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Physicochemical and Antioxidant Properties of Novel Beverage from Mixed of Spices, Herb and Citrus

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Therapeutic benefits of herbs, spices and citrus are well-known and dated back to hundreds of years ago. Their antioxidant capacities and compounds are also well published in scientific literature. Therefore, a ready-to-drink beverage was developed using those ingredients and analyses were conducted to determine its physicochemical and antioxidant properties. Six spices (cinnamon, clove, fennel, cumin, coriander, and star anise) were extracted using decoction method; mixed with galangal, calamansi and lime juice; and then produced into a drink. This beverage contains high vitamin D (16.67 µg/100 mL) which may help in bone health. Different methods of antioxidant analyses exhibited that this novel ready-to-drink beverage has good antioxidant properties. Its β-carotene inhibition, DPPH free radical scavenging activity (expressed as IC_{50}), and ferric reducing power were 86.93%; 6.69 mg/mL, and 0.28 mg ascorbic acid equivalent/mL; respectively. It also contains 31.5 µg gallic acid equivalent/mL for total phenolic content and 27.18 µg rutin equivalents/mL for total flavonoid content. These results revealed that this novel RTD beverage has the potential to become a new functional drink that suitable for human health consumption in their daily diet.

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Introduction

Spice can be defined as an aromatic plant substance in the whole, broken, or ground form, whose characteristics may vary depending on harvest, location or their historical background. In much of the scientific and trade literature, there are no clear distinctions between culinary herbs and spices; with some plants considered to be both. Since millennia, spices and culinary herbs have been included in cooking to add flavour and enhanced palatability in foods. They also played a role in perfumery, cosmetics and medicine due to their essential oils and therapeutic benefits. Traditionally, they were used to relieve bloating, sore throat, asthma, fever, flu, cough, diarrhoea and other mild illness (Dog, 2006; Balasubramanian et al., 2016). Old folks used them as a natural home remedy; an alternative to synthetic medicines pills, tablets or syrups. Many researchers have conducted investigations to explain these effects and reported the findings in numerous studies. They found that spices and culinary herbs had anti-inflammatory, antimicrobial, anti-diabetic, antioxidant, and anti-cancer properties which beneficial to health in improving digestion, cognition, respiratory and cell damage (Dog, 2006; Kaefer and Milner, 2008; Jungbauer and Medjakovic, 2012; Balasubramanian et al., 2016; Bower et al., 2016).

The therapeutic effects were associated with their constituents. They contained vitamins and minerals (Bower et al., 2016; Balasubramanin et al., 2016) that are important for body functions. In addition, their bioactive components act as the key to reducing or preventing diseases. Epidemiological evidence denoted that dietary intake of antioxidants may lower the incidence of morbidity and mortality (Devasagayam et al., 2004) and these compounds were abundant in spices and culinary herbs (Suhaj, 2006; Embuscado, 2015; Shahidi and Ambigaipalan, 2015). USDA's National Food and Nutrient Analysis Program (NFNAP) found that spices (clove, oregano, ginger, cinnamon, and turmeric) were the top five of the top 50 foods with antioxidant capacities (Halvorsen et al., 2006). Eugenol, a phenolic compound from clove was effectively inhibited ascorbate/Fe^{2+}-induced lipid peroxidation in rat liver microsomes in vitro in a dose-dependent manner (Reddy and Lokesh, 1992). Azab et al., (2011) reported that cinnamon extract significantly reduced lipid peroxidation of liver and protein oxidation in radiated rats due to its phenolic and flavonoids antioxidant properties. Besides that, culinary herb such as galangal also exhibited high antioxidant capacities with galangin as the responsible compound (Lu et al., 2011).

Citrus is another group of the plant that frequently used together with spices and culinary herbs in the preparation

of cuisines, drinks or traditional medicines. Health benefits of citrus are well documented including antitumor, antimicrobial and antioxidant (Lou and Ho, 2017). Flavonoids and phenolic acids such as quercetin, hesperetin, kaempferol and coumarin were the main constituents in citrus that accounts for its antioxidant properties. Nakao et al., (2011) found that hesperetin can directly reduce cellular free radical production by inhibiting xanthine oxidase. They also show their antioxidant activities by directly react with ROS and/or reactive nitrogen species (RNS) (Zou et al., 2016).

Since those ingredients showed promising effects to health especially due to their antioxidant properties, they are incorporated to develop a new drink. Furthermore, this kind of beverage is limited in the market; particularly in Malaysia and commonly prepared traditionally at home by old folks. This current study also determines its physicochemical and antioxidant properties as well as its phenolics and flavonoids content to investigate its benefits for consumer consumptions.

Materials and Methods

Raw materials

Dried spices; cinnamon (*Cinnamomum verum*), cumin (*Cuminum cyminum*), clove (*Syzygium aromaticum*), coriander (*Coriandrum sativum*), fennel (*Foeniculum vulgare*) and star anise (*Illicium verum*) were purchased from local spice manufacturer; Faiza Marketing Sdn. Bhd. Young and sound greater galangal (*Alpinia galanga*) was bought from a vendor in the local market (Pasar Larkin). Lime (*Citrus aurantifolia*) and calamansi (*Citrus microcarpa*) pure extracts were obtained from local juice manufacturer; Hamara Trading Sdn. Bhd. Other ingredients for the ready-to-drink beverage such as sugar and stabilizer were procured from local market.

Chemicals

Standards of folic acid, cholecalciferol (Vitamin D_3), rutin hydrate, gallic acid, β-carotene, and ascorbic acid were from Sigma-Aldrich (St. Louis, MO). Folin-Ciocalteu, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), aluminium chloride and all other chemicals were of analytical grade and purchased from Sigma-Aldrich (M) Sdn Bhd.

Preparation of extracts

Dried spices were extracted using decoction method adopted from Tacouri et al., (2013). One part of spices and five parts of boiling distilled water were left to simmer for one hour. The solution was allowed to cool before filtered using muslin cloth. Galangal was peeled, washed and cut into small pieces and extracted using Panasonic MJ-70M juice extractor. Pure lime and calamansi juice were purchased from a local manufacturer; Hamara Trading Sdn. Bhd. All extracts were kept at -18 ºC until use.

Preparation of ready-to-drink (RTD) beverage

Extracts of spices, galangal, lime and calamansi were mixed and sieved using 50 µm Nylon cloth. Then, sugar and stabilizer were added and it was heated until 45 ºC. Next, the mix was transferred to colloid mill (Model 2F Gaulin Corporation, Everett, MA) to reduce its droplet size before pasteurized at 90 ºC for five minutes. Finally, it was hot-filled into the glass bottle and capped. The RTDs were kept at ambient temperature for future use.

Physical properties determination

Total soluble solids (TSS) of the RTD beverage; expressed as ºBrix was determined using handheld digital refractometer ATAGO® Master-M (Made in Japan). The pH value was measured with the help of Eutech Instruments pH meter (Made in Singapore) that was calibrated using buffer 4.0 and 7.0. The colour assessment was conducted using a Chroma Meter CR-400 optical sensor (Konica Minolta Optics, Inc., Osaka, Japan) according to the CIE Lab scale. The parameters were L* (lightness, 0 = black, 100 = white), a* (−green to +red component) and b* (−blue to +yellow component). The instrument was calibrated with black cuvette and white reference standard.

Determination of vitamin D

Vitamin D was quantified according to National Standard for Food Safety of The People's Republic of China (GB-5413.9-2010). 50 g of the sample was weighed in 250 mL conical flask, and 100 mL ethanol solution containing vitamin C was added. After mixing, 25 mL of potassium hydroxide solution was put in and mixed again. The flask was placed in a water bath at 53°C for 45 minutes. Immediately, it was cooled down to room temperature. The solution was rinsed with a small amount of distilled water into 500 ml separatory funnel. 100 mL of petroleum ether was added, gently shake and closed. It was thoroughly mixed for 10 minutes and leave for layers to appear. The solution layer was poured into another 500 mL separatory funnel, and the extraction process was repeated. The ether solution was combined and neutralised with distilled water. Then, it was filtered and dehydrated using anhydrous sodium sulphate. The filtrate is poured into 500 mL round-bottom flask and set up for distillation with nitrogen at 40°C to nearly dry. 2.0 mL of hexane was added to the flask to dissolve the residue. After cooled down to room temperature, HPLC injection was conducted using ZORBAX Eclipse Plus C18 column (150 mm x 4.6 mm, five µm). Methanol was used as the mobile phase, and the column temperature was set at 35°C. Sample injection volume was 100 µL, and the flow rate was one mL/min. The chromatogram was detected at wavelength 264 nm.

Determination of antioxidant capacity

DPPH assay

The free radical scavenging capacity of this beverage towards a methanolic DPPH solution was measured by the decreasing of the absorbance at 517 nm (Lim et al., 2007). The reading was taken after allowing the solution to stand for 30 min and calculated as % inhibition; where

$$\% \text{ inhibition} = [(A_{control} - A_{sample})/A_{control})] \times 100\%$$

Its antioxidant capacity was then expressed as IC_{50}; the amount of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration (0.1 mM). It was derived from the % disappearance vs concentration plot.

Ferric reducing power

An adopted method of potassium ferricyanide–ferric chloride from Yen and Cheng (1995) with some modifications was used to determine the ferric reducing power of this beverage. 1 ml of diluted samples (different concentrations) were added to 2.5 ml 0.2 M phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1%). Then, 2.5 ml trichloroacetic acid (10%) was added, and the mixtures were incubated at 50 °C for 20 min. After that, two and one-half millilitres of the mixture was taken and mixed with 2.5 ml water and 0.5 ml 1% FeCl3. The solution was allowed to stand for 30 min before measuring the absorbance at 700 nm. A graph of absorbance vs sample extract concentrations was plotted to observe the reducing power. Ascorbic acid (0–100 µg/mL) was used as a reference compound, and the results were expressed as milligrams of ascorbic acid equivalent (AAE) per 100 mL sample.

β-Carotene-linoleic acid (linoleate) assay

Antioxidant capacity of this beverage was also determined using β-Carotene-linoleic acid (linoleate) assay that was adopted from Amarowicz et al., (2004). Briefly, 1 ml of β-carotene (0.2 mg/mL) was dissolved in chloroform. After removing the solvent using a rotary evaporator, 20 mg of linoleic acid, 200 mg of Tween 40 and 50 ml of aerated distilled water were added and stirred vigorously. Then, five mL of the prepared emulsion was transferred to tubes containing 2 mg of extract or 0.5 mg of BHA. Next, they were placed in a water bath at 50 °C for two h. The absorbance of the sample in each tube was measured immediately after sample preparation (t=0 min) and at 15-min intervals until the end of the experiment (t=120 min) using a spectrophotometer (UV-1800, SHIMADZU, Kyoto, Japan) set at 470 nm. The rate of β-carotene bleaching was calculated according to this equation:

$$\text{Rate of β-carotene bleaching} = \ln (A_{t=0}/A_{t=t}) \times 1/t$$

where $A_{t=0}$ is the initial absorbance of the emulsion at time 0; $A_{t=t}$ is the absorbance at 15 min time intervals, and t is the time in min.

An average rate was calculated based on the rates determined. The antioxidant activity was expressed as the percent inhibition of the rate of β-carotene bleaching relative to the aqueous control using the equation:

$$\% \text{ β-carotene bleaching inhibition} = 100 \times (R_{control} - R_{sample}) / R_{control}$$

where $R_{control}$ and R_{sample} are the average bleaching rates of β-carotene in the emulsion without antioxidant and with sample extract, respectively.

Determination of total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteu's reagent (Singleton and Rossi, 1965). 0.3 mL of sample was poured together with 1.5 mL of Folin-Ciocalteu's reagent (diluted ten times with water) and 1.2 mL of sodium carbonate (7.5%w/v) into a test tube. The tube was vortexed, covered with parafilm and set aside for 30 min. Absorption of the sample against the reagent was measured at 765 nm using spectrophotometer (UV-1800, SHIMADZU, Kyoto, Japan). TPC was expressed as mg gallic acid equivalents per 100 mL sample. Correction of the absorbance from ascorbic acid was carried out by constructing a calibration curve since ascorbic acid also contributes to the formation of the blue molybdenum–tungsten complex that will affect the results.

Determination of total flavonoid content (TFC)

TFC was quantified using a method adopted from Ismail et al., (2010) with slight modifications. The sample was centrifuged at 3000 rpm (4 °C) for ten minutes. Then, 450 µlL of supernatants were reacted with 450 µlL of 2% aluminium chloride (with methanol). After ten minutes of incubation at ambient temperature, the mixtures were centrifuged for 10 min at 7500 rpm (25 °C). Finally, the absorbance of the supernatant was measured at 435 nm by using a spectrophotometer (Multiskan GO, Thermo Fischer Scientific Oy, Finland). The total flavonoid content was expressed as rutin equivalents in microgram per millilitre sample (µg RE/mL sample).

Statistical analysis

All experiments were carried out in triplicate, and the results were expressed as mean values ± standard deviations. Correlation analyses between antioxidant capacity, TPC and TFC were performed using Pearson's correlation coefficient of MINITAB 14 (MINITAB Inc., USA) statistical software. The significance level was set at $p<0.05$ using Student's t-test.

Results and Discussion

Physicochemical properties

Its low pH (3.83±0.03) was most probably due to the addition of calamansi and lime juice. Cheong et al., (2012) reported that the pH of calamansi juice from Malaysia, Philippines, and Vietnam was in the range of 2.50 to 2.57. Therefore, with the incorporation of other ingredients in this beverage, the pH was indeed higher

than that of the mentioned report. This value also was in line with the findings from Li-Ying et al., (2008) on seven cultivars of orange juice (3.81-4.31). On top of that, organic acids and sugars in the citrus juice were also contributed to the total soluble solids (Li-Ying et al., 2008; Cheong et al., 2012) of this drink (10.15±0.07 °Brix) besides the added sugar. Sucrose, fructose, and glucose were the primary sugars in citrus juice. Colour measurement of this drink (Table 1) exhibited that it had dark yellow colour with low L* (24.24±0.02) and a* (0.63±0.04) values but higher positive b* (5.08±0.02) value. The results were as expected due to the yellow colour of the spices' extract and their high concentration in the drink. It was comparable to pineapple and banana b* values reported in Pastoriza et al., (2017).

Table 1. Physicochemical properties of novel beverage based on spices, herb and citrus

Parameter	Value[a]
pH	3.83±0.03
Total soluble solids (°Brix)	10.15±0.07
Colour	
L*	24.24±0.02
a*	0.63±0.04
b*	5.08±0.02
Vitamin D (µg/100ml)	16.67±0.83
Folic acid (µg/100ml)	160.20±2.97

[a] Values are means of triplicate determinations ± standard deviations

Vitamins (A, B vitamins, C, D, E, and K) content in this drink were also evaluated, and surprisingly it contains all the vitamins. However, only vitamin D was reported in this current study since it was the highest vitamins found in this novel beverage. Vitamin D is associated with bone health and skeletal muscle strength. Its deficiency is a worldwide epidemic and of a public health concern. Recommended dietary allowance (RDA) of vitamin D for adults is 400 IU (10 µg) per day but several studies concluded that it is insufficient as adults need higher levels up to 5000 IU per day (125 µg) (Brincat et al., 2015). Therefore, this new RTD beverage may help in meeting the necessity as its vitamin D content was 666.8 IU (16.67±0.83 µg/100 mL).

Table 2. Content of antioxidants in novel beverage based on spices, herb and citrus

Assay	Unit	Value[a]
DPPH	IC_{50} (mg/ml)	6.69±0.11
Ferric reducing power	mg ascorbic acid equivalent/ml	0.24±5.30
β-carotene bleaching	% inhibition	86.93±5.54
Total phenolic content	µg gallic acid equivalent/ml	31.5±0.16
Total flavonoid content	µg rutin equivalent/ml	27.18±0.2

[a] Values are means of triplicate determinations ± standard deviations

Antioxidant capacity (AC)

Three methods of different mechanisms (DPPH radical scavenging, ferric reducing power, and β-carotene bleaching assay) have been used to assess the antioxidant capacity of this drink. DPPH scavenging assay is based on an electron or hydrogen atom donation of antioxidants to neutralise DPPH free radical that resulting in changes of purple colour ($DPPH^{\bullet}$) to yellow (DPPH2) (Shahidi and Zhong, 2015). On the contrary, ferric reducing power assay; the non-radical redox potential-based method was also conducted since antioxidants also act as metal ions reductants (Mishra et al., 2012). Antioxidants in the sample will cause a reduction of the Fe^{3+} of ferricyanide complex (yellow colour) to ferrous form; Fe^{2+} (green colour). In addition, the ability of antioxidants in this beverage to inhibit the oxidation of unsaturated fatty acids was also discovered by using β-carotene linoleic acid assay (BCB). Decolourisation of β-carotene at 470 nm indicates that antioxidants have donated their hydrogen atoms to quench radicals (Mikami et al., 2009).

Results for those analyses are shown in Table 2. A noticeable amount of antioxidant capacity was obtained. Ingredients of this beverage which consist of an extract of spices, herb (galangal) and citrus were believed to play the role of its antioxidant capacity. DPPH radical scavenging activity of hot water extracts from various spices reported by Kim et al., (2011) demonstrated a range of % inhibition of the spices; with the highest of 84.22% from clove to the lowest of 10.48% from fennel. Cumin and coriander were also included in the report with the moderate amount; 35.02% and 30.40%; respectively. Besides that, Mallick et al., (2016) also investigated the DPPH scavenging activity of coriander and fennel, but they expressed the values as IC_{50} (746.9 µg/mL and 751 µg/mL). The higher IC_{50} value indicates lower scavenging capacity since those values defined as the concentration of substrate that causes 50% loss of the DPPH activity. Other than that, Lu et al., (2011) also analysed antioxidant capacity of spices commonly consumed in China. They found that the % inhibition of DPPH for cumin was lower than fennel with 18.12% for the former and 25.03% for the latter. The values differences were most likely due to the variations of the sample used including seasons, locations, geography, and agricultural practices. Apart from that, high DPPH scavenging activity (> 76%) of other spices such as star anise, cinnamon and galangal were also reported in that study. Therefore, the value of % DPPH inhibition in Table 2 was comparable to those reports as a different proportion of those spices extract were incorporated in this drink.

In another study that conducted by Hinneburg et al., (2006), the antioxidant capacity of fennel and cumin were calculated as ferric reducing power. Their content (~0.1 mmol ascorbic acid equivalent/g extract) were significantly better than parsley, ginger, and cardamom. This finding was in line with Mallick et al., (2016) that noted the highest reducing power in fennel followed by black pepper, coriander and fenugreek. In addition, Syrian varieties of coriander showed remarkable reducing capacity (EC_{50} = 54.20 µg/mL) when compared to

Tunisian and Egyptian varieties although all of them exhibited good reducing power towards the control; ascorbic acid (Msaada et al., 2017). Since those spices contained a considerable amount of reducing power, their inclusion to this new formulated beverage resulting a value shown in Table 1. Besides that, the antioxidant capacity of the drink against lipid peroxidation was also tested by the inhibition of β-carotene bleaching in the presence of linoleic acid radicals. This beverage gave high value for this assay (86.93±5.54%) which demonstrated a promising ability of the antioxidants to act as a free radical quencher. This result might be due to its ingredients such as clove and coriander that expressed higher β-carotene bleaching inhibition compared to their controls in previous studies (El-Maati et al., 2016; Msaada et al., 2017).

Table 3. Correlations (r^a) between antioxidant capacities (DPPH, ferric reducing power, β-carotene bleaching assays) and total phenolics content and total flavonoids content of a novel beverage based on spices, herb and citrus.

	DPPH[b]	FRP[c]	BCB[d]	TFC[e]
TPC[f]	0.849*	0.923*	0.953*	-0.525
	(0.032)	(0.009)	(0.003)	(0.284)
TFC	-0.555	-0.728	-0.706	
	(0.253)	(0.101)	(0.117)	

[a] *r* = correlation coefficient; [b] DPPH radical scavenging activity; [c] FRP = ferric reducing power; [d] BCB = β-carotene bleaching; [e] TFC = total phenolics content; [f] TPC = total phenolics content; * = significant at $p< 0.05$; value in brackets ( ) denoted *p* values

Total phenolic content (TPC)

Since antioxidants in spices and culinary herbs were associated with phenolic compounds (Shan et al., 2005; Wojdylo et al., 2007), its content in this drink was also evaluated. Wide ranges of TPC value of various spices and herbs were reported in previous studies (Ninfali et al., 2005; Hinneburg et al., 2006; Przygodzka et al., 2014; Vallerdú-Queralt et al., 2014). The variations were due to their different varieties, extraction solvents and methods. Among them, star anise, cinnamon and clove were described to contain high TPC with the lower amount of coriander, cumin and fennel. There are abundant of phenolic compounds in those spices, but phenolic acids were the major compounds. Some of them were chlorogenic acid, protocatechuic acid, and ferulic acid. Other than that, culinary herbs also contain high total phenols. Galangal was identified with the highest TPC that defeated the other spices (Lu et al., 2011). The inclusion of different proportions of those ingredients in this novel beverage results in the value shown in Table 2. A possible reason for the low TPC value when compared to individual spice extracts from preceding researches (Tacouri et al., 2013; Przygodzka et al., 2014; Chan et al., 2016) might be due to the low concentration of each spice and herb that were incorporated into the drink.

Total flavonoid content (TFC)

Flavonoids were also the key compounds in spices and culinary herbs. Nine of the 32 investigated plants in Wojdylo et al., (2007) study were higher in flavonoids than phenolic acids. Clove contained no phenolic acids but 100% of flavonoids. Galangin, the constituent that responsible for the highest antioxidant capacity of galangal was also from the group of flavonoids (Lu et al., 2011). TFC of spices were varied in different species (Ninfali et al., 2005; Kim et al., 2011; Tacouri et al., 2013; Mallick et al., 2016). Predominant flavonoids in most of the previous findings were quercetin, rutin and epicatechin (Lu et al., 2011; Vallerdú-Queralt et al., 2014). Table 2 presented the TFC of this novel beverage. The value was quite low, but its comparison with other findings cannot be made as different standard was used to express this result. In spite of this, the flavonoids content still play a part for antioxidant properties of this drink. Besides spices and galangal, citrus also contributes to the TFC (Lou and Ho, 2017).

Correlations between antioxidant capacity (AC), TPC and TFC

TPC was significantly high correlated with DPPH, ferric reducing power and β-carotene bleaching assay (Table 3). These results were in line with many studies (Lu et al., 2011; Kim et al., 2011; Tacouri et al., 2013; Chan et al., 2016) and revealed that phenolics were the dominant antioxidant compounds in this drink. The highest positive correlation, *r* (0.953) and the lowest *p*-value (0.003) between TPC and β-carotene bleaching assay indicated that antioxidants in this RTD novel beverage were prone to retard linoleic acid oxidation rather than reducing ferrous ion and scavenging DPPH radical. In contrast, TFC showed non-significant negative correlations with all the ACs. These poor correlations were also demonstrated in previous reports on spices and herbs (Kim et al., 2011; Tacouri et al., 2013; Chan et al., 2016). Furthermore, TPC and TFC also exhibited weak relationship between them (r = -0.5252; p = 0.284). This result explained that phenols in this drink were presumably not from flavonoids group. Besides that, the drawbacks of the methods used also might be the reason for this finding. Folin-Ciocalteu assay may be overestimated by non-phenolic constituents (Shahidi and Zhong, 2015) while aluminium chloride method was specific only for flavones and flavonols although other flavonoids subgroups were possibly presented in the sample (Meda et al., 2005). The correlative relationships also greatly influenced by the number of tested samples and the ranges of the tested values (Shan et al., 2005).

Conclusions

The results revealed that this novel RTD beverage based on spices, herb and citrus contained high vitamin D with low pH and dark yellow in colour. Three different assays (DPPH, ferric reducing power and β-carotene bleaching)

that have been used to determine its antioxidant capacities (AC) showed the remarkable amount. All of the AC values were significantly high correlated with its total phenolic content (TPC) but demonstrated poor correlation with its total flavonoids content (TFC). These findings exhibited that phenolics were the main contributor for its antioxidant capacities. Since this beverage possessed beneficial properties as reported in this current study, it is recommended to be a new functional drink for human health in their daily diet.

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Conflict of Interest

All the authors declare that they have no conflict of interest.

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