Utilization of lyophilized *Cosmos caudatus* extract as additive in green tea bag to improve its total phenolic contents, antioxidant capacity, physicochemical and sensory properties

*Reihani, S. F. S., Tan, T.C. and Easa, A. M.

Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

Abstract

Different brands of commercial green tea beverages exhibit different total phenolic contents (TPC) and antioxidant activities ($TEAC_{DPPH}$). In the present study, lyophilized *Cosmos caudatus* (ulam raja) extract (UREX) was added to different brands of commercial green tea bags at two levels; 25 mg (GT-LUREX) and 50 mg (GT-HUREX) per sachet as an additive for green tea and its effects on their TPC, $TEAC_{DPPH}$, physicochemical and sensory attributes were investigated. The addition of UREX increased yellowness and chroma values of the green tea beverages significantly (P<0.05), however, lightness was significantly (P<0.05) decreased and no influence on the clarity and turbidity was observed. TPC and $TEAC_{DPPH}$ were significantly (P<0.05) higher in GT-HUREX when compared to GT-LUREX and control. Sensory attributes of GT-HUREX were significantly (P<0.05) improved. Based on the obtained results, UREX can enhance the nutraceutical and functional properties of green tea beverage, as well as its physical and sensory attributes.

Introduction

A direct result of modern life style in developed and developing nations is the increase of cancer, diabetes and cardiovascular diseases (Feskanich *et al.* 2000; Haegele *et al.* 2000; Michels *et al.* 2000). Consistent plant-based diet is believed to assist in preventing degenerative diseases, delaying the sign of aging and improving physical fitness (Key *et al.* 2002). It is now a well known fact that plant-based diets offer nutritional benefits and therapeutic values which are connected with the presence of phytochemical contents, such as flavonoids, lignans, steroids and carotenoids, in plants (Liu *et al.* 2007; Raju *et al.* 2007).

The health beneficial properties of tea *(Camellia sinensis)* have raised its popularity as a functional food or beverage (Graham, 1992). In 2012, the world tea production reached to ~4.68 million metric tons of tea (Statista, 2013). It is the second most common beverage in the world and a rich source of dietary phenolics responsible for its functionalities (Rijken *et al.* 2002). Green tea is consumed mainly in East and South East Asia, especially in China, Japan and Taiwan. It contains mainly flavanols epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) (Jia *et al.* 1998;

Rijken *et al.* 2002; Khan and Mukhtar, 2007). Flavonoids, such as quercetin, theaflavin, rutin, taxifolin and kaempferol, are other contributors of the antioxidant activities in tea (Rijken *et al.* 2002). Green tea flavonoids are not only bio-available, but their functionalities in animal models and humans have also been demonstrated (Choi *et al.* 2001; Rezai-Zadeh *et al.* 2005; Bastianetto *et al.* 2006).

About 2,000 herbs and medicinal plants are identified in Malaysia owing to its rich tropical climate. More than 120 species of these plants have been regarded as fresh salad or traditionally known as ulam. Ulam are freshly eaten salads from various plant families with health beneficial properties (Husain *et al.* 2004). *Cosmos caudatus* (Ulam raja) is considered to be one of the most popular traditional ulam with anti-aging and antioxidant properties (Shui *et al.* 2005; Reihani and Easa, 2012). Nevertheless, urbanization has gradually led to a decline of its consumption. Ulam raja has been reported to be rich in proanthocyanidins, chlorogenic acids, quercetin and its derivatives (Shui *et al.* 2005; Andarwulan *et al.* 2010; Mediani *et al.* 2012). The most abundant flavonoid constituents in ulam raja are quercetin glycosides (Abas *et al*, 2003; Andarwulan *et al.* 2010; Shui *et al*, 2005). It has been suggested that quercetin and its derivatives could prevent osteoporosis

*Corresponding author.
Email: *far.reihani@gmail.com*
Tel: 60-4-6532268; Fax: 60-4-6573678

(Hegarty *et al.* 2000), play a crucial role in the prevention and treatment of peptic ulcer (Alarcon de La Lastra *et al.* 1994) and non-bacterial chronic prostatitis and prostodynia (Shoskes *et al.* 1999; Valentova *et al.*, 2014). Quercetin is also known to influence some carcinogenesis markers (Knekt *et al.* 1997; Caltagirone *et al.* 2000; Soobrattee *et al.* 2006).

Green tea and ulam raja appeared to have similarities in their functional fractions and chromatographic profiles (Shui *et al.* 2005; Andarwulan *et al.* 2010; Khan *et al.* 2011). Nevertheless, quercetin has been reported to be the least constituent among the 10 marker compounds in green tea leaves (Khan *et al.* 2011). Commercial tea products from different sources in the world, exhibited significantly different TPC and antioxidant activities (Chan *et al.* 2007; Chan *et al.* 2010; Dutta *et al.* 2013). Therefore, the aim of the present study was to prepare green tea fortified with a lyophilized ulam raja extract (UREX), containing a high level of quercetin constituents (36.90 mg/g of UREX). The effects of UREX addition on the TPC, antioxidant capacity, physicochemical and sensory attributes of the green tea beverages were investigated.

Materials and Methods

Chemicals

2,2-diphenyl-1 picrylhydrazyl (DPPH·), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid and sodium carbonate were purchased from Sigma Aldrich Chemicals (St. Louis, USA). Folin–Ciocalteu phenol reagent was purchased from R and M Chemicals (Essex, UK), while hydrochloric acid and glacial acetic acid were obtained from Lab Scan Asia Co., Ltd. (Bangkok, Thailand) and QRëC Chemical Co., Ltd. (Chonburi, Thailand), respectively. Methanol was purchased from Systerm (Analytical grade, Selangor, Malaysia) and Merck (HPLC grade, Darmstadt, Germany).

Preparation of UREX

UREX was prepared according to the method reported by Reihani *et al.* (2014). Briefly, fresh ulam raja leaves were purchased from an ulam supplier in Penang; washed thoroughly and the leaves were dried at 45°C for 48 h in a convection dryer (AFOS, Mini Kiln, East Yorkshire, UK). The fully dried ulam raja leaves were then ground into powder using a dry blender (Panasonic, MX 335, Malaysia). UREX was prepared by adding 14 g of dried powder into 100 mL of deionized water and allowed for 30 min at 85°C with occasional shaking for the extraction

to occur. The extract was filtered through filter paper (Whatman, No. 541, Wycombe, UK) and the filtrate was freeze-dried (Millrock Technology, LD53, Kingston, USA) to obtain lyophilized powder. The obtained UREX powder was vacuum packed and stored at −18°C (Toshiba, GR-M48MP, Minato-ku, Japan) until further analysis.

Commercial green tea samples

Two brands of commercial green tea (brand B and L) were purchased from 15 different stores around Penang, Malaysia to conduct the preliminary phase of the study, i.e. comparison of TPC and antioxidant capacity between different brands of commercial green tea. After the preliminary comparison study, three commercial samples consisting of tea leaf bags of three different brands (brand B, D and L) were purchased from stores around Penang Island, Malaysia. The amounts of tea leaf content in each sachet were as 1.5 g in brands B and D, while 2 g in brand L.

Different levels of UREX were added to commercial green tea beverage in order to assess the effect of dosage on the TPC and antioxidant capacity of the tea beverage. Samples were designated as Control: green tea without addition of UREX; GT-LUREX: green tea added with a low level of UREX (25 mg per sachet); GT-HUREX: green tea added with a high level of UREX (50 mg per sachet). Therefore, nine different samples were designated comprising B-control, B-LUREX, B-HUREX; D-control, D-LUREX, D-HUREX; L-control, L-LUREX, L-HUREX. Three replicates per sample were prepared to report mean values of the results. Samples of green tea beverage were prepared according to the common procedure of brewing tea i.e. each sachet was placed in a beaker containing 200 mL of boiling water and allowed to stand for 2 min for brewing.

Total phenolic contents (TPC)

The Folin–Ciocalteu method (Wong *et al.* 2006) with some modifications was used to assess the TPC of the samples. An aliquot (100 µL) of sample was mixed with 2.5 mL of Folin–Ciocalteu phenol reagent (10 times diluted). After 5 min, 2.5 mL of saturated Na_2CO_3 solution was added and allowed to stand for 1 h. The absorbance of the reaction mixture was read at 740 nm after 1 h. Samples were analyzed in triplicates and results were averaged. TPC of samples were calculated using a gallic acid calibration curve with five different concentrations within the range of 1.6–1.8 mM and R^2 greater than 0.98. Results were expressed as mg gallic acid equivalent (GAE)

/ sachet of green tea.

Antioxidant capacity (TEAC$_{DPPH}$)

A method described by Brand-Williams *et al.* (1995) was used with some modifications in order to measure the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) scavenging activity. In this assay, the concentration of DPPH methanolic solution was determined and monitored during the required time after addition of antioxidants. Based on this method, a reduction in concentration of DPPH due to the existence of antioxidants takes place and the absorption at 515 nm gradually disappears. Antioxidant capacity of each sample was determined by using an UV-Vis spectrophotometer (Shimadzu, Ultraspec 1601, Nakagyo-ku, Japan). Initial absorbance of the DPPH solution (0.1 mM) was measured at 515 nm and the absorbance was monitored at the same wavelength throughout the period of assay. An aliquot (20 μL) of sample was added to 1.5 mL of methanolic DPPH solution. The change in absorbance at 515 nm was monitored at 30 min intervals until the reaction curve reached the plateau. Samples were analyzed in triplicates and results were averaged. The antioxidant capacity was expressed as μmol trolox equivalent (TE)/sachet of green tea.

Physicochemical properties: colour, clarity and turbidity

Colourimetry was performed using a colorimeter (Konica Minolta, CM-3500d, Chiyoda, Japan) according to the CIE $L^*a^*b^*$ scale. The instrument was calibrated prior to use. The colour measurement resulted in CIE $L^*a^*b^*$ values for lightness (L^*, L = 100 is white and L = 0 is black), redness (a^*, + red to − green component) and yellowness (b^*, + yellow to − blue component). The tea sample was placed on a petri dish (Konica Minolta, CM-A128 Petri Dish, Chiyoda, Japan). The sample was illuminated with D65-artificial daylight (10° standard angle) according to conditions provided by the manufacturer.

The clarity of the samples was determined by measuring the absorbance at 670 nm with Ultraspec 1601 UV-Vis spectrophotometer, and the results were expressed by the transmittance (%T). Turbidity of the tea samples was determined using a turbidimeter (Hach, 2100P, Loveland, USA). Distilled water was used as blank and turbidity value was expressed in Nephelometric Turbidity Units (NTU) (Singh *et al.* 2013).

Sensory evaluation

One commercial brand of green tea (Brand B) added with UREX was selected for sensory evaluation. Control, LUREX and HUREX samples were analyzed by using the 7-points hedonic scale (1 = Dislike very much, 4 = Neither like or dislike, 7 = Like very much). Green tea samples were presented warm (~70ºC) in a randomized order. The sensory panellists comprised of 30 students of the Food Technology Division, Universiti Sains Malaysia, Penang, Malaysia. Pungent food was avoided before the test. Drinking water was used to rinse the mouth between sample analyses. The evaluated attributes included colour, aroma, taste, astringency, bitterness and overall acceptability.

Statistical analysis

Analysis of variance (ANOVA) and Duncan's test for multiple comparisons were used for analyzing the data. SPSS version 18 (SPSS, Chicago, USA) was used for statistical analysis. Pearson correlation analysis was also carried out to determine the correlation between TPC and DPPH scavenging ability of the extracts.

Results and Discussion

Comparison of TPC and antioxidant capacity (TEAC$_{DPPH}$) in different brands

A preliminary study was designed to show the variation of TPC and TEAC$_{DPPH}$ in commercial green tea. Two selected brands of commercial green tea (brand B and L) from 15 different market sources in Penang, Malaysia were analyzed. Results are shown in Figure 1 for TPC and TEAC$_{DPPH}$. As shown in Figure 1A, the brand B had an average value of TPC equivalent to 86.29 ± 7.18 mg GAE/sachet, while brand L had an average value of 57.82 ± 4.99 mg GAE/sachet. Relative standard deviations (RSD) for both samples were calculated and recorded as 8%. On the other hand, as shown in Figure 1B the mean values of TEAC$_{DPPH}$ in brands B and L were 136.87 ± 6.22 and 95.23 ± 4.44 mmol TE/sachet (n=15, RSD=4%), respectively. As illustrated in Figure 1, there are consistent differences in TPC and TEAC$_{DPPH}$ values between the two selected brands of commercial green tea available in markets of Penang Island.

These findings were in agreement with the findings reported by Chan *et al.* (2007), Chan *et al.* (2010) and Dutta *et al.* (2013). In a comparison study, Chan *et al.* (2007) reported that different values of TPC and antioxidant activities were found in various brands of commercial tea from Malaysia. Recent study by Dutta *et al.* (2013) showed that TPC of two different brands of commercial green tea from Bangladesh were reported to be significantly different; 103 vs
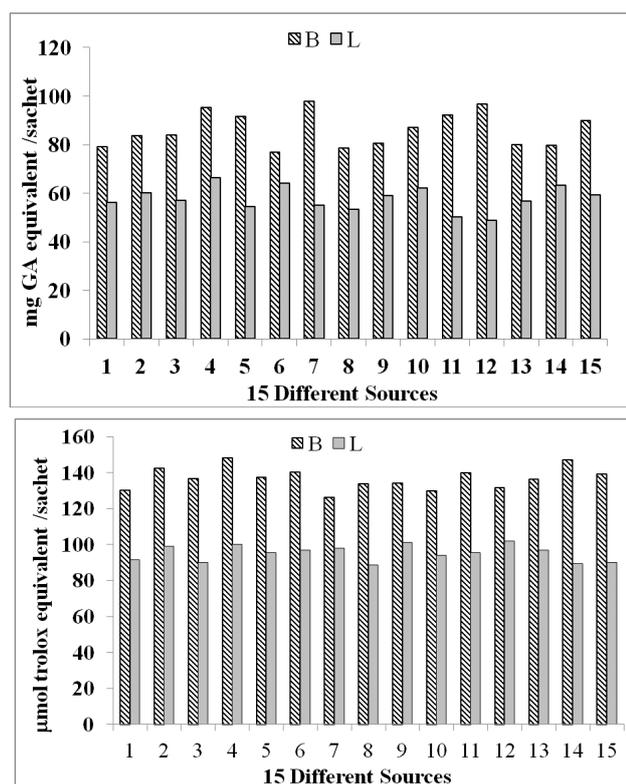
Figure 1. Comparison of (A) total phenolic contents (TPC) and (B) antioxidant capacity (TEAC$_{DPPH}$) of commercial green tea beverage, brand B (downward diagonal bars) and L (grey bars)

Table 1. Total phenolic contents (TPC) and antioxidant capacity (TEAC$_{DPPH}$) of commercial green tea beverage

Tea brands	Added UREX	TPC (mg GA/sachet)	TEAC$_{DPPH}$ (µmol Trolox/sachet)
B (1.5 g/sachet)	Control	94.50 ± 1.64[b]	166.78 ± 2.74[c]
	LUREX	94.50 ± 0.43[b]	170.84 ± 2.09[b]
	HUREX	97.02 ± 0.10[a]	174.07 ± 2.73[a]
D (1.5 g/sachet)	Control	69.36 ± 0.74[c]	153.57 ± 4.30[c]
	LUREX	72.40 ± 1.42[b]	157.25 ± 3.07[b]
	HUREX	81.60 ± 4.60[a]	164.38 ± 3.42[a]
L (2 g/sachet)	Control	49.50 ± 0.76[b]	120.84 ± 2.32[b]
	LUREX	57.06 ± 1.02[a]	122.13 ± 3.76[b]
	HUREX	59.16 ± 3.53[a]	129.02 ± 2.47[a]

Control: without UREX; LUREX: lower level of UREX (25 mg/sachet); HUREX: Higher level of UREX (50 mg/sachet). Comparison within the columns for each brand was shown in the table with the data written as mean ± standard deviation (n=3). Means followed by different lower superscript letter (a-c) are statistically significant at the 5% level.

59 mg GAE/g of green tea. This could be due to the variation in the tea leaves maturity and the drying method used during tea leaves processing (Chan *et al.* 2007). In addition, variation in TPC values in green tea beverages could also be partly due to cultivar and climate that may influence the structure of phenolics and bioactive compounds (Zheng and Wang, 2001; Bolling *et al.* 2010).

Effect of UREX on the TPC and TEAC$_{DPPH}$ of green tea beverages

All the three commercial samples of green tea (brand B, D and L), in which HUREX (50 mg UREX per sachet) was added (i.e. B-HUREX, D-HUREX and L-HUREX) showed significantly higher (P<0.05) TPC compared to their controls (Table 1). However, TPC of brand B and D were not affected (P>0.05) by the addition of LUREX (25 mg UREX per sachet). In spite of containing more amount of tea leaf in brand L, addition with LUREX affected its TPC in considerably higher extents (Table 1). Although all of the three brands showed a similar trend, i.e. increases in TPC level in GT-HUREX, their TPC values at the first place ranged considerably different, from as low as 49.50 mg GA per sachet (B-control) to as high as 94.50 mg GA per sachet (L-control).

Antioxidant capacity of samples was measured by TEAC$_{DPPH}$. This assay is based on the reduction of DPPH radicals in methanol, which causes an absorbance drop at 515 nm. Based on this procedure, the TEAC$_{DPPH}$ of samples may directly be compared against trolox. As shown in Table 1, the addition of HUREX caused a significant (P<0.05) increase in TEAC$_{DPPH}$ of all three commercial green tea beverages. TEAC$_{DPPH}$ value was ranged from 120.84 (brand L) to 166.78 (brand B) µmol trolox per sachet in controls. Nevertheless, using LUREX had no significant impact (P>0.05) on any of the three commercial samples. Similar to TPC value, brand L showed a lower value of TEAC$_{DPPH}$ in spite of its higher amount of tea leaf per sachet (2 g).

UREX was reported to be a potent natural antioxidant and was utilized successfully to enhance the frozen storage stability of beef patties by controlling lipid oxidation (Reihani *et al*. 2014). Thus, its significant influence on the TPC and TEAC$_{DPPH}$ of green tea beverages (at level of 50 mg per sachet) confirmed its potential as a compound with a dominant antioxidant activity properties.

Correlations

Compounds with a high TPC value are generally expected to exhibit high antioxidant activities (Zheng and Wang, 2001; Bolling *et al.* 2010). However, these two assays show no certain correlations in many studies (Wong *et al.* 2006; Tulipani *et al.* 2008; Reihani and Easa, 2012). In this study, correlation analysis was conducted individually for the three brands of commercial green tea beverages. Based on the result of the present study, a significant (P=0.02<0.05) correlation (0.74) between TEAC$_{DPPH}$ and TPC values was observed only in one out of three commercial brands, i.e. brand D. This correlation implied that compounds present in tea leaves are capable of donating hydrogen atoms to DPPH• and

Table 2. Colour parameters, clarity and turbidity of commercial green tea beverage

Brands	Samples	CIE L*	CIE b*	Chroma	Clarity (%T at 670 nm)	Turbidity (NTU)
	Control	85.15 ± 1.76[a]	31.94 ± 0.30[c]	32.01 ± 0.23[c]	82.96 ± 1.01[a]	9.23 ± 0.10[a]
B	LUREX	82.56 ± 2.41[ab]	44.61 ± 2.72[b]	44.65 ± 2.69[b]	83.34 ± 1.02[a]	9.19 ± 0.32[a]
	HUREX	79.16 ± 2.43[b]	53.68 ± 3.17[a]	53.71 ± 3.22[a]	81.63 ±2.01[a]	9.26 ± 0.41[a]
	Control	85.35 ± 1.44[a]	32.98 ± 0.52[c]	40.31 ± 0.43[c]	73.17 ± 1.02[b]	9.52 ± 0.14[a]
D	LUREX	82.75 ± 1.57[ab]	50.30 ± 2.04[b]	50.46 ± 1.96[b]	70.52 ± 2.01[b]	9.64 ± 0.06[a]
	HUREX	79.61 ± 1.82[b]	59.09 ± 2.47[a]	58.43 ± 2.78[a]	78.86 ± 3.02[a]	9.69 ± 0.10[a]
	Control	81.41 ± 0.92[a]	48.57 ± 2.66[c]	48.63 ± 2.69[c]	74.33 ± 0.02[a]	9.45 ± 0.11[a]
L	LUREX	77.97 ± 1.59[b]	58.40 ± 0.96[b]	58.42 ± 1.00[b]	74.35 ± 0.01[a]	9.24 ± 0.26[a]
	HUREX	75.22 ± 1.77[b]	64.20 ± 0.98[a]	64.27 ± 1.11[a]	71.08 ± 0.33[b]	9.59 ± 0.04[a]

Control: without UREX; LUREX: lower level of UREX (25 mg/sachet); HUREX: higher level of UREX (50 mg/sachet).
Comparison within the columns for each brand was shown in the table with the data written as mean ± standard deviation (n=3). Means followed by different lower superscript letter (a-c) are statistically significant at the 5% level.

are also reactive towards the Folin–Ciocalteu reagent. It should be emphasized that TPC may not correlate perfectly with antioxidant activity assays as the presence of certain reducing agents, such as amino acids, carbohydrates and ascorbic acid, which are not antioxidant compounds, can affect the correlation results (Singleton *et al*. 1999; Escarpa and Gonzalez, 2001; Tulipani *et al*. 2008).

Effect of UREX on the physicochemical properties of green tea beverages

The yellow color in green tea is mainly due to the presence of flavonols, such as quercetin, isoquercetin, myricetin and rutin, that consisted between 1.3 to 1.5% of the tea leaves in dry weight basis (Chaturvedula *et al*. 2011). Some flavones (0.02% of the tea leaves in dry weight) including apigenin and isovitexin, as well as their glycosides, also contribute to the yellow color in green tea (Chaturvedula *et al*. 2011). Color of commercial samples of GT-UREX were being significantly (P<0.05) affected in GT-LUREX and GT-HUREX (Table 2). GT-HUREX had lower CIE *L** value in all the three commercial brands tested in the present study. In other words, addition of HUREX led to darker samples. On the other hand, GT-LUREX showed no significant (P>0.05) changes in lightness when compared to control. Nevertheless, yellowness (CIE *b** value) and chroma of all three commercial brands were significantly (P<0.05) affected in GT-LUREX and GT-HUREX. Color saturation could be attributable to the natural yellow pigments of flavonoid compounds in UREX employed (Shui *et al*. 2005; Andarwulan *et al*. 2010; Mediani *et al*. 2012). Yellowness ranged from 31.94 to 48.57 in B-control and L-control, respectively. Other physicochemical

properties, i.e. clarity and turbidity of commercial green tea beverages were not significantly (P>0.05) influenced by the addition of UREX.

Sensory evaluation

The results demonstrated that the addition of UREX enhanced the color, TPC and TEAC$_{DPPH}$ of commercial green tea beverages from different brands in a consistent and similar manner (Table 1 and Table 2). Therefore, only one brand of commercial green tea was used for conducting the sensory evaluation, i.e. brand B. The sensory attributes can be counted as the main indicator of the acceptance of the GT-UREX since the sensory scores reflect the perception of tea consumers towards the product. Except for aroma, all sensory attributes of GT-HUREX scored higher than 5 (Table 3), which indicated a good score for acceptability. As seen in Table 3, GT-HUREX were considered significantly (P<0.05) more acceptable compared to GT-LUREX and control. Moreover, taste and bitterness of GT-HUREX scored higher than 6, indicating the panelists liked the GT-UREX samples more than the commercial green tea. Phenolic compounds of UREX and green tea are reported to be more or less similar; however, catechins and tannin contents are higher in green tea (Andarwulan *et al*. 2010; Khan *et al*. 2011). Interestingly, addition of UREX had a positive influence in consumer perception of bitterness and taste, at the same time enhancing the amount of total phenolic contents present in green tea beverage. Identification and quantification of the marker compounds of green tea added with UREX could provide more detailed information on the enhancements observed in the present study.

Table 3. Sensory parameters of commercial green tea beverage (brand B)

Sensory Parameters	B-Control	B-LUREX	B-HUREX
Colour	3.26 ± 1.5^{b}	5.46 ± 1.9^{a}	5.50 ± 1.6^{a}
Aroma	4.06 ± 2.0^{a}	4.41 ± 1.0^{a}	4.43 ± 1.7^{a}
Taste	4.11 ± 1.6^{b}	4.3 ± 1.9^{b}	6.13 ± 1.4^{a}
Astringency	3.47 ± 1.3^{b}	3.62 ± 2.1^{b}	5.46 ± 1.6^{a}
Bitterness	4.06 ± 1.4^{b}	4.51 ± 1.7^{b}	6.13 ± 1.1^{a}
Overall acceptability	3.74 ± 1.3^{b}	4.02 ± 1.8^{b}	5.66 ± 1.7^{a}

Control: without UREX; LUREX: lower level of UREX (25 mg/sachet); HUREX: higher level of UREX (50 mg/sachet). Comparison within the columns was shown in the table with the data written as mean ± standard deviation (n=30). Means followed by different lowercase superscript letters (a-b) are statistically significant at the 5% level.

Conclusion

As the second most popular beverage worldwide, tea is one of the best sources of flavonoid intake with many proven health beneficial properties. Incorporating green tea with another phenolics-rich extract, i.e. UREX, improved the TPC and $TEAC_{DPPH}$ and provided a wider range of phenolics intake with health beneficial compounds, such as quercetin and its derivatives. On top of that, the addition of UREX improved consumer acceptability in terms of its taste, color, bitterness, astringency and overall acceptability of this health beneficial beverage. Hence, UREX showed great potential to be a good additive in green tea bags due to its potential in enhancing not only the functional properties, but also the physical and sensory attributes of green tea beverage.

Acknowledgements

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