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Evaluation of *in vitro* antioxidant activities of traditional fermented non-alcoholic beverages from Turkey

B. Ceylan*

Department of Pharmacognosy, Faculty of Pharmacy, Harran University, 63050 Haliliye-Sanlıurfa, Turkey

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The aim of this study was to evaluate the *in vitro* potential of different solvent extracts of shalgam juice, hardaliye, boza, ayran (yoghurt drink) and kefir as natural antioxidants. The originality of this study was that different solvents were used for extraction, and according to the extraction yields, total phenolic and flavonoid contents of the extracts and antioxidant activity was determined. Liquid-liquid extraction was applied for sample preparation, which is the preferred extraction technique today due to its simple, fast and efficient procedure to determine antioxidant capacity. The antioxidant capacities of the acetone, ethanol and water extracts of traditional fermented non-alcoholic beverages were estimated using different antioxidant tests, including lipid peroxidation, 1,1-diphenyl-2-picrylhyrazyl (DPPH$^{\bullet}$) free radical scavenging, superoxide anion radical scavenging, 2,2$^{`}$-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS$^{\bullet+}$) cation scavenging activity, hydrogen peroxide scavenging activity and cupric reducing capacity. Results showed that the highest contents of the target components including phenols and flavonoids were found in the water extract. The latter was found to be richer in antioxidant phytochemicals such as phenolics (189.33±2.77 mg PEs/g FW) and flavonoids (321.77±4.03 mg QEs/g FW). This study verified that the water extract with its high level of phenolics and flavonoids can be used as a source of potential antioxidants or functional food materials.

Keywords: Antioxidant activity, Fermented beverages, Phenolic compound, Scavenging activity, Food antioxidant

INTRODUCTION

Free radicals are one of the most important causes of deterioration of food products during processing and storage and are claimed to play an important role in affecting human health by causing many diseases (such as cancer, hypertension, heart attack and diabetes) [1-3]. Dietary intake of phenolic compounds and fermented food products is associated with these diseases and is protective in many health-related properties such as antioxidant, anticancer, antiviral, anti-Alzheimer, antidiabetic and anti-inflammatory activities [4]. Vegetable products and fermented food products are rich sources of antioxidants and are used as food additives to prevent oxidative degradation of fats and oils in processed foods and are compounds that increase shelf life and delay the lipid peroxidation process [5, 6].

Fermentation is one of the oldest (humans consumed 'sour milk' about 2000 years ago) and one of the most economical methods used in food preservation. The beneficial health effects of fermented foods and dairy products on humans are: increased mineral bioavailability, digestibility of proteins and carbonhydrates [7]. In accordance with the awareness of consumers, the trend towards Turkish fermented non-alcholic beverages (shalgam juice, hardaliye, boza, ayran and kefir) has increased, with natural (or slightly processed), high nutritional (due to probiotic properties) and health promoting value. The former ones (shalgam juice, hardaliye and boza) are obtained from vegetables, fruits and cereals, and the latter two (ayran and kefir) are made of milk. Shalgam juice, hardaliye and ayran are produced by lactic fermentation. In boza and kefir, both alcoholic and lactic fermentation occur [8].

In this study the antioxidant activities of acetone, ethanol and water extracts of non-alcoholic beverages (shalgam juice, hardaliye, boza, ayran and kefir) which can be an alternative to synthetic antioxidants (BHA, BHT and α-tocopherol) used in removing free radicals, were investigated using different methods (β-carotene/linoleic acid bleaching assay, ABTS$^{\bullet+}$ cation radical scavenging, DPPH$^{\bullet}$ free radical scavenging assays, superoxide anion radical scavenging, hydrogen peroxide scavenging activity, cupric reducing antioxidant capacity assay). In addition, the extraction method (liquid-liquid extraction) used provides superiority compared to previous studies because it is simple, fast and highly efficient. This study can help in food industry as a natural compound for antioxidant activity, which might be used as an alternative to synthetic antioxidants since it is environmentally friendly and safe for consumption.

* To whom all correspondence should be sent:
E-mail: b.ceylan022@gmail.com

EXPERIMENTAL

Chemicals and reagents

Linoleic acid, α-tocopherol, potassium persulfate, nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), pyrocatechol, quercetin and 3-(2-pyridyl)-5,6-*bis*(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) were obtained from Sigma-Aldrich GmbH, Sternheim, Germany.

Ammonium thiocyanate, ferrous chloride, polyoxyethylenesorbitan monolaurate (Tween-20), tricholoroacetic acid (TCA), ethanol (EtOH) and acetone were purchased from Merck. All other chemicals used were of analytical grade, obtained from either Sigma-Aldrich or Merck. Water was puridifed by Human (Japan) ultrawater purification system.

Material and extraction procedures

Fermented non-alcoholic beverages were purchased from local stores. Since the beverages are in liquid form, they were homogenized (by shaking) before use and used directly without any other pre-treatment. For the preparation of the extracts, 25 mL of the beverage (shalgam juice, hardaliye, boza, ayran and kefir) was incubated in 500 mL of solvent (acetone, ethanol and water) at room temperature (25 ºC) at 100-150 rpm in a shaking water bath for 3 hours. The obtained extracts were filtered through filter paper (Whatman No.1 paper) and the solvents of the filtrates (acetone and ethanol) were evaporated in a rotary evaporator (Büchi R-200, Switzerland) at 40-80 ºC. The resulting water extracts were filtered through filter paper and the filtrate was lyophilized (at 5 μm Hg pressure at – 50 ºC [Labconco, Freezone 1 L]). All extracts were kept at -20 ºC and dissolved in water or solvent before use.

Total phenolic and flavonoid contents

The total phenolic [9] and flavonoid [10] contents of the analysed samples were calculated as equivalent to pyrocatechol and quercetin, respectively. The following equations were used to calculate the total phenolic and flavonoid contents of fermented non-alcoholic beverage extracts:

Absorbance = 0.0413x + 0.0440 pyrocatechol (μg) (r^2 = 0.9975)
Absorbance = 0.0362x + 0.0172 quercetin (μg) (r^2 = 0.9975)

Antioxidant activity

In order to determine the antioxidant activity of the sample six methods were applied: β-carotene/linoleic acid bleaching assay [11], ABTS cation radical scavenging [12], DPPH free radical scavenging assay, superoxide anion radical scavenging [13], hydrogen peroxide scavenging activity [14], CUPRAC (Cupric reducing antioxidant capacity) assay [15]. In oder to calculate IC_{50} (50% inhibition) values of the samples 100, 50, 25 and 10 μg/mL of their concentrations were prepared. The smallest concentration value (10 μg/mL) is the minumum IC_{50} value that can be calculated. That is, at concentrations lower than the smallest concentration (10 μg/mL), the IC_{50} cannot be calculated. In these six antioxidant test methods, BHA, BHT and α-tocopherol were used as standards.

Statistical analysis

Power analysis was performed to determine the number of fermented non-alcoholic beverage extracts. The outcomes were presented as means ± standard deviation (n=3 per each test sample).

RESULTS AND DISCUSSION

Extraction yield, total phenolic and flavonoid contents

The percentage yields of fermented non-alcoholic beverage extracts are shown in Table 1. The highest extraction efficiency was observed in water extracts. The percent extraction yields of the water extracts varied between 38.65% and 30.26%. So the water extract resulted in a higher amount of total extractable compounds. Phenolics or polyphenols are food secondary metabolites and are important by virtue of their antioxidant acitivty by chelating redox-active metal ions, inactiating lipid free radical chains and preventing hydroperoxide conversions into reactive oxyradicals. Polyphenols are known as markers of the nutritional quality of foods. Polyphenols are known for their antioxidant activity as radical scavengers having possible beneficial roles in human health, such as reducing the risk of cancer, cardiovascular disease, and other pathologies. Fermented non-alcoholic beverages containing high amounts of phenolic compounds can be a good source of antioxidants. For this reason, this information has led to the determination of the total phenolic content of the sample under study [16]. The total phenolic content of the ethanol extracts varied between 163.61±0.94 and 110.63±1.58 μg PEs/mg extract. The highest total phenolic content of ethanol

extracts was found in shalgam juice extract (163.61±0.94 μg PEs/mg extract).

Flavonoids are natural phenolic compounds and well known antioxidants. Therefore, dietary intake of flavonoid-containing foods was suggested to be beneficial for preservation from free radical damage. Total flavonoid content of acetone extracts ranged from 210.18±1.83 to 171.90±2.28 μg QEs/mg extract. The highest total flavonoid content of acetone extracts was found in hardaliye extract (210.18±1.83 μg QEs/mg extract). These amounts were comparable with the results described in the literature for other extracts of plant and fruit products [17].

Antioxidant activity

Total antioxidant activity determination

Total antioxidant activity of fermented non-alcoholic beverage extracts was determined by the thiocyanate method (β-carotene/linoleic acid bleaching assay [11]). Fermented non-alcoholic beverages water extracts (shalgam juice, hardaliye, boza ayran and kefir) exhibited effective antioxidant activity.

The effect of the same amounts of water extracts of fermented non-alcoholic beverages (100 μg/mL) on the peroxidation of β-carotene-linoleic acid emulsion are shown in Fig. 1. The effects on lipid peroxidation (IC_{50} value) of linoleic acid emulsion of extracts and standards decreased in the order: KFW (6.78±0.79) > BZW (8.81±1.10) > AYW (11.63±1.40) > HRW (14.66±1.88) > SHW (15.38±1.72) > α-tocopherol (30.34±2.20) > BHT (33.96±2.58) > BHA (35.37±2.08). In previous studies, Ertaş *et al.* reported that the water extract (153.05±0.21) exhibited very strong activity in the β-carotene/linoleic acid bleaching assay [18]. But our results showed stronger activity than this value. The total antioxidant activitiy of fermented non-alcoholic beverages water extracts may be attributed to their chemical composition and the phenolic acid content demonstrated that some bioactive compounds and milk products present in raw materials possessed high total antioxidant activity which was due to the presence of phenolics, carotenoids and flavonoids.

Table 1. Extraction yields and contents of total phenols, total flavonoids in fermented non-alcoholic beverage extracts

Fermented non-alcoholic beverage	Extraction solvent	Abbreviation	Extraction yield (%)	Total phenolic content (μg PEs/mg extract)[a]	Total flavonoid content (μg QEs/mg extract)[b]
Shalgam juice	Acetone	SHA	12.63	133.84±2.82	196.36±2.61
	Ethanol	SHE	21.56	163.61±0.94	284.13±3.93
	Water	SHW	32.44	154.13±1.32	314.43±3.86
Hardaliye	Acetone	HRA	14.38	122.54±2.12	210.18±1.83
	Ethanol	HRE	19.23	145.80±1.38	261.16±3.55
	Water	HRW	38.65	189.33±2.77	321.77±4.03
Boza	Acetone	BZA	11.36	102.29±1.18	188.59±1.99
	Ethanol	BZE	20.88	152.55±3.82	280.69±2.80
	Water	BZW	34.10	177.59±2.43	311.66±3.23
Ayran	Acetone	AYA	10.83	98.35±0.71	171.90±2.28
	Ethanol	AYE	17.68	110.63±1.58	252.90±2.94
	Water	AYW	30.26	146.24±3.40	303.97±3.52
Kefir	Acetone	KFA	13.71	118.66±0.79	202.40±2.12
	Ethanol	KFE	22.16	151.37±1.74	296.33±4.40
	Water	KFW	33.75	166.21±0.87	318.39±2.41

[a]Phenolic content equivalent to pyrocatechol (y=0.021x+0.0396 R^2=0.9993)
[b]Flavonoid content equivalent to quercetin (y=0.021x+0.0396 R^2=0.9993)

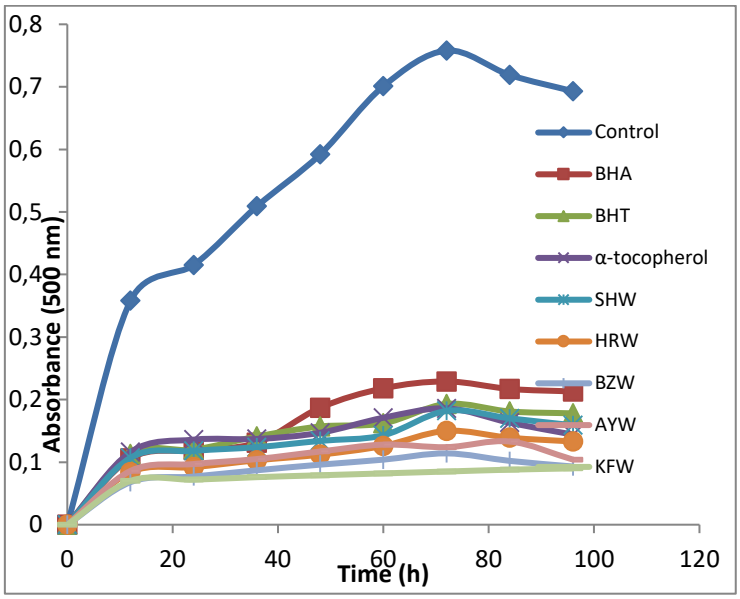

Fig. 1. Inhibitory effect of the water extracts from fermented non-alcoholic beverages on lipid peroxidation. BHA, BHT and α-tocopherol were used as reference antioxidants. Values are means ± SD (n=3)

Table 2. IC_{50} values of DPPH$^{\bullet}$ free radical scavenging activity, ABTS$^{\bullet+}$ cation radical scavenging activity, hydrogen peroxide scavenging activity and superoxide anion scavenging activity of fermented non-alcoholic beverage water extracts (100 µg/mL)

		IC_{50} (µg/mL)		
Extracts and standards	Scavenging ability on DPPH$^{\bullet}$ free radicals	Scavenging ability on ABTS$^{\bullet+}$ cation radicals	Scavenging ability on hydrogen peroxide	Scavenging ability on superoxide anions
SHW	27.84±1.52	17.19±0.13	40.56±3.66	33.15±1.14
HRW	33.67±1.33	12.95±1.10	45.31±2.93	35.88±1.29
AYW	30.88±2.11	14.42±0.06	43.18±2.47	37.60±1.34
BZW	36.45±0.49	21.23±1.75	47.24±2.18	31.27±2.13
KFW	34.35±1.48	22.30±2.01	44.61±3.41	34.83±1.58
BHA	45.80±2.53	38.27±1.49	60.19±2.30	50.24±2.89
BHT	48.28±2.45	40.33±2.52	61.73±1.22	52.33±2.21
α-tocopherol	51.53±3.19	42.81±2.85	64.60±2.32	55.20±2.55

Values are given as the mean and standard deviation of three parallel measurements.

Table 3. CUPRAC test assay of the fermented non-alcoholic beverage water extracts and standards

	Concentrations			
Extracts and standards	10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
SHW	0.150±0.030	0.318±0.085	0.463±0.120	0.808±0.170
HRW	0.123±0.028	0.333±0.074	0.452±0.142	0.826±0.186
AYW	0.135±0.034	0.328±0.060	0.473±0.134	0.859±0.191
BZW	0.142±0.041	0.311±0.077	0.488±0.125	0.840±0.193
KFW	0.130±0.022	0.320±0.091	0.424±0.121	0.820±0.172
BHA	0.352±0.064	0.501±0.110	0.716±0.142	1.215±0.221
BHT	0.385±0.067	0.516±0.119	0.755±0.153	1.236±0.210
α-tocopherol	0.321±0.072	0.511±0.128	0.747±0.150	1.225±0.213

Values are given as the mean and standard deviation of three parallel measurements.

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

The $ABTS^{\bullet+}$ method is widely employed for measuring the relative radical scavenging activity of hydrogen donating and chain breaking antioxidants in many food extracts. $ABTS^{\bullet+}$ cation scavenging activity is best presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of $ABTS^{\bullet+}$ cation present in the test solution (Table 2). A higher $ABTS^{\bullet+}$ cation radical scavenging activity is associated with a lower IC_{50} value. IC_{50} values for SHW, HRW, AYW, BZW, KFW, BHA, BHT and α-tocopherol on $ABTS^{\bullet+}$ radical scavenging activity were found as 17.19, 12.15, 14.42, 21.23, 22.30, 38.27, 40.33, 42.81 µg/mL, respectively. In previous studies, Kolak *et al*. found the IC_{50} value of the $ABTS^{\bullet+}$ cation radical scavenging activity of the compounds they isolated as 78.68±1.32 µg/mL [20]. Fermented non-alcoholic beverages water extracts showed similar $ABTS^{\bullet+}$ cation radical scavenging acitivites compared to the $ABTS^{\bullet+}$ cation radical scavenging activity of the standards.

$DPPH^{\bullet}$ free radical scavenging assay

Antioxidant properties, especially radical scavenging activites, are very important due to the deleterious role of free radicals in foods and in biological systems. Excessive formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance. The model of scavenging the stable $DPPH^{\bullet}$ is that the stable free radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule.

$DPPH^{\bullet}$ free scavenging activity is best presented by the IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of $DPPH^{\bullet}$ present in the test solution (Table 2). A higher $DPPH^{\bullet}$ radical scavenging activity was associated with a lower IC_{50} value. IC_{50} values for SHW, HRW, AYW, BZW, KFW, BHA, BHT and α-tocopherol on $DPPH^{\bullet}$ free radical scavenging activity were found as 27.84, 33.67, 30.88, 36.45, 34.35, 45.80, 48.28, 51.53 µg/mL, respectively. In previous studies, Mavi *et al.* reported that *S. sempervivoides* showed very strong activity - 88.0% inhibition in the $DPPH^{\bullet}$ free radical scavenging assay method at 200 µg/mL concentration [19]. Fermented non-alcoholic beverage water extracts showed similar $DPPH^{\bullet}$ free radical scavenging acitivites compared to the $DPPH^{\bullet}$ free radical scavenging activity of the standards.

Hydrogen peroxide scavenging activity

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells, since it may rise to hydroxyl radicals inside the cell. IC_{50} values for SHW, HRW, AYW, BZW, KFW, BHA, BHT and α-tocopherol on hydrogen peroxide scavenging activity were found as 40.56, 45.31, 43.18, 47.24, 44.61, 60.19, 61.73, 64.60 µg/mL, respectively (Table 2). In previous studies, Yeşiloğlu *et al*. reported that the water extract (78.89±1.3%) exhibited very strong activity in hydrogen peroxide scavenging [21]. Our results are in agreement with previous studies. Fermented non-alcoholic beverages water extracts showed similar hydrogen peroxide radical scavenging acitivites compared to the hydrogen peroxide scavenging activity of the standards.

Superoxide anion radical scavenging activity

Superoxide is a reactive oxygen species, which can cause damage to cells and DNA,bthus leading to various diseases. It was, therefore, proposed to measure the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical. IC_{50} values for SHW, HRW, AYW, BZW, KFW, BHA, BHT and α-tocopherol on superoxide anion radical scavenging activity were found as 33.15, 35.88, 37.60, 31.27, 34.83, 50.24, 52.33, 55.20 µg/mL, respectively (Table 2).

In previous studies, Yeşiloğlu *et al*. reported that the water extract (18.2%) exhibited moderate activity in hydrogen peroxide scavenging [22]. Our results showed very high activity, compared to previous studies. Fermented non-alcoholic beverages water extracts showed similar superoxide radical scavenging acitivites compared to the superoxide anion radical scavenging activity of the standards.

CUPRAC (Cupric reducing antioxidant capacity) assay

The CUPRAC antioxidant determination method was studied at four different concentrations (10, 25, 50, 100 µg/mL) (Table 3). An increase in activity was observed in direct proportion to the increase in concentration. It was found that the water extracts showed moderate activity than the standards. But showed very strong activity compared to other studies. In previous studies, Orak *et al.* found the results of the cupric reducing antioxidant capacity method of dichloromethane, ethanol and methanol extracts of *A. muricata* L. at 100 µg/mL concentration as 0.143±0.020, 0.136±0.060 and 0.063±0.040, respectively [23].

CONCLUSION

Natural antioxidants in fermented products can be used to reduce the harmful effects of free radical

species. Synthetic antioxidants such as BHA and BHT can be used, but the use of these molecules is risky. Therefore, in recent years, restrictions have been imposed on the use of synthetic antioxidants in many countries. Therefore, interest in natural antioxidants has increased and related research has gained momentum. The water extracts of fermented non-alcoholic beverages exhibited different levels of antioxidant activity in all the models studied. In the β-carotene/linoleic acid bleaching assay, SHW (15.38±1.72) showed the closest activity to the standards. In the $ABTS^{\bullet+}$ cation scavenging activity assay, KFW (22.30±2.01) showed the closest activity to the standards. BZW extract showed the highest activity compared to other extracts in DPPH free radical scavenging assays, superoxide anion radical scavenging, and hydrogen peroxide scavenging activity experiments. In the CUPRAC assay, all extracts showed moderate activity compared to the standards. The results revealed that the fermented non-alcoholic beverages had significant antioxidant activity and free radical scavenging activity. The free radical scavenging property may be one of the mechanisms by which these products or beverages are useful as foodstuffs, as well as traditional medicines. However, further investigation of individual compounds, their *in vivo* antioxidant activities and participation in different antioxidant mechanisms is needed. It was concluded that fermented non-alcolic beverages can be used as natural antioxidant sources.

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