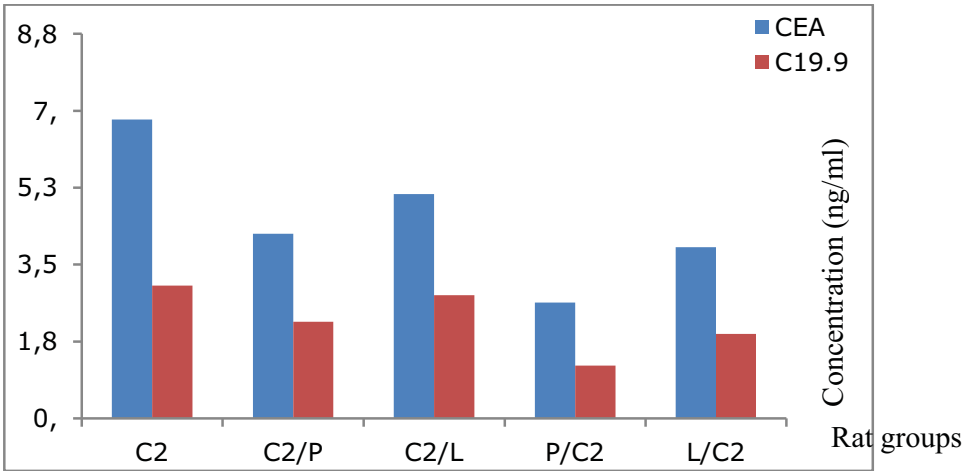
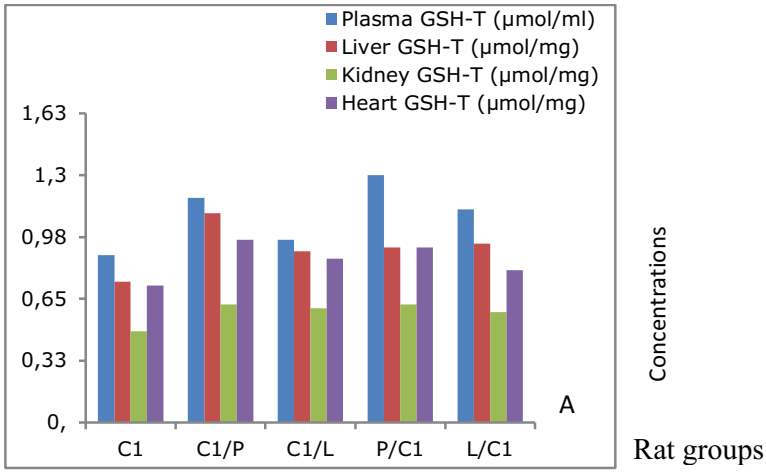


FIGURE 3
CEA and C19.9 levels of experimental rat groups.

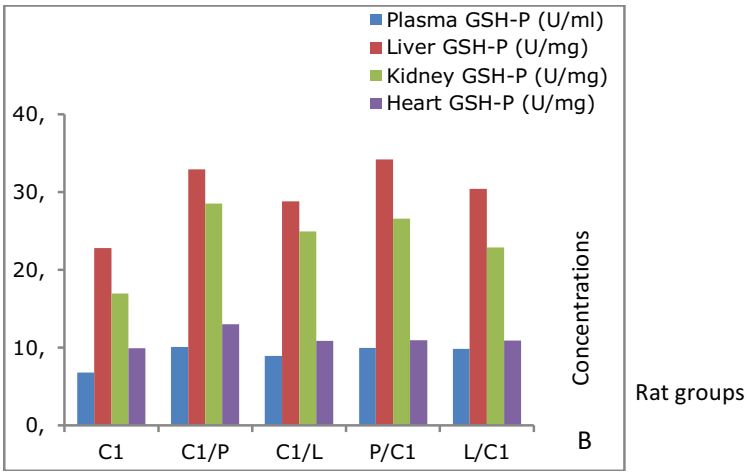
higher levels of LP (4.10±0.60 and 6.20±0.36 respectively) in both carcinogens DMH and DENA rat groups (C1 and C2)as shown in Tables 1,2 [47,62]. These results are in agreement with other investigators [46,87]reported DMH and DENA effects of lipid peroxidation.Significant decreases were observed in the levels of LP in sera of rats administered P and L seed oils compared to those administered DMH and DENA control rat groups (C1and C2). The highest significant decreases in the levels of LP were observed in sera of rats administered L seed oil (C1/L and L/C1 groups) more than those of received P seed oil (C1/P and P/C1 groups) compared to DMH rats group (C1) as shown in Figure (1).These results are in accordance with those reported by other investigators [26,62,98] indicated the decreasedlipid peroxides by cellular accumulation resulting from DMH oxidative stress. A marked significant decreases in the levels of LP were observed in sera of rats administered L seed oil (C2/Land L/C2 groups) more than those of P seed oil (C2/Pand P/C2groups) compared to DENA rats group (C2) as shown in Figure (3). These results are in agreements with those reported by many investigators [81,98,100] reported reduction in reactive oxygen species of DENA that initiates lipid peroxidation and produce lipoperoxides [101].P and L seed oils have antioxidants scavenging free radicals and suppressed lipid peroxidation that protects cells against effect of oxidative stress [102]. Decrease in the levels of LP activityagainst DMH and DENA toxicity may be due to the P and L seed oils antioxidant prevent the formation oflipid peroxidation[9,96,98].

Regarding CEA and CA 19.9 activities the present results revealed significant increases in the levels of CEA and CA 19.9 in será of DMH and DENA rat groups (C1 and C2). These results are in agreements with those reported by other studies [27,103]found the increase in the level of CEA and C19.9 used as a tumor marker for colon and liver cáncer. Abd el Monem et al. [84] and Han et al. (2008) [104]indicated that the CEA and CA19.9 tumor markers are signaling in the promotion, progression and development of cancer. Moreover, CEA and CA-19.9 have many biological aspects as adhesion, metastasis and apoptosis [27, 105].The present data showed significant decrease in the level of CEA and CA19.9 in será of rats treated with P and L seed oils (C1/ P and C1/L) and(C2/ P and C2/L) as compared to group C1 and C2 (Figures 2, 3). A marked reduction in the level of CEA and CA-19.9 in será of rats groups treated with P and L seed oils before induction of colon DMH (P/C1 and L/C1) and liver DENA (P/C2 and L/C2) more than that decreases shown in será of rats groups received P and L seed oils after induction of carcinogenic materials, DMH (C1/P and C1/L) and DENA (C2/P and C2/L). Moreover, all rat groups received P and L seed oils brfore and after DMH and DENA tumors induction are markedly decreased as compared to carcinogenic (DMH and DENA) control rat groups (C1 and C2), indicating the protective and treatment roles of P and L seed oils against chemically induced colon and liver cáncer [46,47].

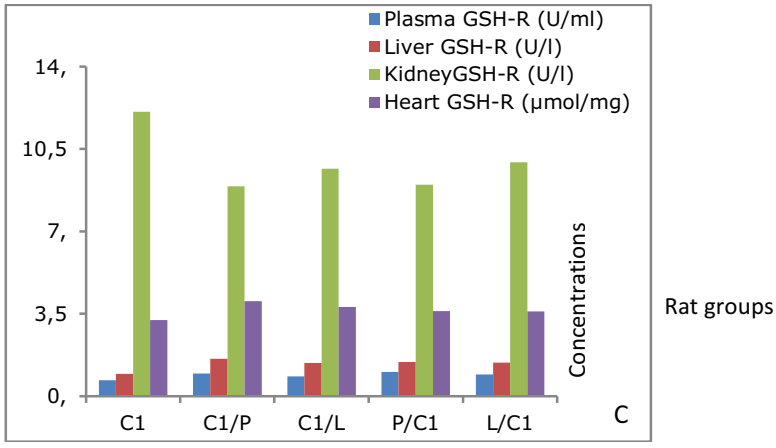
Antioxidant enzymes activities. The present study, focused on investigating the anticancer and antioxidant effects of P and L seed oils againstcolonandliver carcinogénesis of DMH and DENA using experimental rats. Subcutaneous administration of these carcinogéns undergoes metabolic activation in the liver to form different metabolic intermediates and these carcinogens were conversion into DNA reactive metabolites involves the activation and detoxification [81,83,98]. Oxidative stress was involved in the process of tumour development of both DMH and DENA carcinogénesis [82,86]. Oxidation phenomena have been implicated in many illnesses, such as diabetes mellitus, arteriosclerosis and cáncer. Oxidation of DNA, proteins and lipids plays an important role in a wide range of common diseases, including cardiovascular, inflammatory and cancer [43,100].Other investigators [7,98] reported fatty acids of cell membrane is oxidized by reactive oxygen species initiates lipid peroxidation that produces free radicals, toxic substances and lipoperoxides which induces cell proliferation and contributes to cáncer [86,101]. GSH-T, GSH-P, GSH-R and SOD enzymes consider natural defenses antioxidant scavenger free radicals and protect cells against oxidative stress.DMH-induced colon cancer showed significantly decreased in the activities of GSH-T, GSH-P, GSH-R and SOD in plasma and tissue homogenates of liver, kidney and heart of rat group (C1) as shown in Figure (6). Similar results were reported in the enhancement effect of DMH (32,46,82]. The decreased activities of GSH-T, GSH-P, GSH-R and SOD could be due to the dangerous increases in the level of free radical and detoxification of toxic DMH metabolites by tumor cells. These results are in accordance with those reported by other investigators [26,62,98] indicated the DMH oxidative stress by cellular accumulation



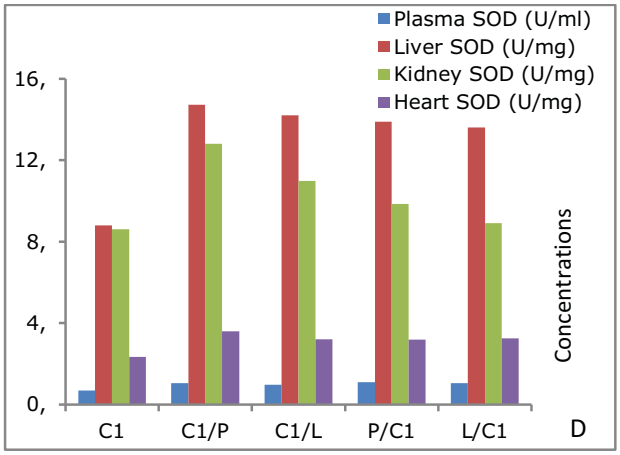
GSH-T levels in plasma, liver, kidney and heart of experimental rat groups.



GSH-P levels in plasma, liver, kidney and heart of experimental rat groups.



GSH-R levels in plasma, liver, kidney and heart of experimental rat groups.



SOD levels in plasma, liver, kidney and heart of experimenal rat groups.

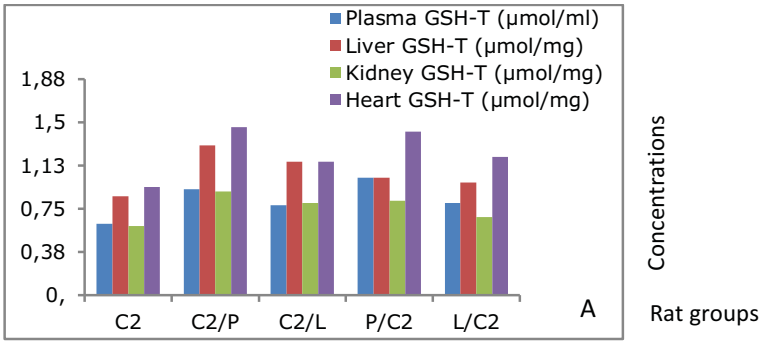
FIGURE 4

Antioxidant enzymes activities in plasma and homogenates of liver, kidney and heart of experimental rat group induced-DMH and treated with P and L seed oils. (Mean value± SE of 7 rats/group).

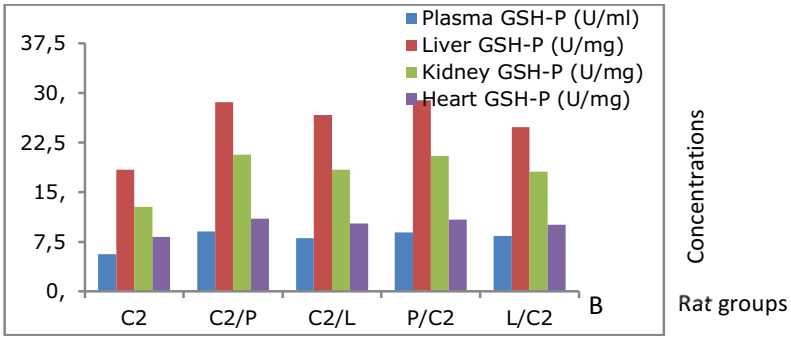
of lipid peroxides leading to decline in GSH-P levels.DENA-induced rats showed significant decreases in the levels of GSH-T, GSH-P, GSH-R and SOD in plasma and tissue homogenates of liver, kidney and heart of DENA rats group C2 (Figure 7). These results are in accordance with those reported by many investigators [8,47,86].The decreased activities of GSH-T, GSH-P, GSH-R and SOD in DENA-treated rats may be due to the enhanced lipid peroxidation or inactivation of the antioxidant enzymes. Similar results were reported by other investigators [7,86].

In the present study, the DMH and DENA carcinogenic substances exhibited nearly similar effects and different percentages of decreases in the activities of GSH-T, GSH-P, GSH-R and SOD levels in liver, kidney and heart tissues of the experimental rat groups (C1and C2). These findings are in accordance with the the findings of other investigators [24,86,101] reported that such subsequent decreases in the antioxidant defense is due to the decreased expression of these antioxidants during hepatocellular damage.The present results showed the activity of GSH-T was significant increases (Figure 6A) in plasma (34 and 9%), liver (49 and 22 %), kidney (29 and 25%) and heart (33and 19%) of rat treated with P and L seed oils (C1/P and C1/L) as compared with DMH rats (C1).GSH-P was significant increases (Figure 6B) in plasma (49 and 31%), liver (44 and 26%), kidney (68 and 47%) and heart (31and 9%) of rat treated with P and L seed oils (C1/P and C1/L) as compared with DMH rats (C1). GSH-R was significant increases (Figure 6C) in plasma (44 and 24%), liver (67and 48%), kidney (26 and 20%) and heart (26 and17%) of rat treated with P and L seed oils (C1/P and C1/L) as compared with DMH rats (C1). On the other hand the activity of GSH-T was significant increases (Figure 7A) in plasma (48 and 26%), liver (51 and 35 %), kidney (50 and 33%) and heart (55 and 23%) of rat treated with P and L seed oils (C2/P and C2/L) as compared with DENA rats (C2). GSH-P was significant increases (Figure 7B) in plasma (63 and 43%), liver (56 and 45%), kidney (61 and 44%) and heart (33and24%) of rat treated with P and L seed oil (C2/P and C2/L) as compared with DENA rats (C2). GSH-R was significant increases (Figure 7C) in plasma (43 and 32%), liver (71and 54%), kidney (26and 17%) and heart (53 and 42%) of rat treated with P amd L seed oils (C2/P and C2/L) as compared with DENA rats (C2). GSH-T is important antioxidant involved of cellular detoxification of endogenous and exogenous compounds and protects cells against effect of oxidative stress by scavenging free radicals and suppressed lipid peroxidation [87,102].GSH-P reduce hydrogen peroxides and protect the cell from peroxidative damage from free radical [25,81]. P and L seed oils improve the levels of antioxidant to exert their scavenging mechanisms and exhibiting their inhibitory effects against colon and liver carcinogenesis.Several investigators [23,31,33] reported the seed oils are provide protection against earlier stages of colon carcinogenesis in rats. P and L seed oils played an important role as a protective factor for DENA-induced toxicityfree radicals[12,50]. Similar results were obtained by other investigators used different seed oils [47,88,101].Moreover, the inhibition of peroxidation by seed oils is mainly attributed to the scavenging of the reactive free radical involved in the peroxidation and disturbing the antioxidant leading to oxidative stress and carcinogénesis [22,88].

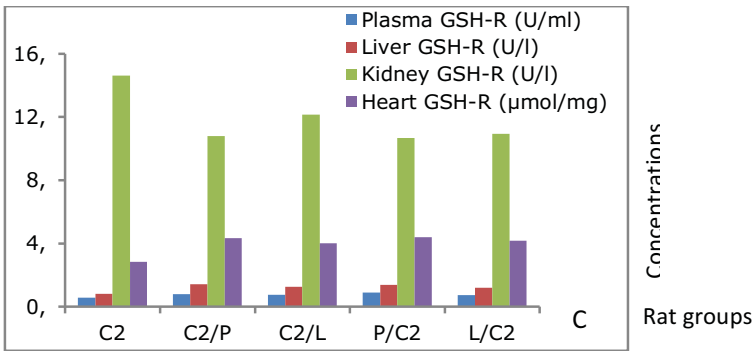
Rats received P and L seed oils (P/C1 and L/C1) showed increase in the activities of GSH-P (47% and 44%) and GSH-R (53% and 36%) in



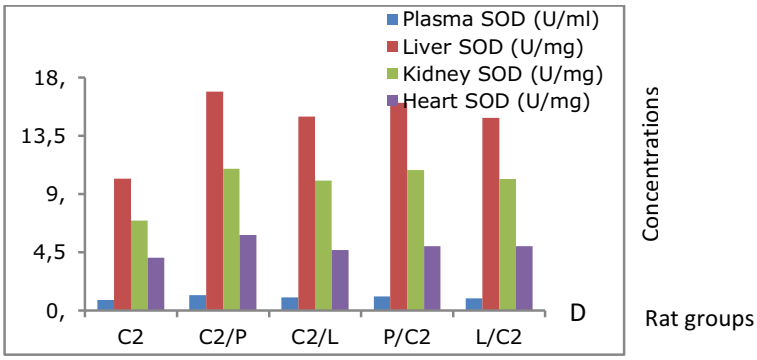
GSH-T levels in plasma, liver, kidney and heart of experimental rat groups.



GSH-P levels in plasma, liver, kidney and heart of experimental rat groups.



GSH-R levels in plasma, liver, kidney and heart of experimental rat groups.



SOD levels in plasma, liver, kidney and heart of experimental rat groups.

FIGURE 5

Antioxidant enzymes activities in plasma and homogenates of liver, kidney and heart of experimental rat group induced-DENA and treated with P and L seed oils. (Mean value± SE of 7 rats/group).

plasma (Figure 6 B, C).GSH-P (20% and 10%) and GSH-R (12% and 10%) in heart (Figures 6 B,C).GSH-R activity was increased in liver (52% and50%) and kidney (26% and18%) in rat groups (P/C1 and L/C1 respectively) compared to those of C1 rats (Figure 6C). GSH-T activity was also increased (Figure 6A) in liver (24% and 27%) and kidney (29% and 21%). GSH-P activity was also increased (Figure 6B) in liver (50% and 33 %) and kidney (57% and 35 %).Rats received P and L seed oils (P/C2 and L/C2) showed increase in the activities of GSH-P (60% and 50%) and GSH-R (61% and 29%) in plasma (Figure 7 B,C), Rats received P and L seed oils (P/C2 and L/C2) showed increase in the activities of GSH-P (31% and 22%) and GSH-R (55% and 47%) in heart (Figure 7B,C).GSH-R activity was increased in liver (65% and46%) and kidney (25% and 26%) in rat groups (P/C2 and L/C2 respectively) compared to those of C2 rats (Figure 7C). GSH-T activity was also increased (Figure 6) in liver (19% and 14 %) and kidney (37% and 13%).GSH-P activity was also increased (Figure 7B) in liver (57% and 35%) and kidney (60% and 41 %). GSH-P isresponsible for most of the decomposition of lipid peroxidation in cells and may thus protect the cell from the deleterious effects of peroxidation. Data in the present study showed the activities of GSH-P and GSH-R were significant increases in liver and kidney of rat groups treated with P and L seed oils (C1/P and C1/L) and (C2/P and C2/L) as compared to DMH and DENA-induced rat groups (C1 and C2) respectively. GSH-P has a high potency in scavenging reactive free radicals in response to oxidative stress and detoxifies peroxides [8,9,47]. P and L seed oils contains antioxidant compounds, makes its an effective antioxidant against DMH and DENA induced free radical generation. These results are in agreements with those reported by other inestigators [22,32,106] showed the constituents of seed oils of polyunsaturated fatty acid andphytochemical contents inhibits the process of carcinogenesis effectively and prevent the development of cancer in vitro and in vivo.In the present investigation, higher GSH-P and GSH-R activities in liver and kidney were observed in rat groups given seed oils (C1/P and C1/L) and (C2/P and C2/L) as compared to those of C1 and C2 rat groups. In heart tissue, however, enhanced lipid peroxidation in rats may be due to lower GSH-P and GSH-R activities as rat group C1 (Figures 6 B, C) and C2 (Figures 7 B, C). The P and L seed oils did not modify GSH-T activity in plasma or liver of the rats.Decrease in the levels of GSH-P and GSH-R activities during DMH and DENA toxicity might be due to antioxidant enzymes resulted during the enhanced oxidative stress and lipid peroxidation[9,23]. This oxidative stress is reduced by action of P and L seed oils leading to a marked increase in the activity of GSH-P compared to rats administered DMH and DENA (C1 and C2 groups) and helping to maintain liver cell integrity and control the level of liver enzymes [8,47,88]. These results are in agreement with other investigators [87,96,99] studied the effects of DMH, DENA and CCl4 on lipid peroxidation and antioxidant enzyme activities of GSH-P, GSH-R and SOD.In the present study, the results showed that, GSH-R concentrations in the liver tissue was significantly higher in rats treated with the P seed oil (C1/P and C2/P) and L seed oil (C1/L and C2/L) than in rats received the carcinogenic material DMH (C1) and DENA (C2) respectively. These antioxidant activities were increased on adminstrations of P and L seed oils, which may be due to the free radical scavenging property of oils and consequently decreased utilization of the antioxidant enzymes.Free radical scavenging and anticarcinogenic properties of P and L seed oil has been associated with their bioactive compound contents[12,62]. Similar results were obtained by other investigators [39,49,50]finding the pumpkin (P) and lettuce (L) seed oils have antioxidant and anticancer activity against colon and liver cancer due to higher polyunsaturated fatty acid contents protect colon and liver from cancer. Results of the present study indicated that P and L seed oils tend to improve the GSH-R concentrations in the rat tissues. These results are in agreement with those reported by other investigators [7,26,65]. The present results showed the activities of GSH-P and GSH-R were significant increases in liver and kidney of rat treated with P and L seed oils compared to DMH-induced colon and DENA- induced liver cancer rat groups (C1 and C2 groups). These results are in the same line with earlier investigation reported by many researchers[8,88,98]. Moreover, the primary radicals, by donating hydrogen radicals, are reduced to non-radical chemical compounds and this action helps in protecting the body from degenerative diseases [62,84,96]. Recent studies on the antioxidant properties of some plant materials revealed their stimulatory action on antioxidative enzymes [32,50,106]reported that the natural products induced significant increases in GSH-P and GSH-R activities and exerted a protective and antioxidant effect. Other studies demonstrate decreases in GSH-P activity and alterations in liver antioxidants in rats [98,107,108]. Results obtained from the present study are very much promising and similar to the observation reported in streptozotocin induced diabetic rats[43,56,100]. In the present study, combined treatment of DMH and DENA separately with each of P or L seed oils resulted in maintained the activities of SOD and GSH-dependent antioxidant enzymes, when compared to C1 and C2 control rat groups, indicating the protective role of P or L seed oils against DMH and DENA-induced oxidative stresses. The chief characteristics of this natural product are that it is rich in poluynsaturated fatty acids, phenolic and flavonoid copmounds exhibits relatively high antioxidant activity.

In the present study the activity of SOD in liver, kidney and heart was investigated. SOD, one of the major antioxidant enzymes, decomposes superoxide peroxide, blocks lipid peroxidation and protects the tissue against oxidative damage [22,83,109].The present results show that the rats received P and L seed oils (C1/Pand C1/L) exhibited higher SOD activity in liver (67% and 61%) and kidney (49% and 16%) respectively as compared to those of C1 (Figure 6 D). Rats received P and L seed oils (P/C1 and L/C1) showed increase in the activities of SOD in liver (58% and 55%) and kidney (49% and 28%) respectively as compared to those of rat group C1 (Figure 6 D).The present results show that the rats received P and L seed oils (C2/P and C2/L) exhibited higher SOD activity in liver (66% and 47%) and kidney (56% and 45%) respectively as compared to those of rats C2 (Figure 7D). Rats received P and L seed oils (P/C2 and L/C2) showed increase in the activities of SOD in liver (58% and 46%) and kidney (56% and 47%) respectively as compared to those of rat group C2 (Figure 7D).Generally, free radicals are produced in the body as the result of metabolic processes. The imbalance between radical-generating and radical scavenging systems produce oxidative stress [12,42,59]. Free radicals are the source of lipid peroxidation derived from oxygen and SOD is the first line of defense [22,47,62]. SOD consider the first line of defense against free radicals derived from oxygen and lipid peroxidation [22,96] shows that the antioxidant substances which scavenge free radicals play an important role in the prevention of free radical-induced diseases.The principal agents responsible for the protective effects could be the presence of antioxidant substances that exhibit their effects as free radical scavengers [12,50,59].A study shows that antioxidant substances which scavenge free radicals play an important role in the prevention of free radical-induced diseases [22,32]. P and L seed oils increases the activity of SOD and it scavenges superoxide radicals and reduces myocardial damage caused

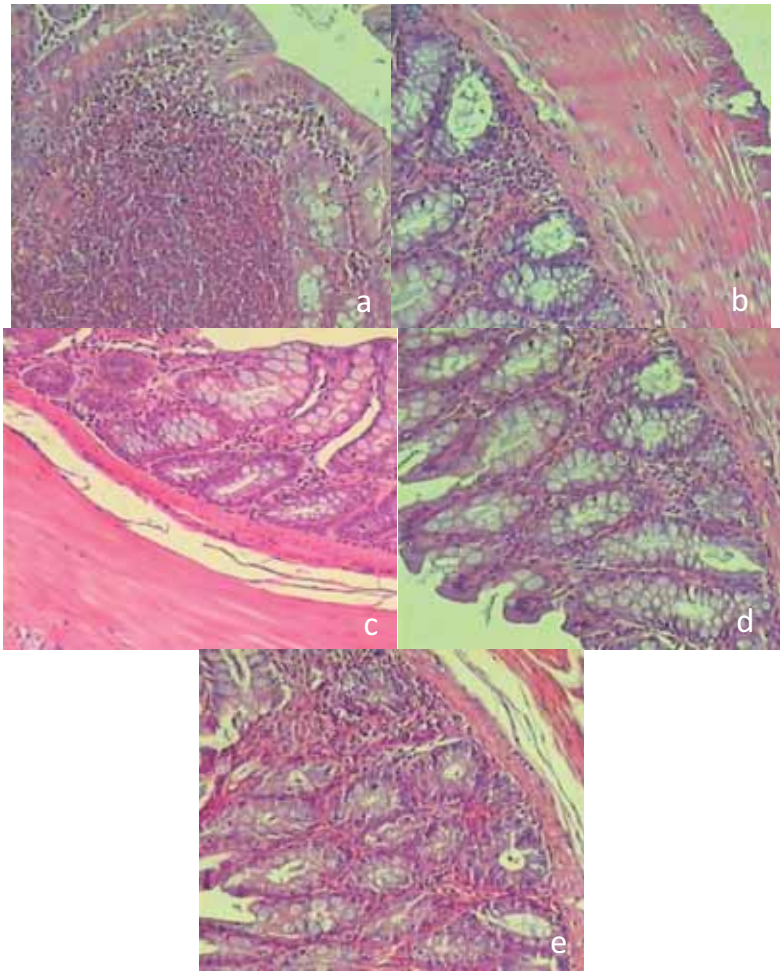


FIGURE 6
Sections of DMH-induced colon cancer rats group (a), treated rats groups(b , c) and (d, e).

by free radicals [12,50] suggested the increased SOD activity in liver ranged from 46% to 67% and kidney ranged from 47 to 59% of rat groups treated with P and L seed oils leads to the absence of accumulation of superoxide anion radical might be responsible for decreased lipid peroxidation in these tissues [59,96,109].This is also evident from the fact that relatively higher decrease in lipid peroxidation in liver and kidney of rats given seed oils being accompanied by the relatively higher increase in SOD activity in these tissues [23,27]. P and L seed oils had antioxidant activity and protect the organs from free radicals and might be retard the progress of the diseases [12,50]. These results are consistent with other investigators demonstrate alterations in the liver antioxidants in rats [23,88,107]. From these results, it appeared that there was a positive correlation with both seed oils contents and SOD scavenging activity [7,50].

Inhibitory effect of P and L seed oils on hepatic enzymatic activities may be due to its acting as a hepatoprotective and antilipid peroxidation agents against the permanent damage caused by DMH and DENA depending on its fatty acids and phytochemical constituents including antioxidants, free radical scavenging and anti-inflammatory properties preventing autoxidation and deleterious destruction of hepatic tissue [7,33,46]. Our findings came in harmony with other investigators [12,79,110] reported the P and L seed oils have the ability to prevent chronic diseases related to oxidative stress, such as cancer and in preventing its progression due to the

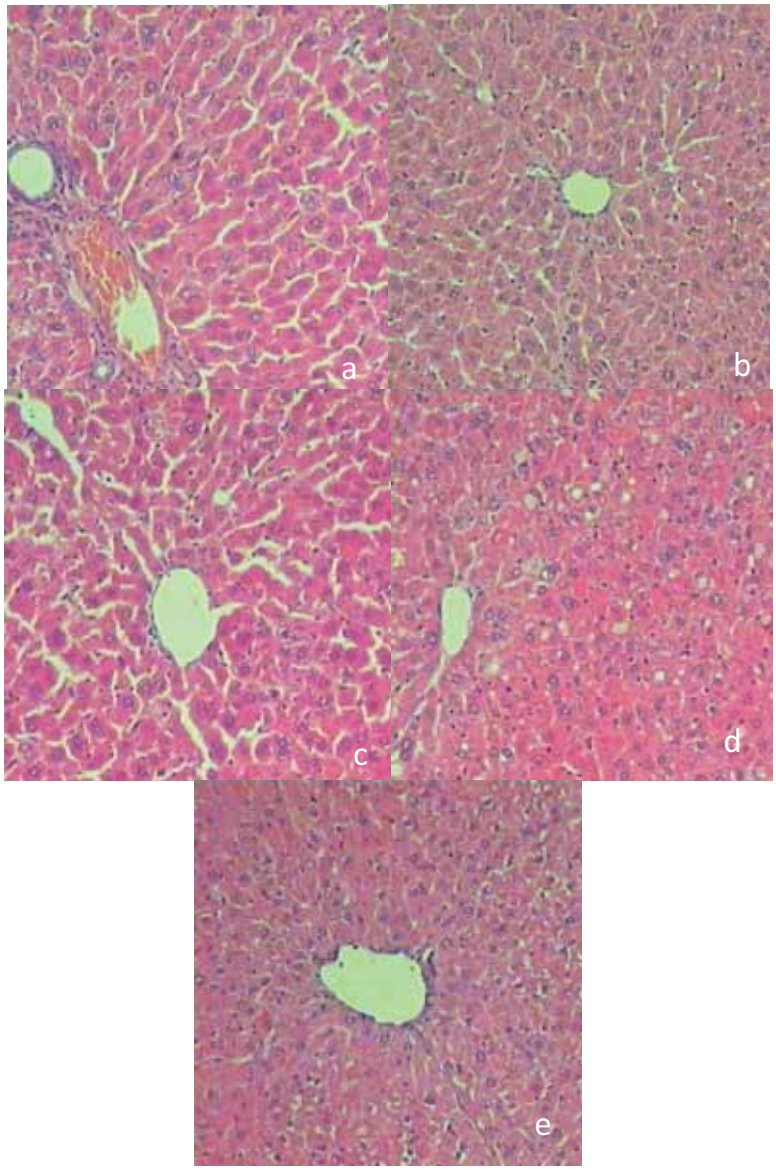


FIGURE 7
Sections of DENA-induced liver cancer rats group (a), treated rats groups (b, c) and (d, e).

their higher contents of polyphenols. Antilipid peroxidation of P and L seed oilsacts against the damaging effects of free radicals produced by DMH and DENA[12,81]. SOD plays an important role in decreasing the free radicals in chemically induced colon and liver carcinogenesis. The superoxide scavenging ability of the P and L seed oils may be due to the presence of fatty acids, phenolic and flavonoid compounds reported by other investigators [49,50,55,78]reported the antioxidant seed oil can be defined as a oil containing significant amounts of natural antioxidants associated to the oil [108]. Many investigators [44,50,57,60] reported the fatty acids, phenolic and flavonoid compounds are important seeds oil constituents that posses antioxidant properties and play an important role as free radical scavengers. However, the chemical properties of P and L seed oils in terms of their availability as radical scavengers predict their antioxidant activity [55,62,78].Moreover, the increases of these antioxidant in rat groups given P and L seed oils indicate the ability of both seed oils to prevent the formation of free radicals, enhance the endogenous antioxidant activity beyond its free radical scavenging property and the reduction of lipoperoxide formations. The above findings correlates with previous report suggested the seed oils posses potent free radical scavenging and antioxidant activities in DMH and DENA induction for colon and hepatocellular carcinogénesis [7,57,62]. Our data suggest that the ability of P and L seed oils to ameliorate DMH and DENA liver injury is associated with its antioxidant and reactive oxgen scavenging properties [7,46,81]. GSH-T, GSH-P, GSH-R and SOD are defense line against reactive oxgen species due to low activity of antioxidant enzymes in some organs and oxidative stress of DMH and DENA-inductions. High levels of antioxidants increase the plasma antioxidant capacity, decreasing tumor growth and inhibiting malignant cells prolifraton [47,87,111]. Therefore, the present results revealed that the P or L seed oils may be used as a protective effect by antagonizing DMH and DENA toxicitys and ameliorated their decreases in the activities of antioxidant enzymes.

Histopathology. Examined sections of colons from DMH rats group (C1) revealed necrosis and showing fibrosis with infiltration of the covering mucosa as recorded in Figure 6 (a). DMH induced rats exhibited proliferation of cells with damaged of mucosa and submucosa. Aberrant crypt foci, and enlargement of nucleus have been reported [27,62]. Colon sections from rat groups treated with P and L seed oils (C1/P and C1/L) showed reduction in fibrosis, normal mucosal lining with mucose secreting cells and the lamina propria showed minimal inflammatory cells (Figure 6 b, c). Colon sections from rat groups received P and L seed oils before DMH administration (P/C1 and L/C1) showed great decreases in fibrosis and cellular infiltration as compared to those of treated rat groups. Submucosa, musclosa and serosa are within normal with no pathological changes (Figure 6 d, e).These finding are similar to previous repoted by other investigators [32,83,84]. Examined sections of rat liver from carcinogenic DENA rat group (C2) revealed the damaging effect of the DENA used in the form of marked lymphoid follicle hyperplasia with infiltration in the portal area. Dilatation with congestion of blood sinusoids and vacuolar degeneration of hepatocytes were observed as shown in Figure 7 (a).These findings are in the same line with those reported by other investigators[82,87] recorded the histopathological examination of DENA rat liver showing large focal area of hepatocellular necrosis infiltrated with mononuclear inflammatory cells.Sections of liver tissue from C2/P and C2/L treated groups showed decrease in lymphoid, normal mucosal lining and the lamina propria showed minimal inflammatory cells. Dilatation and congestion of blood sinusoids is still present (Figure 7 b, c). Sections of liver tissue from a rat received P and L seed oils before DENA administrations (P/C2 and L/C2 groups) showing disappearance of lymphoid and cellular infiltration all over the tissue although mild blood sinusoids dilatation and congestion were observed.Submucosa, musclosa and serosa are within normal with no pathological changes (Figure 7 d, e).These results are in agreement with those findings reported by other investigators [8,47,81].P seed oil showing great decrease of lymphoid and cellular infiltration at portal area in sections of rat liver than that of rats received L seed oil. These results suggested the important use of P and L seed oils as protective agents against DMH and DENA carcinogenesis. Moreover, these histopathological studies indicated the P and L seed oils (200 mg/kg) showed great improves in the histology of colon and liver in rats received DMH and DENA carcinogens.The present study examined possible usefulness of P or L seed oils, as natural antioxidants treated and protected rats from carcinogenic effects of DMH and DENA carcinogens and improve hepatic and antioxidant enzymes that protect the cells from oxidative stresses and lipid peroxidation. However, based on the published studies, administration of P and L seed oils to man is simple, since, they are used as common dietary constituents in many parts of the world. Pumpkin (P) and lettuce (L) seed oils rich in fatty acids and phytochemicals have been used in treated tumours in medicine indicated to production of suitable new pharmaceutical therapeutic drugs used in low dosses for protective and treatment of different human cancers and encourage as nontoxic natural products. According to these observations the present study establishe that the P and L seed oils have appreciable anticancer and antioxidant activities. Further studies including clinical trials with advanced techniques are required for oils or

their constituents to be more effective, targeted and specific treatments and inhibition of carcinogenesis.

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BIOACTIVE COMPOUNDS INVOLVED IN BIOLOGICAL ACTIVITES AND KINETIC BEHAVIOR OF *MORUS ALBA* L. LEAVES GROWING IN SOUTHWEST ALGERIA

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ABSTRACT

The objectives of this study were to evaluate the biological properties of phytocompounds of *Morus alba* L. leaves using different assays. The chloroformic extract contained the highest levels of total phenolic contents 115.55±17.30 mg GAE/gDW, while the flavonoids, tannins and flavonols were high in the acetonic extract. The IC_{50} values based on the DPPH 0.97±0.10 mg/mL, ABTS 0.12±0.03 mg/mL and reducing power 0.70±0.10 mg/mL were high in aqueous extract, this extract exhibited the scavenging activity on hydrogen peroxide 31.51±0.23% better than BHT, while the acetonic extract showed an effective antioxidant activity on TAC and FRAP assay with 1110.82±20.77 mgAAE/g and 460±144.22 mgFe(II)E/g, respectively. The ethyl acetate fraction reaches equilibrium TEC_{50} after 5.18 min better than the ascorbic acid. The tannins showed good inhibition against *B. cereus* and *F. graminearum* with 14.66±0.57 mm and 37.79±11.99% respectively. The obtained results indicate the possibility of pharmacological exploitation of *M. alba* L. extracts as good source of natural antioxidants and antimicrobics.

KEYWORDS:
Antimicrobial activity, antioxidant power, kinetic, *Morus alba* L., polyphenols

INTRODUCTION

Plants constitute a valuable source of natural antioxidants such as vitamins, phenolic compounds, and flavonoids [1]. Because of their potential carcinogenicity, the utilization of synthetic antioxidants is progressively restricted in the food industry. This trend is concomitant with an increasing interest in the identification and valorization of natural antioxidants of plant origin. Moreover, microbial activity is a primary mode of deterioration of many foods and is often responsible for the loss of quality and safety. Concern over pathogenic and spoilage microorganisms in foods is increasing because of the increase in outbreaks of food-borne disease. There is growing interest in using natural antibacterial compounds such as plant extracts of herbs and spices for the preservation of foods because these extracts possess a characteristic flavor and sometimes show antioxidant activity and antimicrobial activity [2].

The mulberry belongs to the genus *Morus* (Family *Moraceae*), including approximately 24 *Morus* species and one subspecies, with at least 100 known varieties [3]. It is distributed in different areas including North America, South America, Africa, Europe, and Asia [4]. In folk medicine, different parts of *M. alba* L. are traditionally used for the treatment of several kinds of diseases [5]. In addition, mulberry leaves have long been used in traditional Chinese medicine to treat fever, improve eyesight, strengthen joints, reduce high blood pressure, high cholesterol, neutral fat, prevent thrombus formation and ageing, treat constipation and diabetes, and to promote urination [6,7].

The assessment of the biological characteristics of this plant in the arid zones in Algeria have not yet been investigated, therefore, the aim of the present study was to screen the chemical composition and to determine the total phenolic contents of *M. alba* L. leaves growing in the Southwest of Algeria (Bechar) as well as to evaluate *in vitro* the biological activities of the different extracts using various models, and to investigate their kinetic behaviour of DPPH radical scavenging activity.

MATERIALS AND METHODS

Plant material. The leaves of *M. alba* L. were harvested in March 2016 from Bechar region, Algeria. The plant was identified in the National Conservation Agency Bechar unit, Algeria. Plant samples were dried in the share and conserved until use.

Phytochemical screening. Chemical tests were carried out respectively on the diethyl ether, methanol and distilled water extracts for the qualitative determination of phytochemical constituents as described in literature [8].

Extraction of chemical compounds. Crude extracts. The leaves of *M. alba* L. (4/30: w/v) were subjected to two types of extraction, decoction with methanol, ethanol and distilled water at 60°C during 3 hours [9] and maceration with acetone, ethyl acetate and chloroform with stirring for 48 hours at room temperature [10]. The extracts recovered by filtration are subjected to evaporation of the solvent under reduced pressure in a rotary evaporator (Buchi), stored at 4°C until it is used.

Flavonoids extracts. A total of 100 g of defatted leaves were contacted with 200 mL of water and 200 mL of ethanol in capped flask with timely shaking and stirring for 4 days at room temperature. The obtained extract was filtered and evaporated to dryness. The residue was dissolved then in boiled water and extracted respectively with diethyl ether, ethyl acetate and *n*-buthanol (4×50 mL). The organic layers were filtered and concentrated to give crude extract of flavonoids [11].

Tannins extracts. A total of 100 g of defatted leaves were contacted with 250 mL of water and 160 mL of acetone in capped flask with timely shaking and stirring for 4 days at room temperature. After filtrating, and evaporating of the acetone, the aqueous layer was extracted respectively with dichloromethane (2x50 mL) and (4x50 mL) with ethyl acetate. The organic layer (AcOEt) was dried over Na_2SO_4, filtered and concentrated to give crude extract of tannins [12].

Determination of total phenolic contents. Total phenolic content in brut extracts was determined using Folin-Ciocalteu reagent method [13]. Briefly, 200 µL of plant extract mixed thoroughly with 1 mL of Folin-Ciocalteu reagent diluted 10 times. After 5 minutes, 0.8 mL of 7.5% Na_2CO_3 solution was added to the mixture. This later was allowed to stand in darkness for 30 min at 25°C. The absorbance was determined using spectrophotometer (UV-VIS 1700 pharma spec SHIMADZU) at 765 nm. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per g of dry weight.

Determination of total flavonoid contents. Total flavonoid content was quantified according to a standard method using quercetin as a standard**.** The plant extract of 500 µL was added to 1.5 mL distilled water followed by 150 µL of 5% $NaNO_2$ solution. After 5 min of incubation at 25°C, 150 µL of 10% $AlCl_3$ was added. After another 6 min, the reaction mixture was treated with 0.5mL 1M NaOH. Then the absorbance was measured at 510 nm. The flavonoid content was calculated from a quercetin curve expressed as mg QRE/gDW [14].

Determination of total condensed tannins. The analysis of condensed tannins was carried out according to the method of Julkunen-Titto [15]. To 400 µL of properly diluted sample; 3 mL of 4% vanillin solution in methanol and 1.5 mL of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min, and the absorption was measured at 500 nm. The amount of total condensed tannins is expressed as mg catechin/g DW.

Determination of total flavonols. Total flavonols in the plant extracts were estimated using the method of Almaraz-Abarca [16]. To 1 mL of sample, 1 mL of 2% $AlCl_3$ in ethanol and 3 mL sodium acetate (50 g/L) solutions were added. The absorption at 440 nm was read after 2.5 hours at 25°C. Total content of flavonols was expressed in terms of quercetin equivalent, mg QRE/g DW.

Antioxidant activity. Thin layer chromatography antioxidant assay. The extracts were subjected to TLC qualitative antioxidant assay on a silica gel plate (20×20 cm, silica gel F254, Merck). The extracts were loaded on CCM plate and were sprayed with 0.004 % (w/v) DPPH reagent prepared in methanol. The spots were observed after they had been heated at 60 °C for 30 min [17].

Free radical scavenging activity, DPPH assay. The free radical scavenging activity was measured by a modified DPPH• assay. Briefly, 100 µL of various concentrations of the extract in methanol was added to 1.9 mL of a methanol solution of DPPH (0.004 %). The mixture was vigorously shaken and then allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was measured at 517 nm. The scavenging activity on the DPPH radical was expressed as inhibition percentage using the following equation:

$$DPPH^{\bullet}_{scavenging\text{-}radical}\ (\%) = [(A_0\ A_s) / A_0] \times 100$$

A_0: is the absorbance of control reaction

A_s: is the absorbance of sample solution containing the test compound.

The ascorbic acid and BHT were used as a positive control. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentrations [18].

Kinetic analysis. Preparation of solution (A) of DPPH (0.004 %) kept in the dark at ambient temperature. Then, solution (B) of extract 1 mg/mL, from this solution, different concentrations were prepared. To follow the kinetic 1.5 mL of solution (B) was mixed with 1.5 mL of solution (A), the absorbance

was measured at 517 nm after each 30s until it becomes constant, results compared with ascorbic acid and BHT. The DPPH• concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression:

$A517\ nm = 17.531 \times [DPPH^{\bullet}]t + 0.039 \quad R^2 = 0.999$

where [DPPH•]t was expressed as mg/ml at *t* time. The percentage of the remaining DPPH• (%$DPPH^{\bullet}_{REM}$) at the steady state was calculated as follows:

$\%\ DPPH^{\bullet}_{REM} = 100 \times [DPPH^{\bullet}]t\ /[\ DPPH^{\bullet}]t=0$

where [DPPH•]t=0 and [DPPH•]t are concentrations of DPPH• at t = 0 and t = t, respectively.

Using various antioxidant concentrations, it was possible to determine the amount of antioxidant necessary to halve the initial DPPH• concentration (EC_{50}).

EC_{50} is expressed in mg of dry extract per g of DPPH. The time needed to reach the EC_{50} concentration, noted TEC_{50}, was graphically determined. The antiradical efficiency (AE) was calculated as follows: $AE = 1/ (EC_{50} \times TEC_{50})$

Authors have classified the kinetic behavior of the antioxidant as follows: fast (TEC_{50} < 30 min), medium (TEC_{50}: 30-60 min) and slow reaction kinetics (TEC_{50} > 60 min) [19]. And the classification of antiradical efficiency was proposed as: ARE < 1×10^{-3} slow, [1×10^{-3}; 5×10^{-3}] intermediate, [5×10^{-3}; 10×10^{-3}] high, and ARE > 10×10^{-3} very high [20].

Reducing power capacity. Various amounts of the extracts in distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide 1% solution. The mixture was incubated at 50°C for 20 min. 2.5 mL of trichloracetic acid 10% were added to the mixture which was then centrifuged for 10 min. The supernatant 2.5 mL was mixed with 2.5 mL of distilled water and 0.5 mL of a freshly prepared $FeCl_3$ 0.1% solution. The absorbance was measured at 700 nm. Ascorbic acid and BHT were used as a positive control. In this method, increased absorbance indicates an increased reducing power [21].

Phosphomolybdenum reduction assay. The assay is based on the reduction of Mo (VI) to Mo (V) by the extracts and subsequent formation of green phosphate/Mo (V) complex at acid pH. 1 mL of extract was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance was measured at 695 nm. The total antioxidant capacity is expressed in milligram equivalents of ascorbic acid per gram of the dry weight (mg EAA/g DW) [22].

Hydrogen peroxide scavenging activity. 1 mL of extract prepared in distilled water was mixed with 2 mL of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance was taken at 230 nm. The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equation:

H_2O_2 radical scavenging activity = {(Abs control Abs sample)/(Abs control)} × 100

Where; Abs control is the absorbance of H_2O_2 radical + methanol; Abs sample is the absorbance of H_2O_2 radical + sample extract or standard [23].

FRAP assay. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM 2, 4, 6- tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3.6H_2O$. Extract 200 µL was allowed to react with 2800 µL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve of $FeSO_4$ was linear. Results are expressed in mg Fe (II)/g dry weight and compared with ascorbic acid and BHT [24].

ABTS radical cation decolorization assay. The ABTS radical was generated during a chemical reaction between the 7 mM aqueous solution of ABTS diammonium salt and the 2.45 mM potassium persulfate. The solution was kept at a room temperature in darkness throughout the night, in order to complete the reaction and to stabilize the ABTS cation-radical. Prior to analysis, the radical solution was diluted with ethanol to obtain final absorbance value of A = 0.70±0.02 measured at 734 nm. 200 µL aliquots of the properly diluted extract or of standards solutions were added to 2 mL of ABTS 0.7 and absorbance value was measured 6 min after mixing. The percentage of inhibition of $ABTS^{\bullet+}$ was calculated using the formula as DPPH method [25].

Screening for antimicrobial activity. Bacteria: *Salmonella typhi*murium ATCC 13311, *Bacillus cereus* ATCC 213332 *Listeria monocytogenes* ATCC 15313 and *Staphylococcus aureus* ATCC 25923 were from the Vet Agro Sup, Veterinary School of Lyon, France.

Yeast: *Candida albican* ATCC 10231, *Candida albican* ATCC 26790 and *Candida albican* CIP444 were performed by the laboratory of biology, Tlemcen (Algeria).

Moulds: Six pathogenic strains of fungi were used in the study, namely, *Aspergillus parasiticus*, CBS 100926T, Biocentrum-DTU Microbial Biotechnology Center, Technical University Denmark,

Aspergillus alliaceus, isolated local wheat durum variety Waha from the region of Setif and molecularly identified by sequencing IT1-ITS2 regions and calmodulin, and four others *Fusarium graminearum*, *Fusarium oxysporum*, *Pencillium expansuim* and *Pencillium glandicola*, isolated from wheat, were provided by Research Laboratory of Valorisation of Plant Resource and Food Security in Semi-Arid Areas of Tahri Mohamed University of Bechar, Algeria. Strains tested were stored in the appropriate medium before use and conserved at 4°C.

Evaluation of the antifungal activity. The antifungal activity was determined by using the agar well diffusion method, 1 mL of extracts tested (12.5-0.78 mg/mL), were added to 19 mL of a solution of sterilized potato dextrose agar acidified (PDAa). The mixtures were cast on the Petri dish. Thereafter, a mycelial disc of approximately 6 mm of diameter, cut from the periphery of new culture in agar 2% of 16 h, was inoculated in the center of each Petri dish, and then incubated at 25±2°C. Blank (DMSO 5%) were added separately into the respective labeled wells, used as negative control. The diameters of growth of the hypha were recorded after 7 days. The antifungal index was determined using the following formula:

Antifungal index (I) = (1 – (Da/Db)) × 100

Where: Da and Db are the diameter of the growth zone in test and in control Petri dish (mm) [26].

Evaluation of the antibacterial activity. The disc diffusion method was used for the determination of the antibacterial activity [27]. Sterile discs, 6 mm of diameter, impregnated with 20 µL of extracts tested, were placed in Petri dishes on Mueller-Hinton agar, which had been surface spread with 1 mL of logarithmic phase bacteria adjusted to a 10^8 UFC/mL fixed by the optical density (0.080.1 at 620 nm) [54]. The Petri dishes were then incubated for 18 h at 37°C. The diameter of the inhibition zone was measured**.** The results of the extracts were compared with negative control (solvent of extract DMSO 5%). The same procedure used for *Candida* strains using Sabouraud agar, adjusted strains to (0.12-0.15) at 530 nm which is equivalent to 10^7 UFC/mL. The Petri dishes were then incubated for 48 h to 72 h at 37°C.

Determination of minimum inhibitory and bactericidal concentration (MIC, MBC). Various concentrations of extracts between 12.50.012 mg/mL were introduced into different test tubes; each tube was inoculated with an overnight culture of bacterial strains diluted to give a final concentration of 10^6 cells/mL. The tubes were incubated at 37°C for 24 h. 2 tubes were used as control. The concentration of the extract that did not permit any visible growth of the inoculated test organism in broth culture was regarded as the minimum inhibitory concentration (MIC) in each case [28]. After culturing the test organisms separately in nutrient broth containing various concentrations of the active ingredients, the broth was inoculated onto freshly prepared agar plates to assay for the bactericidal effect. The culture was incubated at 37°C for 24 h. The lowest concentration of extract tested that does not yield any colony growth on the solid medium after the incubation period was regarded as minimum bactericidal concentration (MBC) [29].

Statistical analysis . The experimental results were expressed as mean ± standard deviation (SD) of 3 replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Duncan's Multiple Range test using the GraphPad Prism software version 5.03 program. *P* Values < 0.05 were regarded as significant. Correlations between the parameters evaluated were obtained using Pearson's correlation coefficient (r).

TABLE 1
Yields, Total phenolics, flavonoids, tannins and flavonols contents of extracts from *M. alba* L. leaves

Extract	Yields %	Total phenolic (mg GAE/g DW)	Total flavonoids (mg QRE/g DW)	Total tannins (mg CAE/g DW)	flavonols (mg QRE/g DW)
Aqueous	30.93± 3.85	28.93±14.81	ND	6.24±5.45	6.53±1.52
Ethanolic	12.11±1.42	19.96±6.20	128.89±23.55	60.44±7.47	58.25±2.94
Methanolic	13.95±3.05	19.16±5.55	141.23±15.52	68.02±16.96	63.09±4.95
Ethyl acetate	4.23±0.51	112.63±57.26	842.86±13,49	285.27±28.19	100.94±1.13
Acetonic	4.09±0.37	101.6±22.40	933.35±6.10	421.94±20.16	113.29±3.42
Chloroformic	4.48±0.41	115.55±17.30	713.58±56.52	277.11±16.24	103.75±3.15
Diethyl ether fraction	0.68±0.34	/	/	/	
Ethyl acetate fraction	057±0.29	/	/	/	/
Butanolic fraction	0.78±0.28	/	/	/	/
Tannins	0.34±0.25	/	/	/	/

RESULTS AND DISCUSSION

Phytochemical screening. Phytochemical screening of the extracts shows that the plant leaves are rich in flavonoids, saponins, steroid heteroside, coumarins, terpenoids, tannins, free quinons, reducing sugars and starch. Andallu and Varadacharyulu (2003) [30] reported that the leaves contain a wide variety of nutrients, including proteins, sugars, polyphenols, flavonoids, steroids, vitamins, and minerals. Also De Oliveira et *al.*, (2015) [31] detected the presence of coumarins, flavonoids, tannins, and triterpenes in the ethanolic extract of *M. alba* L. leaves. The results obtained in those studies are in full agreement with our findings.

Yields and phenolic contents. The yield percent and total phenolic content of extracts obtained from *M. alba* L. leaves using different solvents were determined. The maximum recovery percentage and extractable were recorded in aqueous extract, whereas the methanol and ethanol extracts were found to contain comparable level of percentage. The yield of the maceration extracts (chloroformic, acetonic and ethyl acetate) were much lower and does not exceed 4%. Concerning the distribution of secondary metabolites, we record a high yield of butanolic fraction of flavonoids, followed by diethyl ether fraction. While the yields of ethyl acetate fraction of flavonoids and tannins fractions are very low (Table 1). These results may be compared with a previous report (Kim et *al.*, 2014) [32] revealed that yield ranged between 9.1-10.4% of methanolic extract of 10 different *M. alba* L. leaves collected from seven provinces in Korea.

The amount phenolic contents dependent on solvent of extraction. Generally, the highest total phenolic contents was detected in chloroformic extract followed by the ethyl acetate however, the methanolic and the ethanolic extract had the same value 19 mg GAE/g DW. The flavonoids contents in various extracts showed different results ranging from 128 to 933 mg GAE/g DW, while in the aqueous extract, flavonoids compounds could not be detected. Maximum amount of tannins and flavonols contents were present in the acetonic extract, in contrast the lowest contents of tannins and flavonols were obtained in aqueous extract. Earlier report of (Khan et *al.*, 2013) [33] indicated that methanolic extract of the leaves of *M. alba* L. contained 103.68±17.471 mgGAE/g DW, 6.667±2.45 mgCAE/g DW, 185.48±1.19 mg QUE/g DW and 2.36±0.04 mg CAE/g DW respectively of phenolics, flavonoids, flavonols and proanthocyanidins contents. These results demonstrate clearly that the content of phenolic compounds is dependent on the type of plant material, the chemical nature of the extractable compounds as well as the effectiveness of extraction of solvents to solubilize such compounds [34, 35], in addition to the temperature and time of extraction used.

GAE, gallic acid equivalents; CAE, (+)- catechin equivalents; QRE, quercitin equivalents ND, not determined. Values are mean ± standard deviation of triplicate experiments.

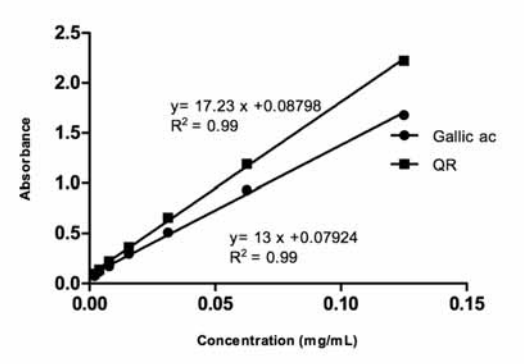

FIGURE 1
Calibration curve for standard gallic acid (total phenolic contents) and quercetin (flavonols contents), R² values represented mean data set of n=3

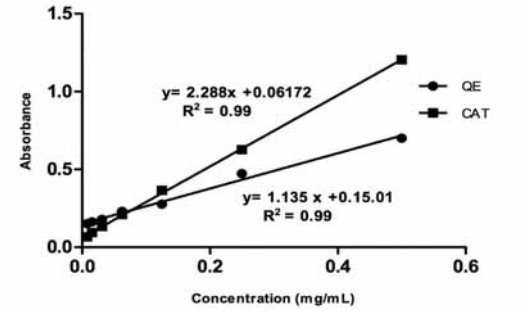

FIGURE 2
Calibration curve for standard catechin (tannins contents) and quercetin (flavonoids contents), R² values represented mean data set of n=3

Thin layer chromatography antioxidant assay. In the present investigation, the data obtained from the TLC antioxidant assay revealed that all extracts as for ascorbic acid exhibited an antioxidant effect. Yellow spots were observed after spraying the TLC plates with DPPH solution. Our results are in accordance with those reported by (Molyneux, 2004) [36].

DPPH radical scavenging activity. The stable DPPH radical is widely used to evaluate the free radical scavenging activity in many plant extracts [37]. The assessment of antioxidant activity showed that the leaves were able to scavenge this radical. After the determination of the IC_{50}, The best free radical scavenging activity was obtained in the ethyl acetate

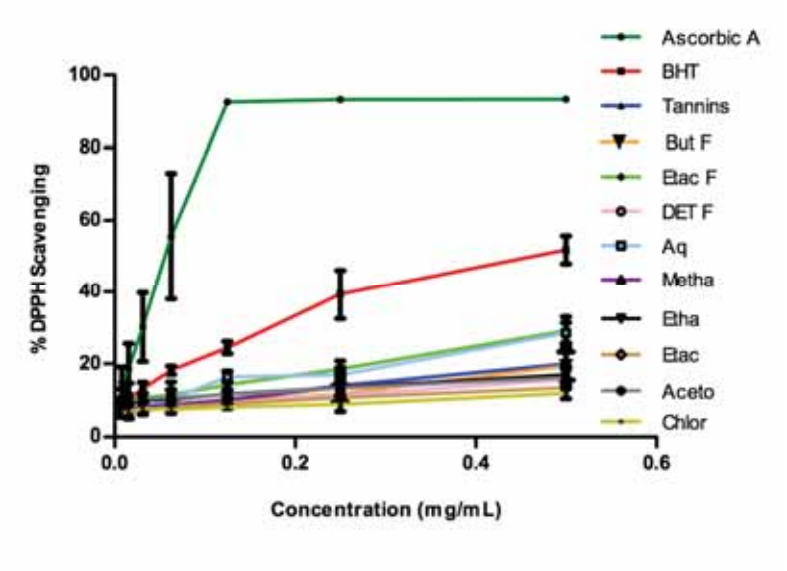

FIGURE 3
DPPH radical scavenging activity of different fractions of *M. alba* L. (I %) at different concentrations. But F, Butanolic fraction; Etac F, Ethyl acetate fraction; DET F, Diethyl ether fraction, Aq, Aqueous; Metha, Methanolic; Etha, Ethanolic; Etac, Ethyl acetate; Aceto, Acetonic and Chlor, Chloroformic. Values are mean ± standard deviation of triplicate experiments.

fraction of flavonoids, while for brut extracts, the decocted aqueous extract displayed a higher activity followed by the macerated extract of ethyl acetate, the lowest value was obtained in chloroformic extract. Although this scavenging effect was lower than that of ascorbic acid and BHT (Figure 3 and Table 3). Higher scavenging activity has been reported in the methanolic and ethanolic extracts of *M. alba* leaves from Pakistan region, where the inhibition percentage was respectively 70.23±1.41% and 58.72±2.32% [53]. A rate almost two times larger than that recorded in our study.

Kinetic of DPPH antioxidant activity. Kinetic curves of degradation of the DPPH radical by the different extracts at different concentrations. Each extract had a distinct behavior according to the concentration. The EC_{50} and antiradical efficiency (AE) values of scavenging DPPH radicals of all extracts ranged from 154.47 to 307532.42 mg of antioxidant/g DPPH$^{\bullet}$ and from 1.24×10^{-3} to 3.09×10^{-9} mg/g.min, respectively for ethyl acetate fraction of flavonoids and ethyl acetate brut extract. Compared with commonly used antioxidants. Taking the classification standards described above as a reference, it is evident that the ethyl acetate fraction of flavonoids react rapidly with the DPPH radical better than standard of the ascorbic acid, while the BHT, was included in the low effectiveness category. Whereas, the ethyl acetate brut extract was considered as reacting at slow speed $TEC_{50} > 60$ minutes (Table 2 and Figure 4).

TABLE 2
Characteristic parameters of the reduction kinetics of DPPH$^{\bullet}$

Extract	EC_{50} (mg Antioxidant/ g DPPH)	TEC_{50} (min)	Kinetics category	ARE $\times10^{-3}$ (mg/g.min)	Efficiency type
Tannins	340.71	30.50	Medium	9.62×10^{-2}	Low
Butanolic fraction	257.99	49.62	Medium	7.81×10^{-2}	Low
Ethyl acetate fraction	154.47	5.18	Fast	1.24	Intermediate
Diethyl ether fraction	635.38	15.28	Fast	0.102	Low
Aqueous	220.49	67.19	Slow	6.74×10^{-2}	Low
Ethanolic	233.33	9.08	Fast	0.471	Low
Methanolic	1156.66	22.83	Fast	3.78×10^{-2}	Low
Ethyl acetate	307532.42	1051.78	Slow	3.09×10^{-6}	Very low
Acetonic	980	14.40	Fast	7.08×10^{-2}	Low
Chloroformic	227.52	15.07	Fast	0.291	Low
Ascorbic acid	3.62×10^{-3}	8.71	Fast	31710	Very high
BHT	15.49	257.87	Slow	0.250	Low

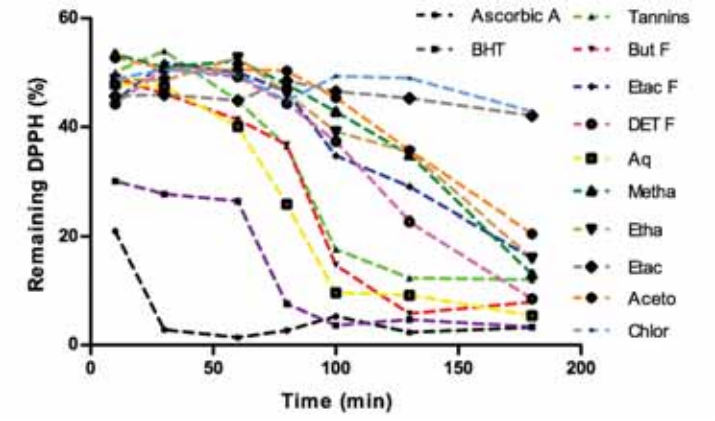

FIGURE 4

Kinetic behaviour of different fractions of leaves of *M. alba* L. at different concentrations. But F: Butanolic fraction; Etac F: Ethyl acetate fraction; DET F: Diethyl ether fraction; Aq, Aqueous; Metha, Methanolic; Etha, Ethanolic; Etac, Ethyl acetate; Aceto, Acetonic and Chlor, Chloroformic

Reducing power assay. The selective exctracts showed more reducing capacity assessment than brut extracts. However, the results depicted that maximum reducing power with IC_{50} 0.700±0.10 mg/mL was exhibited by the aqueous extract as compared to other extracts. Whereas, reducing power of standards and different selective extracts exhibited the following order: ascorbic acid > BHT > Tannins > Butanolic fraction > ethyl acetate fraction > diethyl ether fraction of flavonoids. The methanolic and chloroformic extracts were found to be similar in the capacity of reduction (Table 3). The reducing power was found to be increased with the increase of the concentration of the extract, indicating the presence of some compounds in the extract that is both electron donors and could react with free radicals and to terminate free radical chain reactions [38]. Our results are consistent with the result published of Wattanapitayakul et *al.*, (2005) [39], in which the aqueous extract of *M. alba* L. leaves showed highest antioxidant properties evaluated through ferric reducing/antioxidant power assay.

ABTS radical scavenging activity. All the extracts scavenged ABTS radical in a concentration-dependent way. The selective extracts were fast and effective scavengers of $ABTS^{\bullet+}$ radical. At 0.5 mg/mL the percentage of inhibition ranged from 61.72 to 84.62% in flavonoids and tannins fraction. Nevertheless, BHT and ascorbic acid exhibited 94.47±0.02% and 94.46±0.16% respectively, at the same concentration. Decocted extract exhibited high activity than macerated one. It is to be noted that the percentage of inhibition for the entire fraction tested obtained by ABTS assay were higher with 88 % than those obtained by DPPH assay 29% at the same concentration (Figure 5). In comparison to another study radical scavenging activity of different parts of *M. alba* L. were determined in methanol extract and their fractions dose dependently increased radical scavenging activity of mulberry branches, roots and leaves (more than 70%). Study shows that mulberry fruits exhibited the highest radical scavenging activity [40].

Antioxidant capacity by phosphomolybdenum method. The result of total antioxidant activity demonstrate that, acetonic extract showed the higher levels of TAC at 0.5 mg/mL. However, the ethyl acetate brut extract exhibited similar activity to the BHT. Similarly, the ethyl acetate fraction of flavonoids was found the highest among four selective extracts with 405.67±59.39 mg AAE/g DW at the same concentration. While the aqueous extract was found the lower. The extracts tested in this study were varied significantly among each other (Table 4). The natural antioxidants are most multifunctional, and it might be contributed by the polyphenols [41].

Hydrogen peroxide scavenging activity. Hydrogen peroxide, although not a radical species play a role to contribute oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally-occurring iron complexes inside the cell believed to react with H_2O_2 *in vivo* to generate highly reactive hydroxyl radicals and this may be the origin of many of its toxic effects [42]. Among ten extracts tested, aqueous extract exhibited maximum percentage of inhibition at the concentration of 0.015 mg/mL. In addition, the both of ethyl acetate and butanolic fractions of flavonoids showed good activity in depleting H_2O_2 with 30 % value. The percentage of H_2O_2 scavenging activity of ethanolic extract was found to be similar to the BHT, but the results remain lower than those of standard ascorbic acid (Table 4). Previous studies have suggested that the leaves of mulberry contains higher amount of quercetin which is responsible for reduction of oxidation process *in vivo* and *in vitro* [43-46].

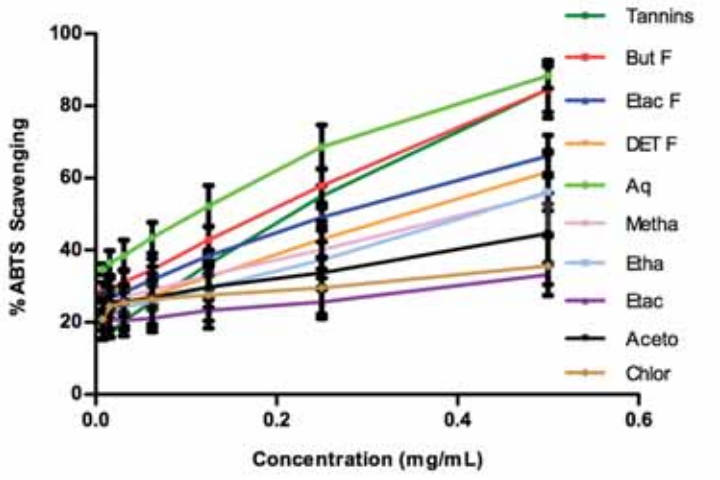

FIGURE 5

ABTS radical scavenging activity of different fractions of *M. alba L.* (I %) at different concentrations. But F, Butanolic fraction; Etac F, Ethyl acetate fraction; DET F, Diethyl ether fraction, Aq, Aqueous; Metha, Methanolic; Etha, Ethanolic; Etac, Ethyl acetate; Aceto, Acetonic and Chlor, Chloroformic. Values are mean ± standard deviation of triplicate experiments

FRAP assay. The results of the FRAP assay indicates that the high amount was noted for acetonic extract and the minimum value was observed in aqueous extract. Whereas, the selective fractions showed a considerable antioxidant effect ranging from 130 to 196.66 mg Fe(II)E/g. Furthermore, the BHT activity was found 266.66 mg Fe(II)E/g. All the samples tested possessed different antioxidant activity power. This difference might be due to composition of the extract or their condensation of chlorophyll which can be increase the absorbance as in the case of macerated extract (Table 3). Halvorsen et *al.*, (2006) [47] suggested most of the secondary metabolites are redox-active compounds that will be picked up by the FRAP assay.

Correlation different antioxidant methods with phenolic contents. In order to more appreciate the relationships between antioxidant capacities and phenolic content of leave extracts, correlations between assays under different extracting conditions were analyzed. A good correlation between flavonoids, flavonols and IC_{50} values for DPPH, ABTS ranged from 0.50 to 0.83. Similarly, a positive correlation was also drawn between flavonols

TABLE 3

Antioxidant effect with IC_{50} values of DPPH, hydrogen peroxide, TAC, and FRAP assay, reducing power assay and ABTS of *M. alba* L. leaves

Plant extracts	DPPH	Reducing power	H_2O_2 (% I)	Total antioxidant (mg AAE/g DW)	FRAP (mg Fe(II)E/g)	ABTS
Tannins	1.71±0.54	0.77±0.09	28.01±0.58	327.72±27.58	196.66±23.09	0.23±0.02
Butanolic fraction	1.83±0.53	0.83±0.09	30.09±0.73	213.24±33.14	143.33±5.77	0.19±0.03
Ethyl acetate fraction	1.05±0.19	0.91±0.06	30.15±0.06	405.67±59.39	150±36.05	0.29±0.04
Diethyl ether fraction	2.33±0.08	1.20±0.08	27.19±0.56	356.95±81.37	130±55.67	0.35±0.06
Aqueous	0.97±0.10	0.70±0.10	31.51±0.23	119.46±22.81	80±00	0.12±0.03
Ethanolic	2.65±0.69	1.164±0.31	29.16±0.82	289.97±78.05	173.33±20.81	0.44±0.06
Methanolic	2.28±0.55	1.48±0.22	28.44±0.54	299.71±51.32	240±26.45	0.42±0.18
Ethyl acetate	2.25±0.32	1.32±0.22	24.69±1.17	846.54±53.06	243.33±32.14	1.19±0.28
Acetonic	3.04±0.33	1.33±0.25	24.47±2.18	1110.82±20.77	460±144.22	0.68±0.24
Chloroformic	4.55±1.15	1.165±0.36	22.88±0.63	844.10±174.08	280.33±26.45	1.09±0.35
Ascorbic acid	0.04±0.01	0.027±0.002	33.06±1.02	-	-	0.009±0.001
BHT	0.44±0.04	0.11±0.007	29.50±0.94	846.54±108.56	266.66±15.27	0.01±0.002

Values are mean ± standard deviation of triplicate experiments.

TABLE 4
Linear Correlation Coefficients (R) between phenolic contents and antioxidant activity of crude extracts

	DPPH		ABTS		reducing power assay	
Phenolic compounds	R^2	*P*	R^2	*P*	R^2	*P*
Polyphenols	0.32	0.2420	0.34	0.2221	0.10	0.5383
Flavonoids	0.50	0.1833	0.76	0.0556	0.0046	0.9130
Condensed tannins	0.42	0.11610	0.44	0.1479	0.25	0.3066
Flavonols	0.82	0.0131	0.83	0.0121	0.59	0.0736

values and reducing power assay (Table 4). Iqbal et *al.*, (2012) [46] also reported a good correlation in three different varieties of Mulberry leaves from Pakistan. In contrast, there is no correlation between total phenolic and tannins contents with the three assays in our study. This moderate correlation could be explained by the facts that there are some anti-oxidative compounds that not only exhibit their antioxidant activity by donating hydrogen but also by scavenging oxygen. The differences in correlation coefficient among different antioxidant methods indicate the fact that single assay may not be used to assess the total antioxidant activity [48].

Antibacterial activity. Different extracts of aqueous, methanolic, ethanolic and tannins fraction were evaluated for their antimicrobial activities using initially by the disk diffusion method against different bacteria. The result of antibacterial activity shown that tannins fraction recorded the maximum inhibitory activity on different concentrations against *B. cereus* up to 14.66±0.57 mm. Aqueous extract showed high activity against *L. monocytogenes* with 11.66±2.08 mm, a good effect exerced on *S. aureus* 10±1.73 mm. It can be noted that the most interesting effect of the methanolic extract was against *S. typhimurium* whereas, weak effect against the other strains. Furthermore, the tannins, aqueous and ethanolic extracts showed no or weak activity against *S. typhimurium* at all the concentrations. Tannins fraction formed the largest average zone of inhibition of 10.66±0.28 mm against *C. albican* ATTC 26790 was obtained. While the methanolic and ethanolic extracts have shown good activity respectively with 10.33±1.04 mm and 9.16±1.89 mm against *C. albican* ATTC 10231. The aqueous extract exhibited high value 10±0.5 mm against *C. albican* ATTC 10231, whereas no or weak antimicrobial activity against *C. albican* ATTC 26790 and *C. albican* CIP444.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). From the results obtained of MIC, we noticed that the absence of turbidity was observed at the concentration of 12.5 mg/ml for the methanolic extract against *S. typhimurium*, and for the aqueous and tannins against *B. cereus*. For the MBC, in solid medium, the total absence of colonies was observed at the same concentration. The CMB/MIC ratios are less than or equal to ≤ 4, it is possible to deduce that the action of the extracts tested on the bacterial strains studied is bacteriocidal.

Antifungal activity. The direct contact technique, which involves both contacting between extracts and microorganisms and observing the growth of the fungal strains tested, suggested that all the extracts under study exerted an inhibitory activity on mycelium growth. Tannins fraction was the most potent against all fungal strains; the high percentage of inhibition was 37.79±11.99% at 3.12 mg/mL against *F. graminearum*, good effect against *P. expansum* 24.41±10.40% at 6.25 mg/mL. In addition, the methanolic extract was more effective against *F. graminearum* and *P. expansum* with 29.06±20.84% and 25.78±12.72% respectively at 3.12 mg/mL. The ethanolic extract provided a better effect 26.98±15.99% at 0.78 mg/mL against *P. expansum*, a low activity against the other fungal strains varied from 6.20 to 15.30%. However the activity of the aqueous extract against *F. oxysporum* was 19.48±2.01% at 1.56 mg/mL, *A. alliaceus* 15.04±2.23% at 0.78 mg/mL and 13.99±8.07 % against *A. parasiticus* at 6.25 mg/mL. While against the other strains the percentage of inhibition does not exceed the 8.66%.

Different species of mulberry leaves exhibited antimicrobial activity to varying extents. These differences can be attributed to the accumulation and distribution of variable contents of antimicrobial agents in different species of Mulberry [49]. Park et *al.*, (2003) [50] studied that mulberry extracts are rich in phytochemicals and have antimicrobial potential against harmful pathogens. Furthermore, it was reported that methanol and chloroform extracts of different species of *Morus* exhibited antimicrobial activity against *Pseudomonas aeroginosa*, *Proteus vulgaris*, *Bacillus subtilis, Salmonella typhi, Shigella flexneri*, *Candida albicans* and *Aspergillus niger* [51]. Another study revealed that the ethanolic extract of *M. alba* L. leaves exhibited stronger antimicrobial activities than the aqueous extracts [52].

CONCLUSION

The result obtained from present study showed that the extracts of *Morus alba L.* leaves has remarkable antioxidant activity as well as potential antimicrobial activity. The aqueous extract had significant

antioxidant activity, which was either comparable or better than the standard. More importantly, the results indicated that the tannins extract had strong microbial activities. However, the components responsible for the activities are currently not known. Therefore, it is suggested that further work be performed on the isolation and identification of the bioactive compounds, which may be useful for therapeutic purpose.

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ENRICHMENT OF CAFFEINE FROM COFFEE USING TWEEZING ADSORPTION BUBBLE SEPARATION

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ABSTRACT

Foam fractionation is a separation method that is suitable for the enrichment of surface-active compoundfrom highly diluted aqueous solution. For non-surface-active compound, enrichment can also be achieved using foam fractionation with the assistance of complexation between the target molecule and a "catcher". Here in the present research, foam fractionation experiments were conducted to enrich caffeine from its aqueous solution without and with catchers, chlorogenic acid and n-octylcaffeate. The results indicated that chlorogenic acid enhanced the enrichment of caffeine as high as nearly 4 times as that without catcher, while n-octylcaffeate did not increase the enrichment of caffeine at all. The comparison of the two catchers suggested the importance of the hydrogen bonds in the stabilization of the complex between caffeine and its catcher. A catcher that is both surface-active and be able to firmly complex with the target molecule is essential in the process of complexation base foam fractionation.

KEYWORDS:
Foam fractionation, Caffeine, Caffeine-chlorogenic acid complex, Complexation

INTRODUCTION

Natural bioactive products represent an inexhaustible source of valuable precursors for pharmaceutical and functional products, for their unparalleled chemical and bioactive diversity. Obtaining of these bioactive substances from plants, microbes or marine organisms is no longer problematic for people at present, since a lot of extraction and separation methods have been developed successfully [*1, 2*]. However, these procedures are frequently long and tedious [*1*] and accompanied with extensive consuming of organic solvents, and therefore are serious threats to environment and human health, and costly *[3]*. It was reported that the costs for separation in the bioprocesses account for up to 90% of the whole production expenses, and even more when using the newer separation techniques [*4*]. Meanwhile, the sensitive constituents may degrade or be inactivated during these long and harsh separation procedures.

Being of a new trend in separation techniques, foam fractionation represents a method that is environmentally friendly, cost effective and of simplicity in equipment and maintenance, and therefore receives increasing attention and interests from researchers. Foam fractionation is started with the continuous introduction of inert gas into an aqueous solution containing surface-active substances, during which, bubbles are formed and stabilized by the surface-active substances and move up along the riser. Separation is accomplished through the collection of the foam containing the substances that are preferentially adsorbed on to the gas-liquid interface according to the difference in their surface activity.Therefore, the most surface-active substances will be enriched into the foamate (collapsed foam) firstly, and the ones that are not surface-active will stay in the bulk solution. For the substances that are not surface-active, separation or enrichment can also be achieved by foam fractionation if they are able to form a complex with the others, which was named as "catcher", to form a surface-active product.A lot of parameters such as pH value, initial concentration, flow rate, height of the column, and temperature may all influence the processes of drainage and coalescence, which occur when the foam rises up a column, and finally determine the foaming efficiency.
Caffeine (1,3,7-trimethylxanthine), the most widely consumed stimulant drug in the world nowadays, has a controversial effect on human health. Caffeine is proved to be able to keep people awake and improving mental alertness after fatigue [*5*], and also, it is believed to help to reduce the risk of metabolic syndrome, including type 2 diabetes mellitus and obesity [*6*]. On the other hand, people think that consumption of caffeine may have some adverse effects on human health, like palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure and insomnia [*7, 8]*. Therefore, there is a public desire to enrich or eliminate caffeine from the products people daily consumed.

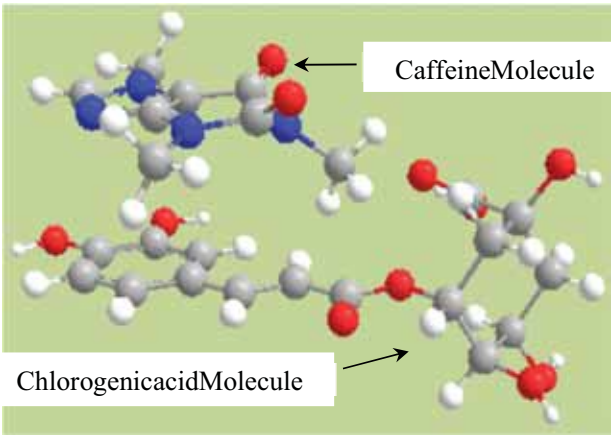


FIGURE 1
A possible schematic representation of caffeine-chlorogenic acid complex.

It is well known that caffeine complexes with polyphenols and aromatic hydroxyl acids molecules in aqueous solution [*9, 10]*.The complex between caffeine and chlorogenic acid (3-o-caffeoylquinic acid)was firstly isolated from green coffee beans almost one century ago[11]. Sondheimer et al.[*12*] proposed that the complex is formed by caffeine and chlorogenic acid in the ratio of 1:1 in aqueous solution. Horman and Viani[13] described the complex as a 1:1 hydrophobically-bound π-molecule, in which the plane of caffeine is parallel to the plane of the caffeoyl aromatic ring, and the five and six membered rings of caffeine are equally involved in the complex formation. However, other researchers believed that beside the π stacking interaction, hydrogen bonding is also responsible for the stabilization of the complex [*14]*. A proposed possible schematic representation of the complex is exhibited in Figure 1.

Theoretically, caffeine cannot be enriched in the foamate through foam fractionation, since it is non- surface-active. However, would it be quite a different story if it is assisted by complexation? Here in the present research, we tried to foam caffeine first, and then with two different catchers, chlorogenic acid and n-octylcaffeate, to illustrate the possibilities of enrichment of caffeine using foam fractionation.

MATERIALS AND METHODS

Reagents and standards. 1-Octanol was purchased from Riedel-de Haën (Seelzer, Germany). Distilled water was obtained from an in house distillation system in the department. HPLC water was obtained from a Milli-Q185 Plus device, Millipore (Darmstadt, Germany). Caffeine,chlorogenic acid and the other chemicals both for synthesis and analysis were all purchased from Sigma-Aldrich (Steiheim, Germany). All the chemicals were used without further purification.

General Experimental. Nuclear magnetic resonance (NMR) experiments were conducted on a Bruker DMX 500 spectrometer (Billerica, MA, USA).The electrospray ionization (ESI) experiments were conducted on an HCT Ultra electrospray ionization- Ion Trap Mass Spectrometerfrom BrukerDaltonics (Bremen, Germany); the high resolution mass spectrum (MS)was recorded on a Bruker Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI-FT-ICR-MS) from BrukerDaltonics (Bremen, Germany)

Preparation of caffeine-chlorogenic acid complex. The caffeine-chlorogenic acid complexwas prepared according to the method from literature [15]. Seventy-five mg of chlorogenic acid and 50 mg of potassium acetate were dissolved in ethanol (95%), and followed by 150 mg caffeine. After storage in the fridge at 0 °C for 48 hours, the mixture of the solution was filtered, anda product of light yellow needle crystal was obtained, with a yield of 65.7%. The NMR data are shown as follows: ^{1}H NMR in caffeine part (500 MHz, MeOD) δ: 3.192 (3H, s, 1-CH_3), 3.357 (3H, s, 3-CH_3), 3.817 (3H, s, 7-CH_3), 7.762 (1H, s, H-8); ^{13}C NMR in caffeine part (125 MHz, MeOD) δ: 29.259 (1-CH_3), 31.2 (3-CH_3), 34.751 (7-CH_3), 108.864 (C-5), 144.534 (C-8), 149.232 (C-4), 153.424 (C-2), 157.026 (C-6). ^{1}H NMR in chlorogenic acid part (500 MHz, MeOD) δ: 2.049, 2.247 (2H, m, H-2), 2.085, 2.192 (2H, m, H-6), 3.915 (1H, dd, *J*=3.5, 10.0 Hz, H-4), 4.295 (1H, dd, *J*=3.5, 6.5 Hz, H-5), 5.363 (1H, ddd, *J*=11.5, 6.5, 5.0 Hz, H-3), 6.187 (1H, d, *J*=16.0 Hz, H-8'), 6.715 (1H, d, *J*=8.0 Hz, H-5'), 6.803 (1H, dd, *J*=2.0, 8.0 Hz, H-6'), 6.837 (1H, d, *J*=2.0 Hz, H-2'), 7.364 (1H, d, *J*=16.0 Hz, H-7'); ^{13}C NMR inchlorogenic acid part (125 MHz, MeOD) δ: 38.787 (C-6), 39.964 (C-2), 72.251 (C-5), 72.686 (C-3), 74.404 (C-4), 78.316 (C-1), 115.405 (C-2'), 115.610 (C-8'), 116.944 (C-5'), 123.381 (C-6'), 127.727 (C-1'), 145.534 (C-3'), 146.923 (C-7'), 148.340 (C-4'), 170.292 (C-9'), 182.279 (-COOH). Our NMR data are in well consistent with the one reported [*10]*.

Synthesis of n-octylcaffeate. n-Octylcaffeatewas synthesized according to the method reported in the literature [*16]*, with little modification. 1.02 g (5.6 mmol) caffeic acidwas dissolved in 25 ml dioxane, followed by 0.6 ml (8.2 mmol) $SOCl_2$ under N_2. The mixture was stirred at 100 °Cfor 3 hours. Then 1.33 ml (8.4 mmol) 1-octanolwas added dropwise to the mixture, and stirred for another 6 hours. After the remove of the solvent under reduced pressure, the residue was subjected to a silica gel column chromatograph, using n-hexane/acetone (3:1) as a mobile phase, to give the crude caffeic acid ester: n-octylcaffeate. The pure product was obtained as a light yellow needle crystal after recrystallization from n-hexan/diethyl ether solvent system, with a yield of 41.5%. The structure was confirmed by NMR and MS experiments. The NMR data are shown as follows: ^{1}H NMR (500 MHz, MeOD) δ: 0.895 (3H, t, J=7.0 Hz, H-8'), 1.330 (10H, m, H-3' – H-7'), 1.678 (2H, m, H-2'), 4.144 (2H, t, J=6.5 Hz, H-1'), 6.252 (1H, d, J=15.5 Hz, H-8), 6.760 (1H, d, J=8.0 Hz, H-5), 6.932 (1H, dd, J=8.5, 2.0 Hz, H-6), 7.028 (1H, d, J=2.0 Hz, H-2), 7.520 (1H, d, J=15.5 Hz, H-7); ^{13}C NMR (125 MHz, MeOD) δ: 14.534 (C-8'), 23.798, 27.160, 29.879, 30.464, 30.464, 33.061 (C-2' – C-7'), 65.581 (C-1'), 114.903 (C-2), 115.027 (C-8), 116.388 (C-5), 122.992 (C-6), 127.574 (C-1), 146.813 (C-7), 146.825 (C-3), 149.602 (C-4), 169.403 (C-9). Important ESI-MS data are as follows (m/z, (fragment, %)): 291.00 ($[M-H]^-$, 100) in the ESI-MS negative mode; 315.20 ($[M+Na]^+$, 100) in the positive mode; 291.00 ($[M-H]^-$, 51), 178.80 ($[M-H-C8H16]^-$, 100), 160.80 ($[M-H-OC8H16-2H]^-$, 58), 134.80 ($[M-H-COOC8H16]^-$, 62) in the ESI-MS/MS negative mode. ESI-FT-ICR/MS for $[M-H]^-$ ($C17H23O4$): calculated 291.15964, found 291.16018.

Evaluation of foamability and foam stability. Two ml of catchers' aqueous solution (with different concentration from 10^{-7} to 10^{-3} M and pH value from 2 to 10, and a limited amount of organic solvent may added in terms of a poor water solubility) in a 5 ml volumetric flask was shaken for 1 minute. The height of the foam was recorded as the index of foamability, and afterwards, the height of the foam recorded at two minute was taken as the index of foam stability of the catcher.

Spectrophotometric titration of catchers using caffeine. As one of the unique properties of a given substance, molar absorption coefficient (E_λ, $M^{-1}\cdot cm^{-1}$)was calculated using Beer-LamberLaw and the shifts of the value during the spectrophotometric titration process was taken as a direct evidence of the formation of the complex in the solution. Caffeine stock solution was prepared by dissolving a weighted amount in the 0.1 M phosphate buffer (pH 7.0), to obtain a caffeine aqueous solution of 50 mM. A catcher solution of 0.25 mM was prepared in the same way, and limited amount of organic solvents maybe added in terms of a poor water solubility of the catcher. The mixtures for spectrophotometric titration were prepared by adding an increasing amount of caffeine stock solution into 1 ml of catcher solution, and the total volume of the test solution was maintained as 4 ml, byadding various amount of buffer solution.The absorption spectrumof each mixture wasrecorded on a UV-1800 spectrometer from Shimadzu (Tokyo, Japan), with UV Probe 2.34 software for Windows, at 0.5 nm intervals and stored in a digital form, which were converted into the form of E_λ. The spectrum was presented as a graph of molar absorption coefficient versus wavelength.

Foam fractionation apparatus and operation. The apparatus for foam fractionation adopted in the present research is illustrated in Figure 2. The foaming system is composed of six parts: N_2 tank with switch and valve; N_2 inlet copper tube with an inner diameter of 0.3 cm; flowmeter; bubble frit consisting of a glass tube and a fused porous glass end (porosity grade 3: 16 − 40 μm); foam riser or column with an inner diameter of 1.8 cm; and foam collector. In this apparatus, the foam collector is settled upon the foam riser, while the frit is fixed at the bottom of the riser. N_2 flow is inlet into the column continuously through the frit,delivered by the tube connected with the N_2 tank, controlled by a switch and valve and monitored by a flowmeter.

The stock solution of caffeine and the catchers for foam experiment was prepared by dissolving weighed amount into the distilled water, and limited amount of organic solvent was added in terms of a poor solubility of n-octylcaffeate in water. The stock solution was stored at 4 °C, and re-prepared every three days. The bulk solution for foam fractionation was obtained by dilution of the stock solution of caffeine and its catchers using distilled water, to form a solution with a certain concentration. The foaming experiment was started by inlet of N_2 continuously into the bulk solution from the bottom of the riser. The bulk solution of caffeine was foamed firstly, then the bulk solutions of caffeine and its catchers, chlorogenic acid and n-octylcaffeate, were foamed, to compare the efficiency of the catchers. To investigate the influence of parameters on the foaming efficiency, all the parameters, namely pH value, flow rate, amount of saponin, height of column, temperature and so on, were all varied in the foaming experiments. All the foam experiments were conducted at least in duplicate.

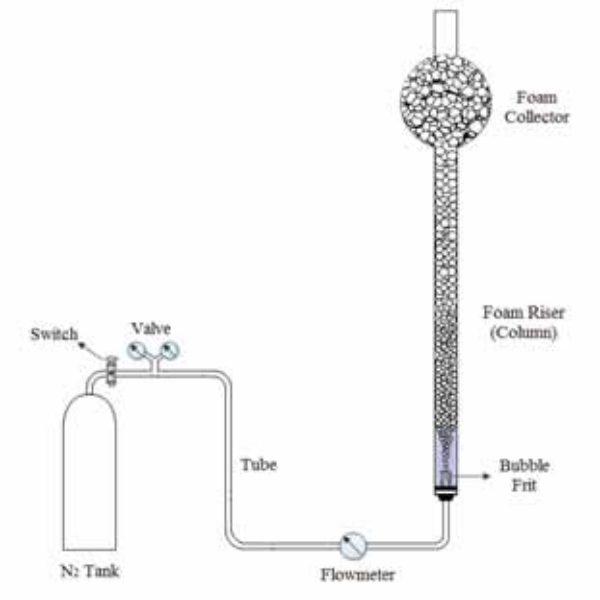
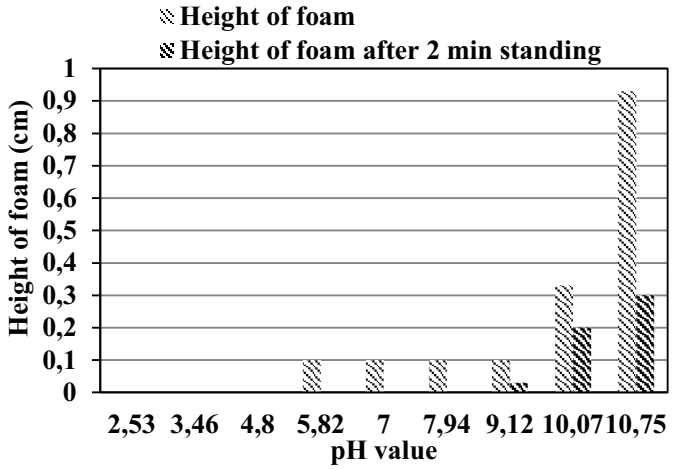
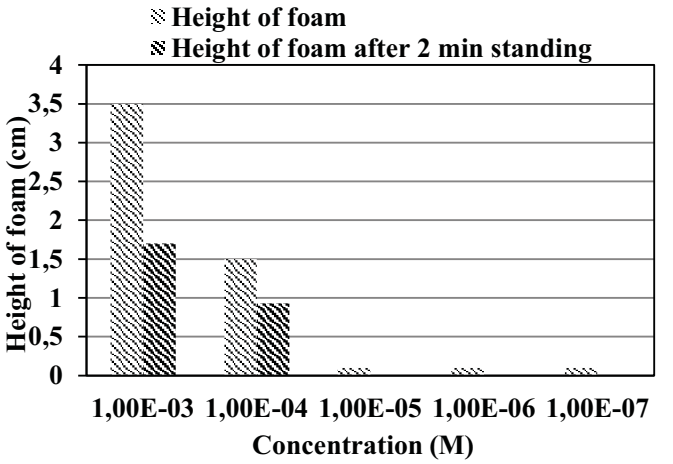


FIGURE 2
Diagram of batch mode foam fractionation apparatus.



A



B

FIGURE 3
Foam properties of n-octylcaffeate DMSO/water solution:(A)concentration, 3×10^{-5} M, depending on the pH value; (B) pH 10.5, depending on the concentration.

HPLC analysis. The HPLC analyses were conducted on an UltiMate 3000 HPLC system from Thermo Fisher Scientific (Sunnyvale, United States), which was coupled with a diode array detector (DAD) and Chromeleon 6.8 software for Windows. A Thermo ODS Hypersil column (150×4.6 mm, 5 µm) was used for the analysis. The HPLC analysis for caffeine, and caffeine-catchers, were optimized as follows:

HPLC analysis for caffeine: the flush of the column was lasted for 10 min, using a combined eluent of 75% water and 25% methanol (volume to volume, V/V), at a flow rate of 1.0 ml/min at 20 °C. Caffeine was detected at a wavelength of 280 nm.

HPLC analysis for caffeine and chlorogenic acid: the eluent was a combination of 0.1% (V/V) formic acid in water and 0.1% (V/V) formic acid in water-acetonitrile (40:50, V/V). The flush was finished in 10 min at a flow rate of 1.0 ml/min, with a decreasing amount of 0.1% (V/V) formic acid in water from 88% to 83% (V/V) at 20 °C. Two wavelengths were set to monitor caffeine and chlorogenic acid respectively: 280 nm, 320 nm.

HPLC analysis for caffeine and n-octylcaffeate: the flush of the column was conducted with a varied combination of acetonitrile and water (V/V) at a flow rate of 1.5 ml/min at 20 °C. The proportion of acetonitrile was maintained at 20% in the first 3 min, then increased to 70% at 5 min and maintained up to 10 min. Two wavelengths were set to monitor caffeine and n-octylcaffeaterespectively: 280 nm, 320 nm.

Evaluation of the foam fractionation efficiency. The efficiency of foam fractionation is evaluated by comparison of the concentration of the target components that are transferredin the foamate with those in the bulk solution before foaming. For these purpose, enrichment ratio (*ER*)was used for the comparison of the concentration in the present research:

Where:

C_f is the concentration of the target component in the foamate

C_i is the concentration of the target component in the initial bulk solution

For *ER*, a value < 1 means the concentration of the component in the foamate is lower than that in the bulk solution; therefore, no enrichment is achieved. An *ER* value > 1 means the concentration in the foamate is higher than that in the bulk solution, and the component is enriched. A high value of *ER* indicates a high foaming efficiency.

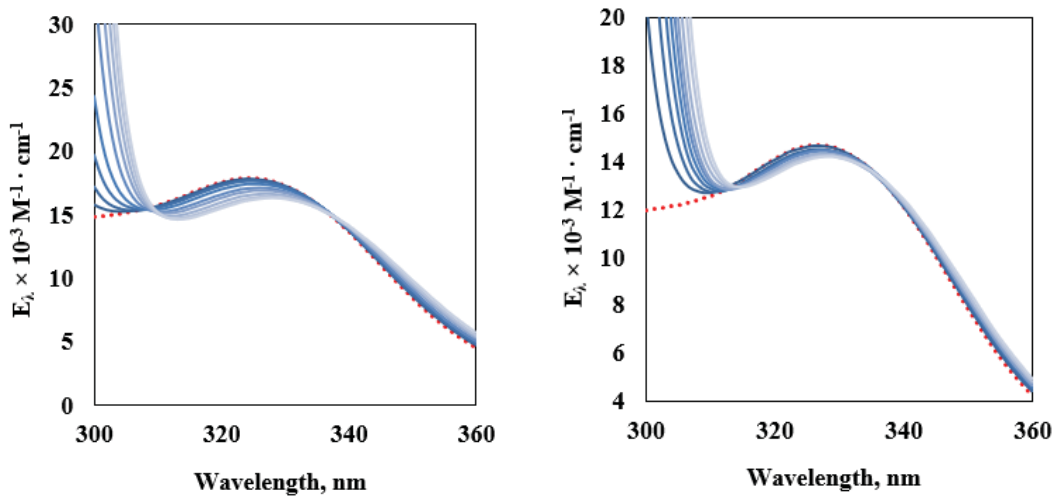
Statistical analysis. All the results were analyzed using IBM SPSS 13.0 software (Chicago, USA) to compare the means. The Duncan's Multiple Range post hoc test was selected above the other post hoc tests due to its descriptive presentation of significant differences between group means. Significance level was established at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Evaluation of the foamability and foam stability of caffeine and its catchers. The tests showed that the aqueous solution of caffeine with a different concentration (10^{-7} M to 10^{-3} M) and different pH value (from 3 to 10) was not able to produce any foam, or collapsed very quickly if any. The same situation was observed for chlorogenic acid. The foamability and foam stability of n-octylcaffeate depending on the pH value and concentration was shown as follows (Figure 3A and 3B).

Because of a poor solubility of n-octylcaffeate in water, a portion of weighted amount was dissolved in a small amount of DMSO, and then diluted with distilled water to a concentration of 3×10^{-5} M, with a pH value around 6. The pH value of the diluted solution was then adjusted to a range of 2.53 to 10.75. Both the foamability and foam stability of n-octylcaffeate solution (3×10^{-5} M) were very weak except the ones at the pH value around 10 (shown in Figure 3A). The poor solubility of n-octylcaffeate below pH 7 was indicated by tiny particles noticed in the process of pH adjusting. It was also noticed that the color of the solution changed gradually from colorless to light yellow, until light green when the pH was increased from 7 to 10.75. The evaluation of the foam properties of n-octylcaffeateat different concentrations was conducted at pH 10.5, in order to observe a best foaming performance.The results in Figure 3B indicated that the foamability and foam stability of n-octylcaffeate increased significantly with an increased concentration. However, it was proved in former research [17] that compounds such as chlorogenic acid and caffeic acid that containing a caffeic acid based structure are not stable at pH above 7. And the change of the color in our test above pH 7 from light yellow to light green could also be an indication of the n-octylcaffeate degradation. Therefore, all the experiments afterwards were conducted below pH 7.

Spectrophotometric titration of chlorogenic acid and n-octylcaffeate with caffeine. The absorption spectra of chlorogenic acid and n-octylcaffeate titrated with caffeine are presented in Figure 4A and 4B, respectively, in the form of molar absorption coefficient (E_λ). Wavelength ranging from 320 nm to 350 nm was chosen as the band to reflect the absorption changes of chlorogenic acid/n-octylcaffeateonly, as caffeine has negligible absorption over 320 nm. The bathochromic shifts (or red shifts) are clearly visible in these spectra, suggesting that aromatic chromophore interactions happened between caffeine and chlorogenic acid/n-octylcaffeate molecules, and a new absorbing component (complex of caffeine and chlorogenic acid/n-octylcaffeate) appeared in the mixture. The presence of an isosbestic point at around 336 nm in both Figure



A B

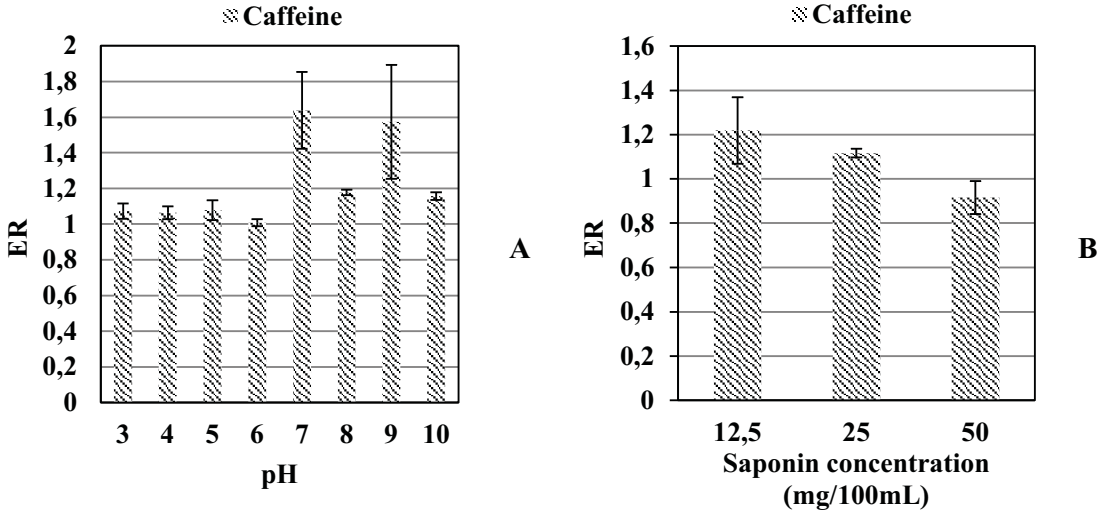
FIGURE 4

Spectrophotometric titration of chlorogenic acid and n-octylcaffeate with caffeine

(A), chlorogenic acid (initial concentration 0.042 mM) titrated with caffeine (concentration ranging from 0mM to 15 mM); (B), n-octylcaffeate (initial concentration 0.042 mM) titrated with caffeine (concentration ranging from 0mM to 31.25 mM). The fading blue solid lines represent the spectra of the mixtures with an increasing concentration of caffeine, and the dotted red lines represent the spectrum of free chlorogenic acid in (A) and free n-octylcaffeate in (B).

4A and 4Bin the spectra indicates that two components of chlorogenic acid/n-octylcaffeate were predominantly present in the mixture of the titration solution: monomer of chlorogenic acid/n-octylcaffeate and the caffeine-chlorogenic acid/n-octylcaffeate complex.

Foam fractionation experiments and foam efficiency. Foam fractionation of caffeine. For the foam experiments of caffeine aqueous solution, all the experiments were conducted based on the experimental condition: pH, 7.0; caffeine initial concentration, 1×10^{-5} M; saponin concentration, 25 mg/100ml; flow rate, 12 ml/min; height of the column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. In each series of experiments, there was one and only one parameter that was varied in a proper range, in order to evaluate its influence on the foam efficiency. The fluctuation of the enrichment ratio of caffeine based on the variation of pH value, saponin concentration, caffeine initial concentration, flow rate, height of column, temperature and concentration of NaCl were schematically represented in Figure 5A, 5B, 5C, 5D, 5E, 5F, and G, respectively.



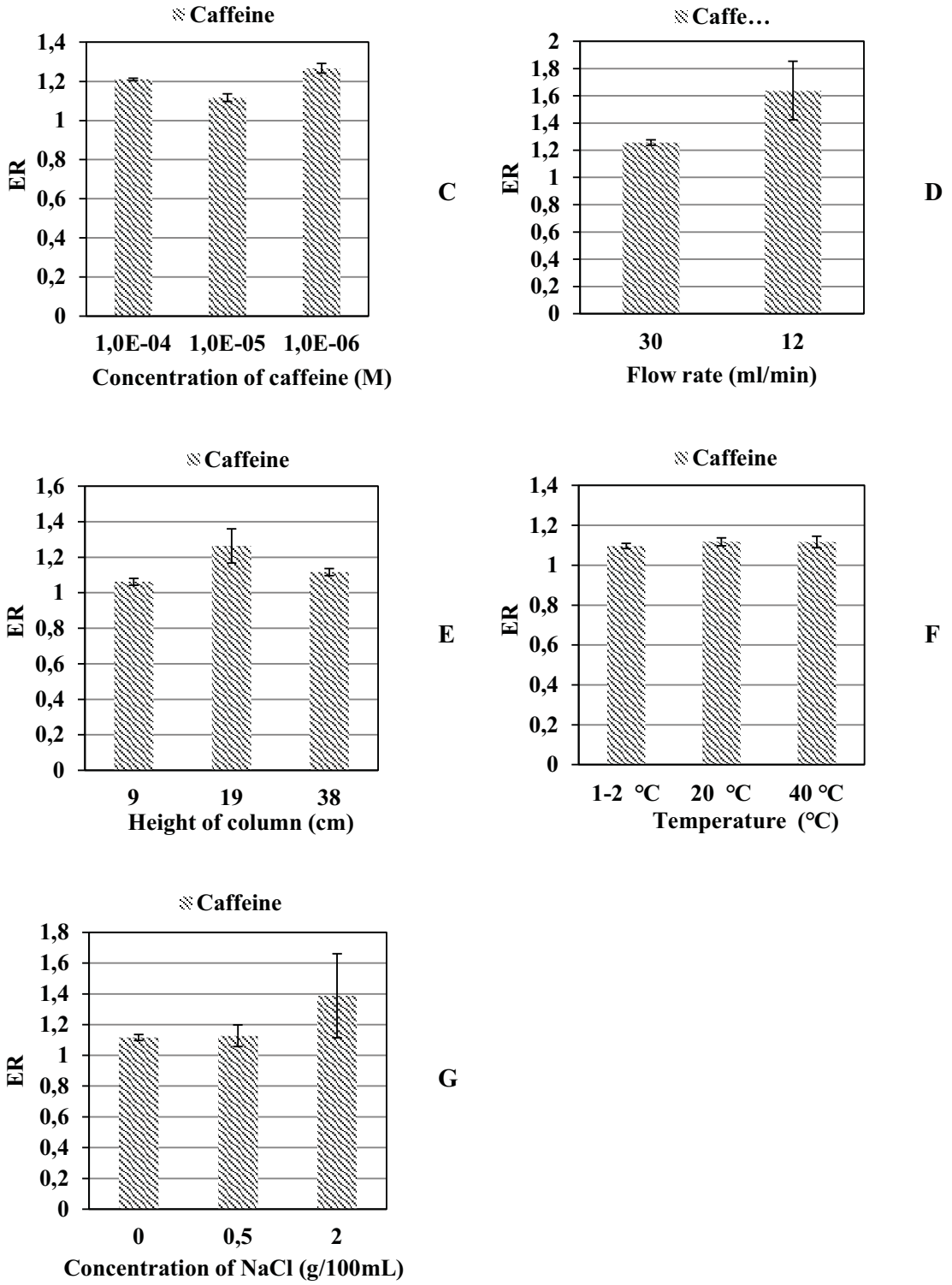


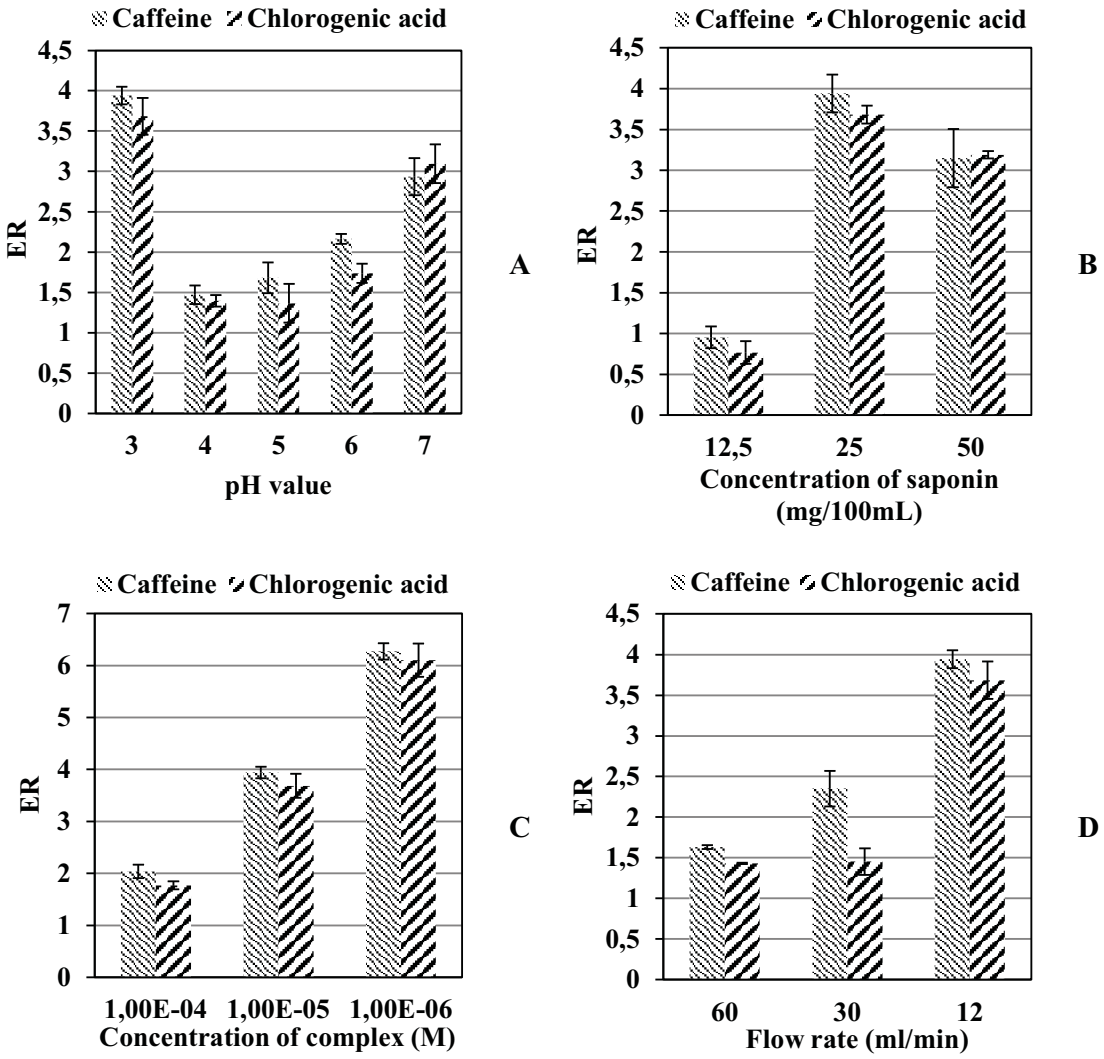
FIGURE 5

Enrichment ratio (*ER*) fluctuation of caffeine based on the variation ofdifferent parameters

pH value (A), saponin concentration (B), caffeine initial concentration (C), flow rate (D), height of column (E), temperature (F) and concentration of NaCl (G). These experiments were conducted based on the conditions as follows: pH, 7.0; caffeine initial concentration, 1×10^{-5} M; saponin concentration, 25 mg/100ml; flow rate, 12 ml/min; height of the column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. One and only one of the parameters were varied in each series of experiments.

As indicated in Figure 5, all the enrichment ratios of caffeine were around 1, which means that caffeine was poorly enriched into foam under the experimental conditions adopted here in this research. Among all the parameters investigated, only three parameters exhibited slight influence on the enrichment of caffeine: pH value, initial concentration, and flow rate(summarized in Table 1.). And these influences were negligible; even they were proved to be statistically significant.

Foam fractionation of caffeine-chlorogenic acid complex. For the foam experiments of caffeine-chlorogenic acid complex aqueous solution, all the experiments were conducted based on the experimental condition: pH, 3.0; caffeine-chlorogenic acid complex initial concentration, 1×10^{-5} M; saponin concentration, 25 mg/100ml; flow rate, 12 ml/min; height of the column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. In each series of experiments, there was one and only one parameter that was varied in a proper range, in order to evaluate its influence on the foam efficiency. The fluctuation of the enrichment ratio of caffeine and chlorogenic acid based on the variation of pH value, saponin concentration, caffeine-chlorogenic acid complex initial concentration, flow rate, height of column, temperature and concentration of NaCl were schematically represented in Figure 6A, 6B, 6C, 6D, 6E, 6F, and 6G, respectively.



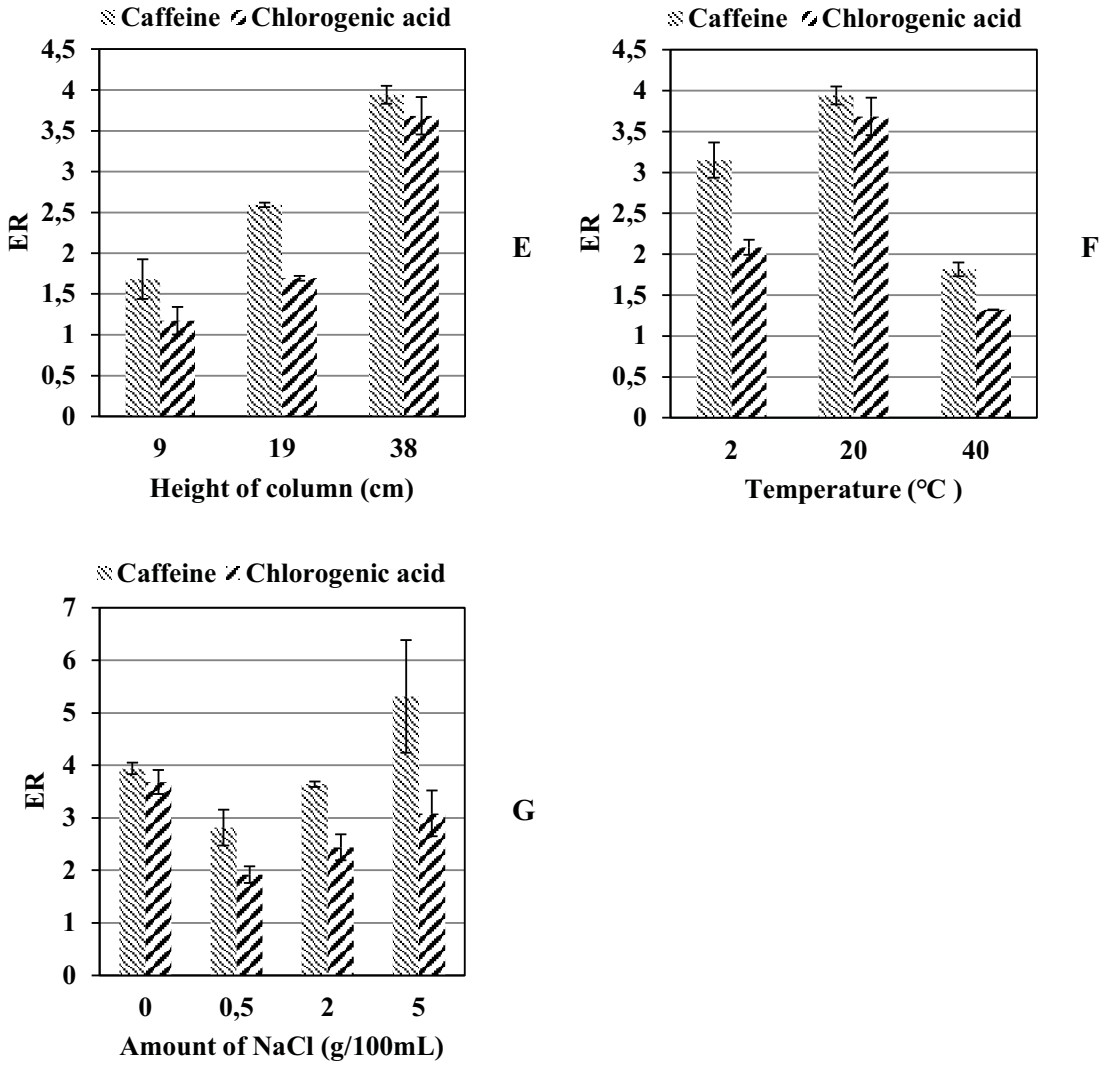


FIGURE 6
Enrichment ratio (*ER*) fluctuation of caffeine and chlorogenic acid based on the variation of different parameters

pH value (A), saponin concentration (B), caffeine-chlorogenic acid complex initial concentration (C), flow rate (D), height of column (E), temperature (F) and concentration of NaCl (G). These experiments were conducted based on the conditions as follows: pH, 3.0; caffeine-chlorogenic acid complex initial concentration, 1×10^{-5} M; saponin, 25 mg/100ml; flow rate, 12 ml/min; height of the column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. One and only one of the parameters were varied in each series of experiments.

The results exhibited in Figure 6 indicated that all the parameters investigated had evident influence on the enrichment of caffeine (also summarized in Table 1.) and chlorogenic acid, which were proved by statistical analysis.

The best foaming efficiency for both caffeine and chlorogenic acid were obtained at a pH value of 3, with an enrichment ratio of 3.94 and 3.68, respectively. At pH 4, the foaming efficiency of both caffeine and chlorogenic acid decreased to 1.5, and increased gradually to about 3 at pH 7. What very interesting shown in Figure 6A is that caffeine and chlorogenicacid are sharing a similar fluctuation trend along with the variation of pH, since the enrichment ratio for both caffeine and chlorogenic acid are very close to each other at each pH value investigated, which may indicate that caffeine and chlorogenic acid were enriched in the form of complex, but not separately.

Limited amount of saponin was added into the bulk solution, in order to produce enough foam and make the foam fractionation be in progress. The results in Figure 6B showed that the enrichment ratio

of both caffeine and chlorogenic acid were all enhanced by adding more saponin(25 mg/100 ml) into the bulk solution compared with that of 12.5 mg/100 ml. And 50 mg/100 ml was proved to be too much, since the enrichment decreased slightly compared with the ones at 25 mg/100ml. The stability of the foam is largely depending on the surface activity of the foam or the number or concentration of the surfactant on the gas-liquid interface. Bubbles with more surface-active surfactants or higher concentration of surfactants will form more stable foam, and therefore would prevent the occurrence of the drainage process by reducing the collapse and coarsening of the bubbles, and result in decreased enrichment ratio. We also noticed that the enrichment ratio of both caffeine and chlorogenic acid were quite close to each other in this series of experiments.

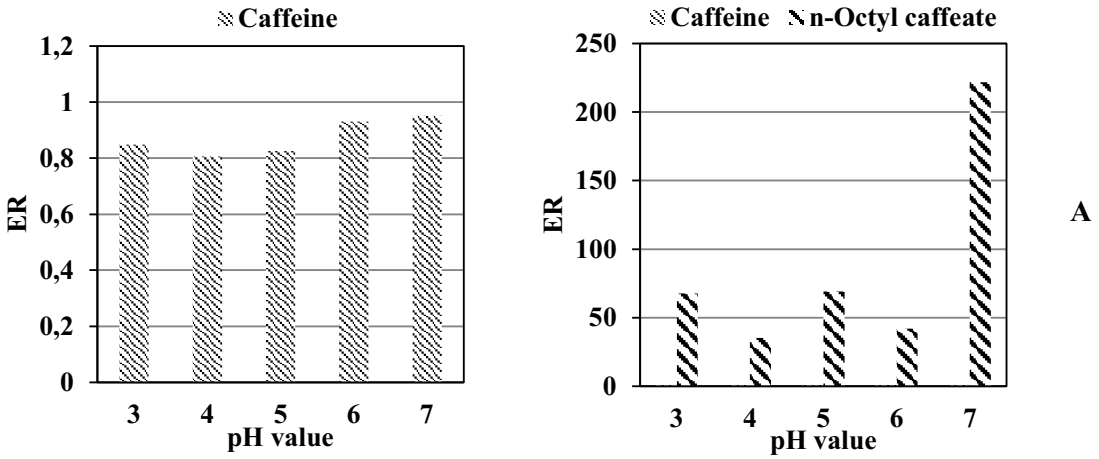
The concentration of the complex was also proved to be able to influence the enrichment of caffeine and chlorogenic acid (shown in Figure 6C). Specifically, lower concentration of the complex enhanced the foam efficiency of both: the enrichment ratio of caffeine was increased from 2.04 at the complex concentration 1×10^{-4} M to 6.27 at the complex concentration 1×10^{-6} M, while, the enrichment ratio of chlorogenic acid was also increased from 1.77 to 6.10 when the complex concentration decreased from 1×10^{-4} M to 1×10^{-6} M.

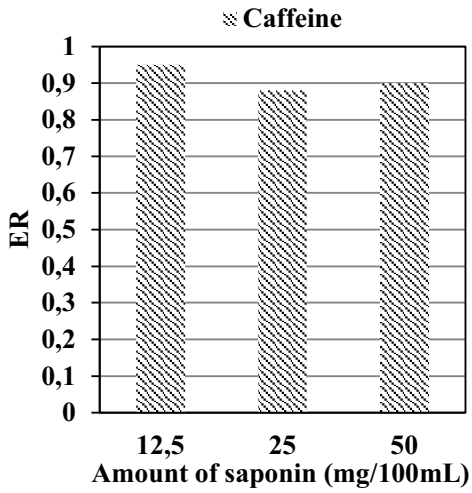
A lower flow rate was also proved to be beneficial for the foaming efficiency of both caffeine and chlorogenic acid (shown in Figure 6D): the enrichment ratio was increased from 1.63 to 3.94 for caffeine, while increased from 1.43 to 3.68 for chlorogenic acid when the flow rate decreased from 60 ml/min to 12 ml/min. Theoretically, a lower gas flow rate provides more time for drainage, and therefore, giving more time for the liquid flowing back

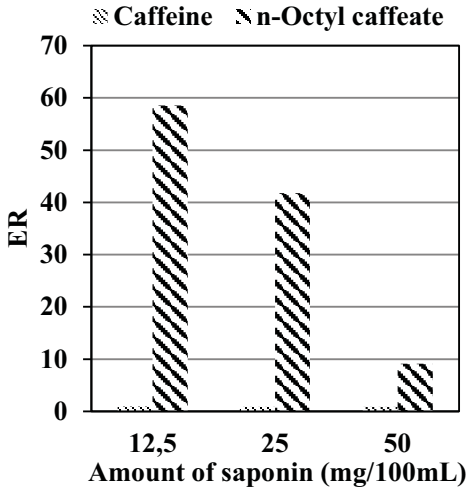
TABLE 1
Enrichment of caffeine when foamed alone or with different catchers, depending on different operating parameters

	Caffeine alone	Chlorogenic acid	n-Octylcaffeate
pH	7 (1.64) [a] 9 (1.57)	3 (3.94)	— [c]
Saponin amount (mg/100ml)	—	25 (3.94) [b] 50 (3.15)	—
Caffeine initial Concentration (M)	1×10^{-6} (1.27)	1×10^{-6} (6.27)	—
Flow rate (ml/min)	12 (1.64)	12 (3.94)	—
Height of column	—	38 (3.94)	—
Temperature (°C)	—	20 (3.94)	—
NaClconcentration (mg/100ml)	—	5 (5.31)	—
Total	(3.01)	(11.22)	N.D.

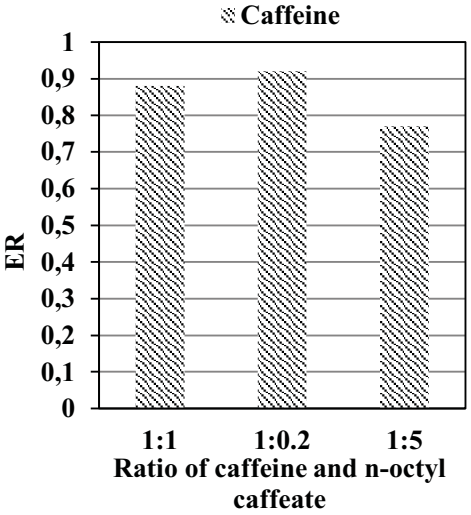
[a] The value before the brackets is the value of the parameter, at which the foam experiment was conducted, and the value in the brackets is the enrichment ratio of caffeine obtained at the value of the corresponding parameter;
[b] these values of enrichment ratio in the same cell were proved to be no difference statistically;
[c] —, no enrichment was obtained for caffeine; N.D., not done; and the enrichment with a same value (such as 3.94 and 2.72) was obtained in the same foaming experiments, which was done first and used as a reference for the comparison later on.

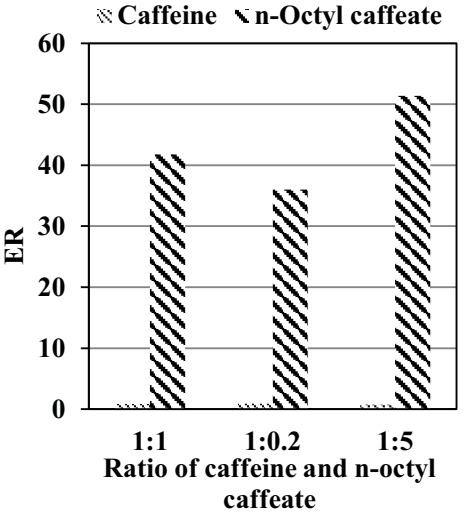



Caffeine
ER
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
12,5
25
50
Amount of saponin (mg/100mL)

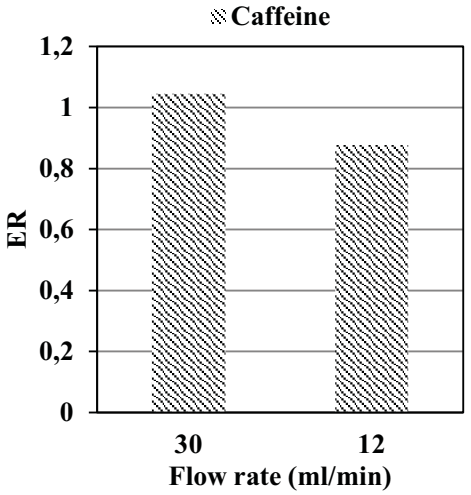

Caffeine
n-Octyl caffeate
ER
70
60
50
40
30
20
10
0
12,5
25
50
Amount of saponin (mg/100mL)

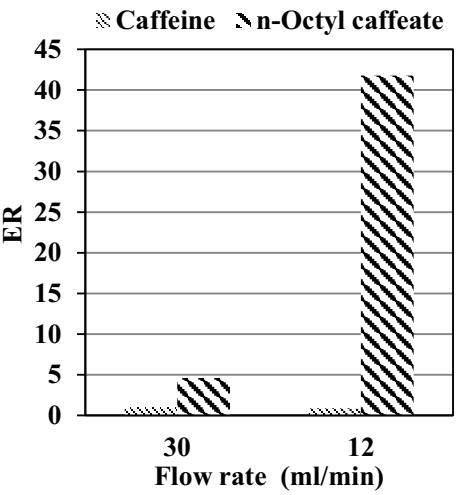
B


Caffeine
ER
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
1:1
1:0.2
1:5
Ratio of caffeine and n-octyl caffeate


Caffeine
n-Octyl caffeate
ER
60
50
40
30
20
10
0
1:1
1:0.2
1:5
Ratio of caffeine and n-octyl caffeate

C


Caffeine
ER
1,2
1
0,8
0,6
0,4
0,2
0
30
12
Flow rate (ml/min)


Caffeine
n-Octyl caffeate
ER
45
40
35
30
25
20
15
10
5
0
30
12
Flow rate (ml/min)

D

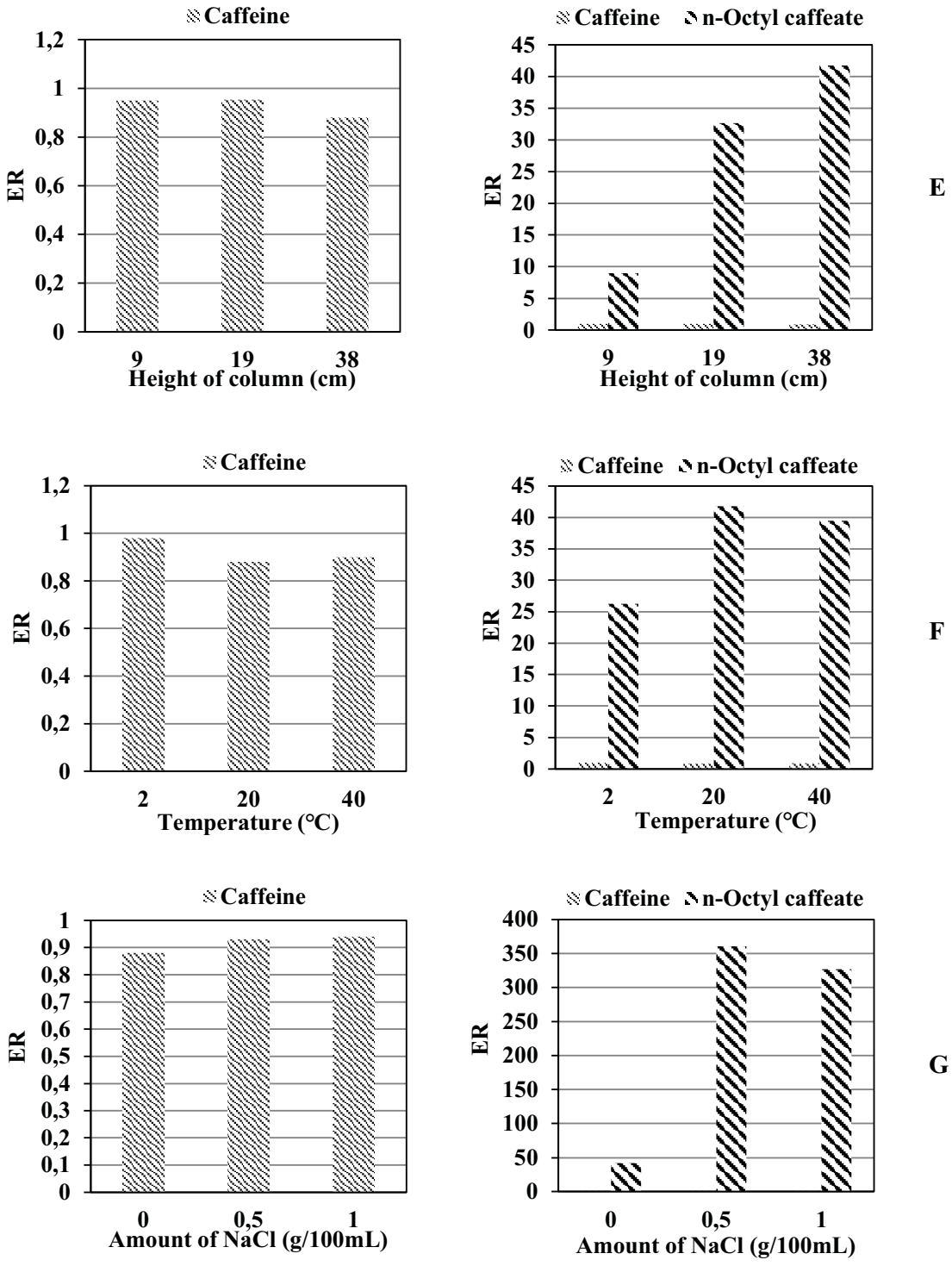
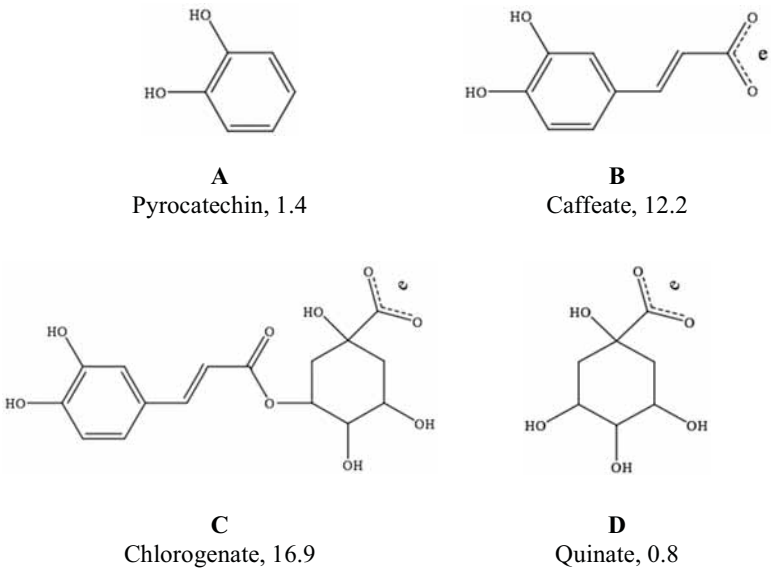


FIGURE 7
Enrichment ratio (*ER*) fluctuation of caffeine and n-octylcaffeate based on the variation of different parameters

pH value (A), saponin concentration (B), ratio of caffeine and n-octylcaffeate (C), flow rate (D), height of column (E), temperature (F) and concentration of NaCl (G). These experiments were conducted based on the conditions as follows: pH, 5.8; ratio of caffeine and n-octylcaffeate 1 to 1, based on a caffeine concentration of 1×10^{-5} M without change; saponin concentration, 25 mg/100ml; flow rate, 12 ml/min; height of the column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. One and only one of the parameters were varied in each series of experiments.



A
Pyrocatechin, 1.4

B
Caffeate, 12.2

C
Chlorogenate, 16.9

D
Quinate, 0.8

FIGURE 8
Association constants for the formation of complexes between caffeine and compounds studied (13).

to the bulk solution, but leaving the surface-active targets in the foam phase. However, foam fractionation with a further decrease of the flow rate wasnot conductedsuccessfully, since it was not able to maintain the production of enough foam, or took too much time to produce enough foam for collection.

As shown in Figure 6E, the enrichment ratio of caffeine and chlorogenic acid were both increased at the column length of 38 cm, compared with those at a column length of 9 cm.A longer column also provides more time for drainage, and as a result, is beneficial for the enrichment. Therefore, it is quite possible that an even longer column would enhance the enrichment ratio of both caffeine and chlorogenic acid further.

In terms of the influence of bulk solution temperature, a better enrichment was obtained at around the room temperature, 20 ℃, compared with the situation at 2 ℃ or 40 ℃. Temperature has proved to have a very complicated influence on the foam fractionation: it may affect the stability of foam through many aspects, such as adsorption, surface elasticity and viscosity. A lower temperature (20 ℃) would increase the elasticity and viscosity of the solution, and as a result, the bursting and drainage would be decreased, which would depress the efficiency of foam fractionation. On the contrary, a higher temperature (40 ℃) may decrease the stability of the foam, which is also not favorable for foam fractionation. Here in our experiments, the complexation may also be influenced by temperature. Since the temperature may influence the stability of π stacking interaction as well as hydrogen bonding interaction [13, 14], which are mainly responsible for the formation and stability of the caffeine-chlorogenic acid complex.

Beside the temperature, ion concentration will also affect the formation of the hydrogen bond, and therefore will have an influence on the stability of the complex. High concentration of ions in the aqueous solution will depress the formation of the hydrogen bond, and hence decrease the stability even the total amount of the complex. Meanwhile, these ions will also enhance the adsorption of the components at the gas-liquid interface, and therefore enhance the foaming efficiency of both caffeine and chlorogenic acid. Here in this research, the enrichment ratio of both caffeine and chlorogenic acid decreased when 0.5 g/100ml NaCl was added into the initial solution, and then both increased when more NaCl was added, while thedifference of enrichment ratio between them was enlarged. Apparently, the result observed here is a joint consequence of the impactsof both hydrogen bonding depression and adsorption enhancement caused by the increaseof ion concentration in the initial bulk solution: the depression of the hydrogen bonding decreased the stability of the complex, and consequently, the enrichment of caffeine and chlorogenic acid as a complexdecreased(ion concentration 0.5 g/100ml); however, the increase of the ion concentration in the initial bulk solution enabled the impact of the adsorption enhancement to dominate in the enrichment process gradually.As a result, both the enrichment ratios were increased but with different extent,since caffeine and chlorogenic acid were mainly not enriched as a complex but separately according to their different properties.

Since all the parameters investigated were proved to have an influence on the foam efficiency of caffeine and chlorogenic acid, further foam experiments combined all the optimized parameters were conducted: pH value, 3; saponin amount, 25 mg/100ml; caffeine-chlorogenic acid initial concentration, 1×10^{-6} M; flow rate, 12 ml/min; column

height, 60 cm, temperature, 20 °C, and NaCl concentration, 5 g/100ml. After 45 min foaming, an enrichment ratio of 11.22 and 7.9 for caffeine and chlorogenic acid were obtained, respectively.

Foam fractionation of caffeine and n-octylcaffeate. For the foam experiments of caffeine and n-octylcaffeate aqueous solution (with limited amount of DMSO to improve the solubility), all the experiments were conducted based on the experimental condition: pH, 5.8; ratio of caffeine and n-octylcaffeate 1 to 1, based on a caffeine concentration of 1×10^{-5} M without change; saponin concentration, 25 mg/100ml; flow rate, 12 ml/min; height of the column, 38 cm; initial solution volume, 40 ml; temperature, 20 °C. In each series of experiments, there was one and only one parameter that was varied in a proper range, in order to evaluate its influence on the foam efficiency. The fluctuation of the enrichment ratio of caffeine and n-octylcaffeatebased on the variation of pH value, saponin concentration, ratio of caffeine and n-octylcaffeate, flow rate, height of column, temperature and concentration of NaCl were schematically represented in Figure 7A, 7B, 7C, 7D, 7E, 7F, and 7G, respectively.

As indicated in Figure 7, most of the values of enrichment ratio of caffeine were below 1 when it was foamed with n-octylcaffeate under all the conditions investigated. An enrichment ratio below 1 means that caffeine was not able to enriched at all, which is even worse than that when caffeine was foamed alone. Compared with caffeine, n-octylcaffeate exhibited very high enrichment efficiency. The highest enrichment ratio for n-octylcaffeate (221.7) was obtained at a pH value of 7 in the experiments of pH variation. Saponin was also added into the initial bulk solution since n-octylcaffeate was not able to produce enough foam for the experiments. The best enrichment ratio for n-octylcaffeate was obtained when 12.5 mg/100mlsaponin was added, and the enrichment decreased significantly when more saponin was added. Besides, a lower concentration of n-octylcaffeate (caffeine:n-octylcaffeate = 1:5), lower flow rate (12 ml/min), higher column (38 cm), a temperature of 20 °C, and a NaCl concentration of 0.5 g/100ml, were all proved to increase the enrichment ratio of n-octylcaffeate.

The results of the foam experiments conducted in this research were generalized in Table 1. It is quite clear that the enrichment ratio of caffeine behaved significantly different when it was foamed alone, with chlorogenic acid or n-octylcaffeate.

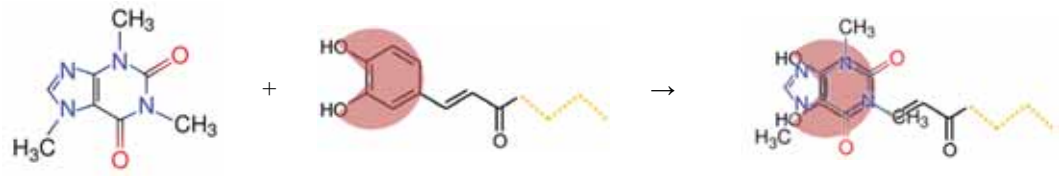
Only three parameters, namely pH, initial concentration and flow rate, were proved to have a slight influence on the enrichment of caffeine when caffeine was foamed alone. Quite different situation happened when caffeine was foam with different catchers: all the parameters were proved to have a moderate influence on the enrichment of caffeine when caffeine was foamed with chlorogenic acid, while no parameters influenced the enrichment ratio of caffeine when it was foamed with n-octylcaffeate.

As mentioned in the former part in this paper, the complex between caffeine and chlorogenic acid was isolated almost one century ago, and it was believed that the complex is a 1:1 hydrophobically bound π-molecular. Besides the hydrophobic interaction, hydrogen bonding was also believed to stabilize the complex [13, 14]. In the research of Horman and Viani, pyrocatechin, caffeate ion, chlorogenate and quinate were investigated for their ability to complex with caffeine in aqueous solution, and a corresponding association constant of 1.4, 12.2, 16.9 and 0.8 was obtained respectively (Figure 8.). They ascribed the enhancement of the association ability of chlorogenate, compared with caffeate, to the stabilizing effect contributed by the hydrogen bonding induced by quinate moiety, which was only weakly complex with caffeine. Actually, the hydrogen bonds in the complex of caffeine and chlorogenic acid are both contributed by the hydroxyls from the caffeic acid moiety and the quinic acid moiety in chlorogenic acid [14].

It is not difficult to understand now that the absence of the quinic acid moiety in n-octylcaffeate would certainly decrease the stability of the complex formed with caffeine or attenuate the interaction between them. As a result, the complex between caffeine and n-octylcaffeate are more likely to be foamed separately but not together in the form of complex. Meanwhile, the strong hydrophobicity of the 8-carbon alkyl group in n-octylcaffeate make it adsorbed at the gas-liquid interface and enriched in the foam highly effectively, but leaving caffeine molecules in the bulk solution, as the results indicated in Figure 7.

Here in the present research, we illustrated the possibilities of the enrichment of caffeine, a non-surface-active compound,from a diluted aqueous solution using complexation based foam fractionation. Similar with other regular foam fractionation experiments, a lower concentration of the molecule, a lower flow rate, a longer column, and the increase of ionic strength was proved to begenerally beneficial for the enrichment of the target molecules.Compared with the foam experiments of caffeine alone, the formation of a complex between caffeine and chlorogenic acid enhanced the enrichment ratio of caffeine moderately. This result indicates us the importance of the hydrogen bonding in the stabilization of the complex between caffeine and chlorogenic acid, and meanwhile addressed the prerequisite of the foam experiments assisted by complexation: stability of the complex. A “catcher” that is both highly surface-active and is able to complex with the target molecule firmly is needed in the trials afterwards.

GRAPHIC FOR TABLE OF CONTENTS



Caffeine "Catcher" Complex that can be enriched by foam fractionation

ABBREVIATIONS

Nuclear magnetic resonance, NMR; Electrospray ionization, ESI; Mass spectrum, Mass spectrometer, MS; Fourier Transform Ion Cyclotron Resonance Mass Spectrometry, FT-ICR-MS; Molar absorption coefficient, E_λ; Enrichment ratio, *ER*; Volume to volume, V/V.

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