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Assessment of antioxidant potential using total phenolic content and DPPH assay of a Sri Lankan 'spice' mixture used to impair obesity

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Abstract
In 2012 21 % in men and 32.5 % in women were affected by excess weight in Sri Lanka. It was said that excessive oxidative stress on tissues is one of the main problems in obesity. Further, studies have also revealed that anti-oxidants impair obesity. The aim was to determine the anti-oxidant contents in a native 'spice' mixture that was used to impair obesity. The 'spice' mixture contains 5 spices commonly used in Sri Lanka: *Cinnamomun zeylanicum, Cuminum cyminum, Piper nigram, Murraya koenigii* and *Allium sativum.* Total Phenolic Content (TPC) and DPPH assays were used to determine the anti-oxidant potential of the 'spice' mixture. TPC was determined using Folin-Ciocalteu (FC) assay. Working standards of 100, 200, 300, 400, 500 µg / mL of concentrations of Gallic acid were prepared by diluting the stock solution with appropriate amount of distilled water to plot the standard calibration curve. A 1 mL of the samples (both extracts) was added to 5 mL of the diluted FC reagent. After 5 min 4 mL 7.5 % Na_2CO_3 was added, incubated and absorbance was recorded. For DPPH assay dilutions serious were prepared by aqueous, Methanol extracts of the 'spice' mixture. A 160 µL of each concentration (25, 50, 100, 200, 400, 800 µg) from both samples were pipetted out to the wells of the micro plate. Then a 0.25 mM DPPH (40 µL) was added to the wells and this was kept in dark at 30^0C for 15 minutes and the absorbance was measured. Average TPC of methanolic and aqueous extracts were 3.06 and 1.84 mg / g respectively. DPPH assay resulted that the ID_{50} of the both extracts of the spice mixture are not significant different ($p < 0.05$) with the percentage inhibition of the standard Butylated Hydroxy Toluene (BHT). Hence, the study concludes that 'spice' mixture used to impair obesity possesses substantial amount of anti-oxidant potentiality.

Keywords: anti-oxidant, 'spice' mixture, obesity

Introduction
Most of the Asian countries the prevalence of overweight and obesity has increased many folds in the past few decades [1]. In south East Asia, where the percentage of overweight and obesity tripled from a lower starting point of 7 % to 22 % in between 1980 – 2008 [2]. A study revealed that the percentages of Sri Lankan adults in the overweight, obese and centrally obese categories were 25.2 %, 9.2 % and 26.2 %, respectively [3]. In addition to this, another study reveled in 2012 that the prevalence of obesity in 21 % for men and 32.5 % for women [4]. Obesity is a multifactorial disease influencing biological, genetic, behavioral, social and environmental factors [5]. Apart from the above factors Unani Medicinal concept says, poor metabolism is also one of the important factor to obesity. Therefore to optimize the weight reduction, Unani text says to select herbs which should be potentiated the powers of digestion and metabolism. Concern on this Unani concept herbs were selected possessing the activity of digestion and metabolism. A clinical trial was carried out to reduce excess weight using Sri-Lankan available natural spices. This spice mixture contains 5 spices which has been commonly used in Sri Lanka such as *Cinnamomun zeylanicum, Cuminum cyminum, Piper nigram, Murraya koenigii* and *Allium sativum.*

Further, it is said in the modern science that the expansion of visceral adipose tissue in obese individuals. Consequence of this visceral adipose expansion, adiposities generate excess reactive oxygen species (ROS) [6, 7]. This excessive amount of ROS may be a primary cause of bimolecular oxidation lead increase oxidative stress in the tissues. On the other hand antioxidants are inhibitors of lipid peroxidation and also as a defense mechanism of living tissues against oxidative damages [8, 9]. Potentiality of antioxidant is widely considered as a parameter for medical bioactive components and different methods are now used to determine the antioxidant activity of plant phenolic compounds. A DPPH radical scavenging method is common spectro-photometric procedures for determining the antioxidant levels of components [10]. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution [11]. Hence, in this study, the above said 'spice' mixture was analyzed to determine the anti-oxidant properties that was used to impair obesity.

Objective
Determine *in vitro* anti-oxidant contents in a native 'spice' mixture by using TPC and DPPH radical scavenging assay methods.

Materials and Methods
Selection of plant material for the 'spice' mixture
Bark of *Cinnamomun zeylanicum*, Seeds of *Cuminum cyminum*

and *Piper nigram*, *leaves of Murraya koenigii* and bulb of *Allium sativum* were used in this 'spice' mixture. All were taken as dried form under the shade of sunlight and mechanically powdered (Centrifugal Mill, Retsch, Germany).

Preparation of Aqueous extract of the 'spice' mixture

Mechanically powdered 'spice' mixture (100 g) was mixed with distilled water (0.25 L) and placed on orbit shaker at room temperature for 24 hours. The filtrate was frozen at -40 ^{o}C and dried for 48 hours using a freeze dryer (Labconco, Model No 7670560, USA). The dried extract was stored at temperature below 0 ^{O}C.

Preparation of Methanolic extract of the 'spice' mixture

Mechanically powdered (Centrifugal Mill, Retsch, Germany) 'spice' mixture (100 g) was mixed with distilled MeOH (0.25 L) and placed on orbit shaker for 24 hours. The filtrate was evaporated under reduced pressure using a rotary evaporator (BUCHI Rotavapor R-114). Nitrogen gas was passed and then it was kept in a vacuum drying oven for 24 hours to remove any remaining solvent residue.

Determination of Total phenolic content

Total phenolic content (TPC) of samples were determined using Folin-Ciocalteu (FC) assays as described by Singleton & Rossi, (1956) with a slight modification. All samples and readings were prepared and measured in Triplicate. 1:10 diluted Folin-Ciocalteu reagent and 7.5 % Na_2CO_3 were prepared. Gallic acid was used as the standard to plot the calibration curve. Gallic acid stock solution (500 µg / mL) was made by dissolving 5 mg of Gallic acid in 10 mL of methanol. Working standards of 100, 200, 300, 400, 500 µg / mL of concentrations of Gallic acid were prepared by diluting the stock solution with appropriate amount of distilled water to plot the standard calibration curve.

A 1 mL of the sample (1 mg / mL) was added to 5 mL of the diluted Folin-Ciocalteu reagent. After 5 min 4 mL 7.5 % Na_2CO_3 was added and incubated for 2 hours at room temperature. The absorbance of the sample was measured at 765 nm using visible (Perkin Elmer Lambda 25 UV/Vis) spectrophotometer and calibration curve was plotted using gallic acid at the different concentration. The results were expressed as mg Gallic acid equivalents (GAE) /g dry weight.

DPPH radical scavenging activity Assay

The DPPH radical scavenging activity assay used by Chan *et al*. (2007) was adopted with a slight modification. A sample of aqueous extract of the 'spice' mixture (4 mg) and a sample of methanolic extract of 'spice' mixture (4 mg) were dissolved in 2 mL of pure methanol, separately. For each stock solution a dilution series was prepared for 7 different concentrations (25, 50, 100, 200, 400, 800, 1600 µg / mL). A known volume (160 µL) of each concentration from both samples (aqueous and methanol) were pipetted out to a 96 well micro plate, separately. Each concentration was run in triplicate. A 0.25 mM DPPH (40 µL) was added to each well of the micro plate which contains different concentrations of the extracts. Then the mixture was kept in dark at room temperature for 15 minutes. The absorbance was measured using the spectrophotometer (Perkin Elmer Lambda 25 UV/Vis) at 517 nm wave length against the blank (pure methanol). Butylated Hydroxy Toluene (BHT) was used as a standard. The percentage of radical scavenging activity was calculated using the standard formula given below

% Radical scavenging = $[(A_O - A_1 / A_O) \times 100]$

Where A_0 is the absorbance of the control and A1 is the absorbance of the sample. Lower absorbance values show higher free radical scavenging activity.

Results and Finding

Present TPC study shows a linear calibration curve of Gallic acid with r^2 value of 0.999 was obtained (Figure 1).

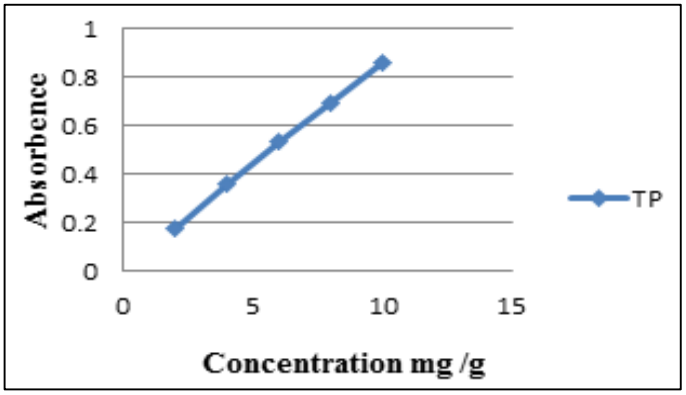

Fig 1: Calibration curve of Gallic acid (GAE)

TPC of the 'spice' mixture extracts measured using the GAE. Here the methanolic extracts of the 'spice' mixture had the highest TPC (3.06 mg / g) when compared to that of the aqueous extract (1.84 mg / g) of the 'spice' mixture.

DPPH assay shows that the methanol and aqueous extracts of the 'spice' mixture in different concentrations exhibited varying degree of anti-oxidant activity is as comparable to the standard BHT. The result of the DPPH assay is given in Table 1, 2 and figure 2.

Table 1: Absorbance and percentage inhibition by Aqueous and MeOH extracts of the 'spice' mixture in the DPPH assay

Amount (µg)	% inhibition		
	MeOH extract	Aqueous extract	BHT
25 µg	5	13	13
50 µg	18	28	26
100 µg	35	42	49
200 µg	58	64	58
400 µg	73	75	72
800 µg	77	81	80

Number of replicate = 3

Table 2: *In vitro* anti-oxidant activity of MeOH extracts and aqueous extract of the 'spice' mixture

Extracts	ID_{50} Antioxidant activity
BHT (+ve control)	0.27 ± 0.14^a
Methanol Extract	0.38 ± 0.20^a
Aqueous extract	0.32 ± 0.18^a

Letters indicate significant differences between on one sample *t* test analysis ($p < 0.05$)

ID_{50} – 50 % inhibition dose

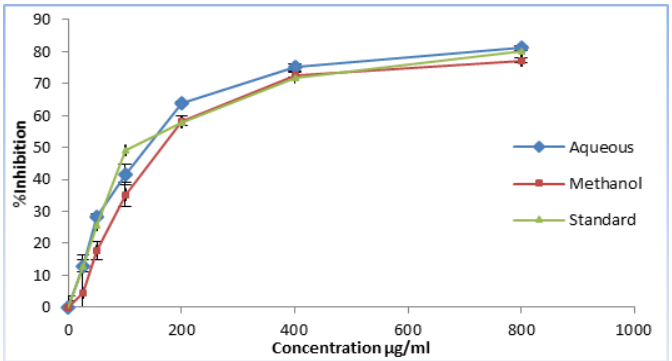

Fig 2: Antioxidant activity of the aqueous and methanol extracts of the 'spice' mixture in the DPPH assay

Further the above graph shows that the percentage inhibition of the aqueous and methanol extracts of the spice mixture is not significant different ($p < 0.05$) with the percentage inhibition of the standard BHT. Hence, the extracts of the above spice mixture possess significant anti-oxidant activity.

Discussion

A study revealed that *Chukrasia tabularis* (belonging to the family Meliaceae) is one of the richest sources of phenolic compounds (17.2 mg GAE / g) than the other herbs [12]. In the present study the phenolic content in methanolic extract of the 'spice' mixture and aqueous extract of the 'spice' mixture were 3.06 mg / g and 1.84 mg / g GAE, respectively.

Oxygen free radicals are formed naturally in the body and play an important role in many normal cellular processes. However, over production of oxygen free radicals will damage major components of cells. Interestingly our body has enzymatic antioxidant defenses include *catalase* (CAT), *superoxide dismutase* (SOD) etc. Although synthetic antioxidants are also available, most of them show serious side effects such as carcinogenic [13]. In contrast, natural antioxidant components derived from plants are pharmacologically potent, effective and there are no reported side effects [14]. Phenolic compounds from the plants stilbenes, alpha tocopherols, tocotrienols etc, ascorbic acid, beta carotene and micronutrient elements like zinc and selenium are reported as natural antioxidants [15].

In recent decades dietary intake of plant based antioxidants has been increased due to their abundant sources and least side effects [16]. Plants contains a wide variety of free radical scavenging molecules, such as phenolic compounds, nonphenolic compounds such as vitamins are rich sources of antioxidant activity [17]. Further a study revealed that *Chukrasia tabularis* (belonging to the family Meliaceae) is one of the richest sources of phenolic compounds (17.2 mg GAE / g) than the other herbs.

Conclusion and recommendation

Our ancestors used plenty of spices in different forms in culinary purposes but they are not utilizing properly by recent decades. The present study revealed that 'spice' mixture had antioxidant property which is comparable with standard BHT. Many studies revealed that anti-oxidants can ameliorate the oxidative stress in the obesity. It is concluded that consumption of right amount of spices daily would work for weight reduction in obesity.

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