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ORIGINAL ARTICLE

Safety and Antioxidant Potential of Traditional Thai Poly-Herbal Tea "Phy-Blica-D" Used as a Rejuvenation Formula

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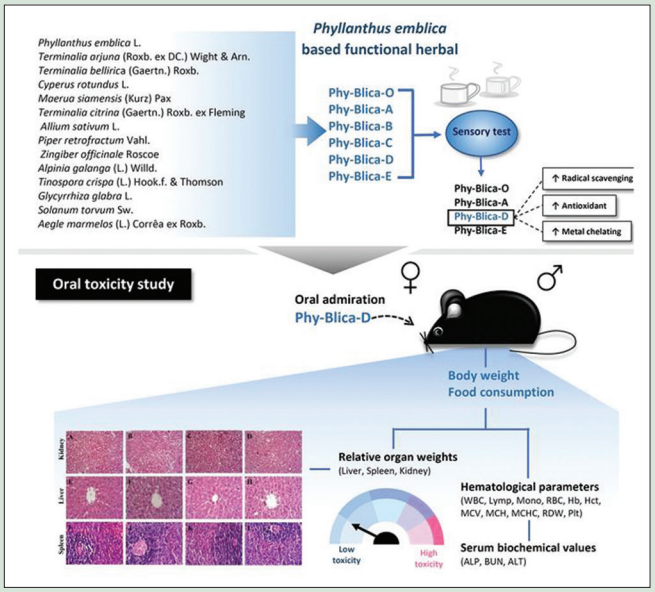
ABSTRACT

Background: The rising popularity of phytonutrient consumption may be due to a vast number of scientific studies that have revealed their health benefits; however, concerns regarding the medical safety of herbal-based products are increasing. Phy-Blica-O is *Phyllanthus emblica*-based herbal tea used in Thai traditional medicine as a rejuvenating remedy. However, its consumption has been limited due to its strong bitter taste with unpleasant odor. **Objective:** The objective of this study is to evaluate the safety and antioxidant potential of Phy-Blica-D, the modified formula of Phy-Blica-O which gave high sensory acceptability scores. **Materials and Methods:** Subacute toxicity studies of Phy-Blica-D infusion was conducted by repeated oral administration of the extract at doses of 5, 50, and 300 mg/kg/day in Sprague-Dawley rats. **Results:** The formula exhibited antioxidant activity with an IC_{50} of 0.243 ± 0.006, 0.486 ± 0.002 and 0.108 ± 0.004 mg/mL using 1,1-diphenyl-2-picrylhydrazyl, 2,20-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid and metal chelating assays, respectively. There was no significant treatment-related toxicity as indicated by clinical signs, body weight, food consumption, serum biochemical and hematological parameters, organ weight, and histopathological examination of the animals treated with Phy-Blica-D infusion. These results suggest that the oral no-observed-adverse-effect-level of Phy-Blica-D extract is >300 mg/kg body weight/day, or ~3.7 L/kg body weight/day for both sexes. The calculated human equivalent dose value is 48.39 mg/kg/day, or ~600 mL/kg body weight/day. There were no target organs affected. **Conclusion:** This study demonstrates that Phy-Blica-D infusion can be regarded as safe and could potentially be used as a functional ingredient to reduce oxidative stress in non-communicable diseases.

Key words: Antioxidant activity, folkloric infusion, functional beverage, *Phyllanthus emblica*, subacute toxicity study

SUMMARY

- *Phyllanthus emblica*-based herbal tea is widely used in traditional medicine throughout Thailand as rejuvenation beverage
- Using a 9-point hedonic scale, panelists rated Phy-Blica-D infusion significantly high in taste and overall acceptability
- Phy-Blica-D infusion is rich in phenolic and have high antioxidant capacities
- The no-observed-adverse-effect level for Phy-Blica-D obtained from the results of the present work was 300 mg/kg body weight/day, or ~3.7 L/kg body weight/day for both sexes.



Abbreviations Used: ABTS: 2,20-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid; DMSO: Dimethyl sulfoxide; DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: Ferric reducing antioxidant power assay; GLP: Good Laboratory Practice; HED: Human equivalent dose; NOAEL: The oral no-observed-ad verse-effect-level; OECD: The Organization for Economic Cooperation and Development; TFC: The total flavonoid content; TPC: Total phenolic content.

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INTRODUCTION

There is growing demand for premium-positioned functional foods due to increasing consumer awareness of their roles in benefiting health. Previous reviews have estimated that the global market of functional foods was <50 billion US$ in the early 2000s and increased to ~125 billion US$ in 2015, with Asia Pacific countries being the largest market segment, followed by North America, Latin America, and Western Europe.[1] Since health claims are becoming key factors for the development of the functional food market, scientific studies are actively being conducted to document the traditional knowledge of plant species used as herbal teas in several countries.[2-4] These herbal-based beverages are considered

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to have multiple health benefits such as providing invigoration (as a tonic), relief of fatigue and stress, protection against the common cold, and boosting the immune system. Thus, there is an opportunity for both consumers and food companies for the development of functional beverages.

Despite the increasing popularity of herbal-based functional beverages and their presumed superior safety to chemical-based products, adverse effects and potential toxicity remain major safety issues for natural substances.[5] Our previous studies of Thai traditional polyherbal infusions used as rejuvenating formulas demonstrated that some formulas possess promising biological activities, including antioxidant activities, and anti-hyperlipidemic effects.[6-8] Among these formulae, *Phyllanthus emblica*-based functional herbal tea (PeHT) named THP-R016 or Phy-Blica-O possessed notable antioxidant properties and exhibited a noncytotoxic effect toward Vero cells. Numerous studies have confirmed *in vivo* the pharmacological properties and their value in clinical use of several herbal constituents of Phy-Blica-O;[9-13] however, there is no toxicological information available for this infusion. Moreover, *Allium sativum* and *Tinospora crispa* present in Phy-Blica-O cause a strong bitter taste with unpleasant odor, which limits its application in functional food industries.

The purpose of this study was to develop a new PeHT with high antioxidant power and improved consumer acceptability. In addition, qualitative and quantitative investigations were performed after 28-day repeated oral administrations of a promising PeHT in rats to obtain information on the oral no observed adverse-effect level (NOAEL) and toxicology of target organs for this polyherbal formula, which could be utilized as a novel naturally occurring antioxidant beverage.

MATERIALS AND METHODS

Preparation of PeHT

Medicinal ingredients of PeHT [Table 1] were purchased from a local licensed medicinal plant store, Triburi Orsot (Songkla, Thailand).[6] *Glycyrrhiza glabra*, *Solanum torvum,* and *Aegle marmelos* were selected based on their information as taste-improving medicinal plants or using as health-promoting herbs[14] and mixed with the original formula. All plant materials were identified against reference specimens of the Materia Medica at the Faculty of Traditional Thai Medicine, Prince of Songkla University, Songkhla, Thailand, by a botanist, Dr. Katesarin Maneenoon, Assistant Professor. The herbal tea was manufactured specifically for the present study by the Traditional Thai Medicine Hospital (Hat Yai, Thailand). The cleaned, dried, and milled plant parts were mixed in varying proportions to obtain five different formulations which have been chosen based on the consumer preference toward approximately 40 Phy-Blica-O-based herbal infusions. Samples (1 g) were bagged in rectangular infusion tea bags (5 cm × 8 cm). All bagged formulas were stored in a sterilized amber glass jar at room temperature.

Tea infusions were prepared by the addition of 1 tea bag per 120 mL of freshly boiled water at 98°C ± 2°C and allowed to brew for 3 min without stirring.[8] Consumer acceptability of *P. emblica* herbal teas was assessed using an untrained panel (n = 30) in the Department of Food Sciences and Nutrition, Faculty of Science and Technology, Prince of Songkla University. Sensory characteristics assessed included color, odor, taste, and overall acceptability using a 9-point hedonic scale (1 = dislike very much; 5 = neither like nor dislike; 9 = like very much).

Radical scavenging activity

The herbal teas were prepared as described above and subjected to freeze-drying. The extraction yield of each dried powdered sample was calculated, and the herbal infusion extracts were stored in a sterilized amber tube at −20°C until analysis for up to 3 days.

For both 2,20-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging mixed mode assays, the extracts were dissolved appropriately with 50% (v/v) dimethyl sulfoxide and serially 2-fold diluted to obtain concentrations of 2500-1.22 μg/mL. Trolox (2-fold dilution; 1250-0.61 μg/mL) was used as an antioxidant standard. The procedures were performed according to a previously published method[15,16] with some modifications as described below. The free radical scavenging ability of the extracts and Trolox are reported as inhibition concentration (IC_{50}; mg/mL), causing 50% inhibition of DPPH/$ABTS^+$ radicals.

An aliquot of 180 μL 80 μM DPPH solution in 80% (v/v) ethanol was added to a 96-well plate containing either 20 μL antioxidant standard or the sample. The plate was mixed well, covered with aluminum foil, and incubated at room temperature in the dark for 30 min. The absorbance of the reaction mixture was determined at 492 nm.

The solution of $ABTS^+$ cation radicals was prepared by the reaction between 2 mM ABTS in water and 2.45 mM potassium persulfate solution in a volume ratio of 1:1, which was kept in the dark at room temperature for 16 h. The solution was then diluted with ethanol to obtain an absorbance of 0.70 ± 0.05 at 734 nm before use. An aliquot of 20 μL of each sample was added to 200 μL of the diluted $ABTS^+$ solution in a 96-well plate. The mixture was then incubated at room temperature for 6 min after initial mixing, and the absorbance at 734 nm was measured.

Ferric reducing antioxidant power assay

The reducing power of the extract was estimated using a single-electron transfer-based assay, ferric reducing antioxidant power assay (FRAP). An

Table 1: Composition of *Phyllanthus emblica* based functional herbal tea with different proportions of herbs and spices (per 150 g formulation)

Scientific names	Parts used	Phy-Blica-O	Phy-Blica-A	Phy-Blica-B	Phy-Blica-C	Phy-Blica-D	Phy-Blica-E
Phyllanthus emblica L.	Fruit	14	30	7	11	18	6
Terminalia arjuna Wight and Arn.	Fruit	14	30	7	11	11	6
Terminalia bellirica (Gaertn.) Roxb.	Fruit	14	30	7	11	11	6
Cyperus rotundus Linn.	Rhizomes	14	10	7	11	4	6
Maerua siamensis (Kurz) Pax.	Root	14	10	7	11	4	6
Terminalia citrina Roxb. ex Fleming	Fruit	14	10	7	11	4	6
Allium sativum L.	Bulb	14	1	1	1	1	1
Piper retrofractum Vahl.	Fruit	14	10	7	11	4	6
Zingiber officinale Roscoe.	Rhizomes	14	10	7	34	4	19
Alpinia galanga (L.) Willd.	Rhizomes	14	10	7	23	4	12
Tinospora crispa (L.) Miers ex Hook.f. and Thoms.	Stem	14	1	1	1	1	1
Glycyrrhiza glabra Linn.	Bark	-	-	78	11	47	68
Solanum torvum Swartz.	Fruit	-	-	7	-	4	6
Aegle marmelos (L.) Correa ex Roxb.	Fruit	-	-	-	-	36	-

aliquot of 30 µL of the extract was mixed with 270 µL of FRAP reagent and monitored by measuring the change in absorbance at 596 nm after 30 min. A freshly prepared working solution of $FeSO_4$ was used for the standard curve, and the result is expressed as M $FeSO_4$ equivalent/g extract.[15,16]

Metal chelating activity

The method described previously by Wong *et al.*[17] with slight modifications was applied to determine the iron chelating power of the herbal tea extracts. Briefly, 12.5 µL of 2 mM $FeCl_2$ was mixed with either 125 µL of the extract at different concentrations (2-fold serial dilution; 2500-1.22 µg/mL) or a chelating agent (ethylenediaminetetraacetic acid [EDTA]), followed by 25 µL 5 mM ferrozine. The mixture was then incubated at room temperature for 10 min and the absorbance of the water-soluble Fe^{2+}-Ferrozine complex was recorded at 562 nm.

Quantitative phytochemical analysis

Total phenolic content

The total phenolic content (TPC) of the extracts was spectrophotometrically measured using Folin–Ciocalteu reagent according to the method described earlier.[15,16] In brief, 15 µL of 2.5 mg/mL extract was mixed thoroughly with 125 µL Folin–Ciocalteu reagent for 5 min, then 125 µL 20% (w/v) sodium carbonate was added to each well. The solution was kept in the dark for 60 min at room temperature and the absorbance was measured at 725 nm. The TPC was calculated from the calibration curve of the gallic acid solution and is presented as mg gallic acid equivalent (GAE)/mg extract.

Total flavonoid content

The total flavonoid content (TFC) of the sample was evaluated using the Aluminum chloride colorimetric method. A 20 µL aliquot of the extract (2.5 mg/mL) was transferred to a 96-well microtiter plate, followed by 6 µL of 5% $NaNO_2$ and 6 µL of 10% $AlCl_3$. After 6 min of incubation, 40 µL of 1 M NaOH was added, and the absorbance of the test solution was measured at 510 nm against blank solution. TFC/mg extract was determined from a standard curve prepared with catechin. The content is expressed as mg catechin equivalent (CAE)/mg extract.[15,16]

Preliminary profiling of the chemical constituents of Phy-Blica-D

Analysis was performed using an Agilent 1290 Infinity ultra-high-performance liquid chromatography (HPLC) system equipped with a binary pump, diode array detector (DAD), auto-sampler, and Agilent 1290 Infinity II Multicolumn Thermostat G7116B column (160 mm × 435 mm × 436 mm) at 30°C coupled with an Agilent 6545 Q-time of flight (TOF). Mass spectrometry (MS) was performed with an electrospray ionization source (ESI) in negative ion mode and scanned from m/z 100 to 1700 in auto MS mode. Ion source parameters were as follows: Gas temperature was 325°C at a flow rate of 11 L/min, nitrogen was used as the nebulizer at 35 psi, and the capillary voltage was 3.5 kV. The mobile phase consisted of a linear gradient of 0.1% (v/v) aqueous formic acid (A) and acetonitrile (B): 0–5.0 min, 7% B (v/v); 5.0–35.0 min, 7-50% B (v/v); 35.0–40 min, 50%–80% B (v/v); and 40.0–45.0 min, 7% B (v/v). The flow rate was 0.5 mL/min and the injected volume was 5 µL. All data were recorded and processed using Agilent Mass Hunter Workstation software (Version B.04.00), Agilent MSC software (Version B.07.00), and the online METLIN database. The accuracy error threshold was set at a 5 ppm.[18] Putative compounds by screening against the METLIN personal compound database (79,609 compounds), accurate mass Q-TOF MS/MS library reference spectra (9452 compounds), and accurate mass and retention time (680 compounds) were reported.

Subacute oral toxicity study

Experimental animal husbandry

Adult Wistar rats (300–350 g male rats and 200–250 g female rats) were purchased from the National Laboratory Animal Center (Nakhon Pathom, Thailand). Twenty animals of each sex were housed individually in stainless steel wire mesh cages in a ventilated room at a temperature of 23°C–25°C and a relative humidity of 50%–55%, with a 12 h light/dark artificial photoperiod (150–300 Lux) and 10–20 air exchanges per hour. Animals were provided with irradiation-sterilized pellet feed (No. CP 082, Perfect Companion Group Co., Ltd., Bangkok, Thailand) and purified water *ad libitum*.

The experiment was conducted in compliance with the Good Laboratory Practice and Test Guidelines of the Organization for Economic Cooperation and Development (OECD) test guideline 407, Repeated Dose 28-day Oral Toxicity Study in Rodents (OECD, 2008)[19] at the Southern Laboratory Animal Facility (Prince of Songkla University). All possible efforts were made to minimize suffering of the animals and to reduce the number of animals used. The Animals Ethical Committee of Prince of Songkla University (MOE 0521.11/711) approved the study protocols.

Study design

After a 2-week quarantine and acclimatization period, 40 healthy Wistar rats were randomly divided into four groups (5 males and 5 females per group). The group mean body weight was within 5% of the overall mean for each sex. Group 1 served as the control group, which received distilled water (vehicle) by gastric intubation daily throughout the course of the experiment. Experimental groups (Group 2–4) were orally administrated with Phy-Blica-D extracts at doses of 5, 50, and 300 mg/kg body weight/day, respectively, for 28 days.

Observation and examination

Clinical signs and mortality

All animals were observed for mortality and morbidity once daily for 28 days, especially after dosing for the first 1 h and every h up to 6 h. The day of administration was set as day 1. Clinical signs of toxicity, including general behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes, and changes in skin and fur texture were observed daily as recommended in OECD guideline 407. Abnormality type, date of occurrence, and the severity of signs were recorded individually.

Body weights and food consumption

Body weights and consumption of food were recorded prior to dosing on day 1 and then checked daily during the experimental period. Food consumption was calculated as the difference between the amount of food before refilling and the food remaining the next day and is expressed as g/head/day.

Relative organ weight, blood sampling, hematological parameters and biochemical analysis

On the 29th day, all animals were anesthetized by an intraperitoneal injection of 100 mg/kg thiopental sodium (Scott-Edil Pharmacia Ltd., Chandigarh, India). Blood samples were collected by cardiac puncture into EDTA-containing tubes (for hematological analysis) and nonheparinized tubes (for biochemical analysis).

Hematological parameters, including white blood cell count (WBC; lymphocyte, monocyte, and granulocyte), red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin (MCH), MCH concentration, red cell distribution width , and platelet count, were measured using an ADVA 2120i Haematology

system. Further analysis of WBCs, differential counts, and examination of RBC morphology were performed by microscopy of blood smears. Blood collected in nonheparinized tubes was centrifuged at 3000 RPM for 10 min. Plasma biochemical parameters, including alkaline phosphatase (ALP), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine, were analyzed on a Cobas® 6000 analyzer (Roche Diagnostics, Penzburg, Germany).

After the blood collection, the animals were then euthanized and their internal organs (liver, spleen, and kidney) were carefully dissected. The absolute weights of the internal organs were measured and relative organ weights were calculated. Organs were then observed for any gross lesions and preserved in 10% buffered formaldehyde solution for histopathological study.

Histological analysis

Microscopic investigations were carried out on the preserved organs and tissues taken from all animals. The organs and tissues were fixed in 10% formalin solution for 24 h. The fixed specimens (1 cm × 1 cm × 1 cm) were dehydrated using a Leica ASP300 automated tissue processor (Wetzlar, Germany), cleaned with xylol and infiltrated with molten paraffin wax at 50°C. The samples were sectioned using a microtome and the slides (3–4 μm) were stained with hematoxylin-eosin for microscopic studies. The sections were examined under a light microscope at ×40. Microscopic observations were performed by initial unblinded comparison of all control and treated groups. Blind and/or semiquantitative scoring was applied when changes were suggested by the initial inspection.

Statistical analysis

Data are expressed as mean ± standard error or mean ± standard deviation. Antioxidant capacity as well as sensory data, body weights, food consumption, relative organ weight, and hematological and biochemical parameters were assumed to be normally distributed and the differences between groups were analyzed using one-way ANOVA followed by Bonferroni's *post-hoc* comparisons tests. Values were considered statistically significant at $P < 0.05$.

RESULTS

Consumer acceptability of PeHT

The PeHT samples were made with different combinations of medicinal plants [Table 1]. The formulated samples (Phy-Blica-A, B, C, D, and E) were compared sensorially with the original polyherbal tea (Phy-Blica-O). Mean scores calculated for each attribute showed no significant differences in odor, taste and overall acceptability among the PeHT formulations, except for Phy-Blica-O [Table 2]. Phy-Blica-D ranked the highest in taste and overall acceptability at 5.50 ± 0.35 (like slightly) and 5.60 ± 0.37 (like slightly), respectively, which was significantly higher than the values obtained for taste and overall acceptability of Phy-Blica-O (2.70 ± 0.34 and 2.90 ± 0.36, respectively). It was observed that PeHT-containing *A. marmelos* had higher scores for taste, appearance, and overall acceptability. Panelists preferred Phy-Blica-D (32.5%) to Phy-Blica-B (30%), Phy-Blica-E (26%), Phy-Blica-C (9%), and Phy-Blica-A (2.5%). Considering the results of consumer acceptability, Phy-Blica-B, Phy-Blica-D, and Phy-Blica-E were evaluated for their antioxidant capacity in comparison with Phy-Blica-O.

Antioxidant capacity

Both DPPH and ABTS radical scavenging assays, expressed as IC_{50}, were performed to evaluate the free radical scavenging properties of the herbal teas [Table 3]. Among PeHT, a similar dose dependent trend was observed in both types of radical scavenging activity assays. The IC_{50} of the tested samples were in the range of 0.122–2.612 and 0.472–1.468 mg/mL for DPPH and ABTS assays, respectively. Phy-Blica-O possessed the highest free radical scavenging activity, followed by Phy-Blica-D, whereas Phy-Blica-B exhibited the lowest activity. The values of the FRAP assay are regarded as significant indicators of their electron-donating antioxidant capacity [Table 3]. The highest FRAP value was observed for Phy-Blica-O (664.36 ± 5.71 μM $FeSO_4$/mg sample) followed by Phy-Blica-D (522.99 ± 3.07 μM $FeSO_4$/mg sample) and Phy-Blica-E (455.99 ± 4.94 μM $FeSO_4$/mg sample), which comports with the results obtained from both the DPPH and ABTS free radical scavenging assays.

Table 2: Sensory data for *Phyllanthus emblica*-based functional herbal tea with different proportions of herbs and spices

Samples	Appearance	Odor	Taste	Overall acceptability
Phy-Blica-O	6.10±0.28^{ab}	5.20±0.36^{a}	2.70±0.34^{b}	2.90±0.36^{b}
Phy-Blica-A	6.90±0.29^{a}	5.57±0.32^{a}	5.07±0.39^{a}	5.27±0.36^{a}
Phy-Blica-B	5.53±0.24^{b}	5.43±0.30^{a}	5.23±0.36^{a}	5.23±0.31^{a}
Phy-Blica-C	5.83±0.23^{b}	5.40±0.32^{a}	5.33±0.40^{a}	5.13±0.35^{a}
Phy-Blica-D	6.60±0.24^{ab}	5.37±0.36^{a}	5.50±0.35^{a}	5.60±0.37^{a}
Phy-Blica-E	5.70±0.24^{b}	5.77±0.30^{a}	5.30±0.37^{a}	5.27±0.34^{a}

$^{a-d}$Values are presented as mean±SD. Means in a row sharing a common superscript letter are not significantly different ($P<0.05$) as analyzed by one-way ANOVA followed by Bonferroni's *post hoc* comparison tests. SD: Standard deviation

The metal chelating activities of PeHT were examined to assess their ability to inhibit the induction and formation of free radicals as well as to prevent the interactions between metals and lipids. Overall, iron chelating activities were in the range of 0.108–0.175 mg/mL, whereas the activity in all samples was less than that of the positive control, EDTA. Phy-Blica-D showed the highest significant chelating effect (0.108 ± 0.004 mg/mL) of all the samples.

Antioxidant-related active ingredients

Phenolic compounds, in particular flavonoids, are the most effective antioxidative chemical constituents that contribute to the antioxidant activity in both plant-originated functional food and herbal medicine. As expected, the content of phenolics and flavonoids in the samples showed similar tendencies with the antioxidant activities [Table 3]. The highest levels of phenolic and flavonoid contents were found in Phy-Blica-O, reaching 0.170 ± 0.002 mg GAE/mg extract and 0.103 ± 0.003 mg CAE/mg extract, respectively, followed by Phy-Blica-D (0.118 ± 0.001 mg GAE/mg extract for TPC and 0.055 ± 0.003 mg CAE/mg extract for TFC).

Considering the results for both consumer acceptability and antioxidant capacity, Phy-Blica-D was further analyzed by HPLC-DAD-ESI-MS in negative mode. Eighteen proposed compounds, including 6-galloylglucose (RT [Retention time] =0.558), 1-O-galloylglycerol (RT = 0.848), fertaric acid (RT = 0.913), vanilpyruvic acid (RT = 1.727), (2S)-5,7,3',4'-tetrahydroxyflavanone 6-C-glucoside (RT = 6.969), naringerin (RT = 10.066), agecorynin B (RT = 10.276), castavinol (RT = 10.647), chalconaringenin 2'-rhamnosyl-(1->4)-xyloside (RT = 10.863), beta-rhodomycin (RT = 11.739), sericoside (RT = 15.674), alliosterol 1-(4"-galactosylrhamnoside) 16-galactoside (RT = 20.056), licorice saponin A3 (RT = 20.648), asparasaponin II (RT = 20.722), licorice saponin G2 (RT = 21.429), betavulgaroside II (RT = 22.876), glycyrrhizic acid (RT = 24.475), and 6-gingerol (RT = 25.347) were identified as the predominant compounds of Phy-Blica-D.

Subacute oral toxicity

In-life parameters

In the 28-day repeated oral dose toxicity study, no treatment-related mortality was observed. No behavioral changes or visual symptoms of toxicity related to the administration of Phy-Blica-D were observed in the animals during the entire treatment period. There were no significant changes in body weight or food consumption for both sexes at all doses during the study [Figure 1].

Relative organ weights

The results show that the repeated oral administration of Phy-Blica-D (5, 50, and 300 mg/kg) did not induce any structural morphology changes in the liver, kidney, and spleen in the treated rats compared to the control rats [Table 4]. No significant changes in the relative weights of liver, kidney, and spleen were observed for all Phy-Blica-D-dosed rats compared to the control rats of both sexes.

Hematology

Statistical analysis of the hematological parameters shows no significant differences between the treated and the control groups of both genders at the end of the treatment period [Table 5].

Biochemical parameters

There were no significant differences in the plasma levels of ALP, ALT, BUN, and creatinine between the treatment and the control groups for both sexes of animals that repeatedly received oral doses of Phy-Blica-D at 5, 50, and 300 mg/kg over the 28-day study period [Figure 2].

Kidney, liver, and spleen histopathology

Histopathological examination of the vital organs including kidney, liver and spleen displayed no consistent treatment-related macroscopic or histological changes in either sex of animals that were treated with Phy-Blica-D [Figure 3]. Histological sections of the liver showed normal architecture with hepatocytes arranged around central veins, with no evidence of necrosis, lesions, or any pathological damage. Observation of histological sections of kidneys of Phy-Blica-D-treated rats showed adequate glomeruli and normal tubules, with no evidence of glomerular damage or lumen casts. There were no test article-related histopathological lesions found in the spleen, which showed normal histology of white pulp, red pulp, and central arterioles.

Table 3: Extraction yield and antioxidant activities of *Phyllanthus emblica*-based functional herbal tea

Herbal infusion	Yield (percentage; w/w)	Free radical scavenging assay IC_{50} (mg/mL)		Ferric reducing antioxidant power $FeSO_4$ equivalent (μM $FeSO_4$/mg sample)	MCA	Active constituents*	
		DPPH	ABTS		IC_{50} (mg/mL)	Total phenolic content	Total flavonoid content
Phy-Blica-O	14.56	0.122±0.002^{ab}	0.472±0.005^{b}	664.36±5.71^{a}	0.127±0.001^{c}	0.170±0.002^{a}	0.103±0.003^{a}
Phy-Blica-B	22.2	2.612±0.111^{d}	1.468±0.011^{d}	442.04±19.20^{c}	0.121±0.005^{c}	0.102±0.004^{c}	0.022±0.004^{c}
Phy-Blica-D	8	0.243±0.006^{b}	0.486±0.002^{b}	522.99±3.07^{b}	0.108±0.004^{b}	0.118±0.001^{b}	0.055±0.003^{b}
Phy-Blica-E	20.2	0.370±0.006^{bc}	1.350±0.007^{c}	455.99±4.94^{c}	0.175±0.004^{d}	0.066±0.003^{d}	0.020±0.004^{c}
Positive control$^{\#}$		0.061±0.001^{a}	0.078±0.002^{a}	-	0.007±0.000^{a}	-	-

$^{a-d}$Values in the same column with different superscripts are significantly different (P<0.05) analyzed by one-way ANOVA followed by Bonferroni's *post hoc* comparisons tests. *TPC is expressed as mg GAE/mg extract and TFC is expressed as mg CAE/mg extract, $^{\#}$Positive controls were trolox for DPPH and ABTS assays and EDTA for MCA assay. TPC: Total phenolic contentl; GAE: Gallic acid equivalent; TFC: Total flavonoid content; CAE: Catechin equivalent; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,20-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid; MCA: Metal chelating activity; EDTA: Ethylenediaminetetraacetic acid

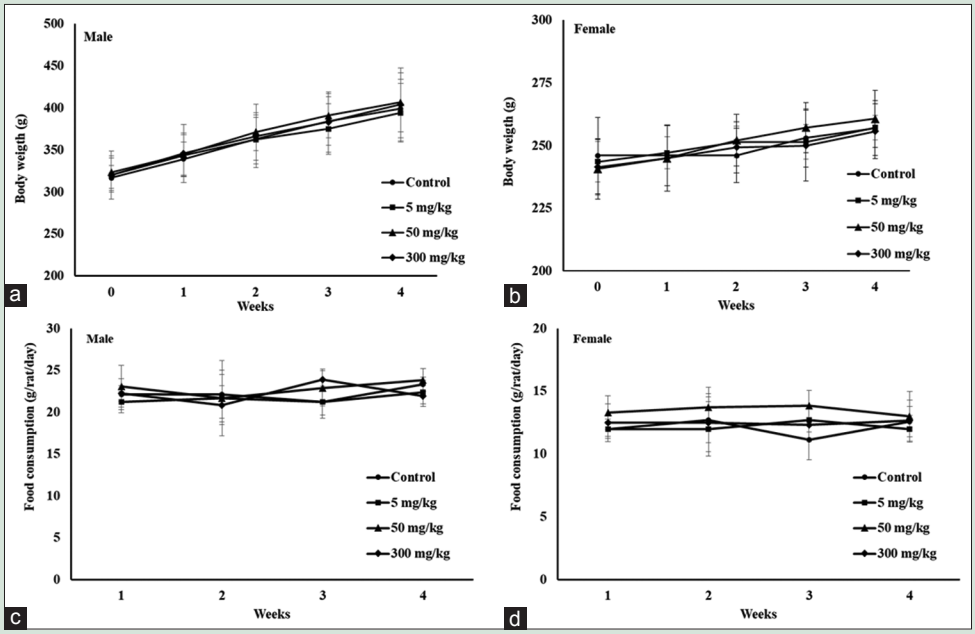

Figure 1: Changes in body weight (a and b) and food consumption (c and d) of male and female rats exposed to 5, 50 and 300 mg/kg/day in the 28-day repeated oral dose toxicity study of Phy-Blica-D extract. Values are presented as mean ± standard deviation and n = 5

Table 4: Relative organ weights of male and female rats treated orally with 5, 50, and 300 mg/kg/day Phy-Blica-D extract for 28 days

Organ	Control	Percentage of relative organ weight (mean±SD)		
		Dose of Phy-Blica-D extract (mg/kg/day)		
		5	50	30
Male rats (*n*=5)				
Liver	3.271±0.080	3.303±0.176	3.213±0.116	3.260±0.072
Spleen	0.213±0.011	0.206±0.030	0.210±0.020	0.206±0.015
Kidney	0.543±0.070	0.546±0.025	0.553±0.028	0.541±0.010
Female rats (*n*=5)				
Liver	3.016±0.170	3.022±0.165	2.974±0.103	3.074±0.178
Spleen	0.266±0.020	0.254±0.020	0.266±0.027	0.256±0.027
Kidney	0.552±0.027	0.532±0.032	0.538±0.033	0.550±0.036

SD: Standard deviation

Table 5: Hematological parameters of male and female rats treated orally with 5, 50, and 300 mg/kg/day Phy-Blica-D extract for 28 days

Parameters (unit)*	Control	Dose of Phy-Blica-D extract (mg/kg/day)		
		5	50	300
Male rats (*n*=5)				
WBC (10^3/mm^3)	2.85±0.85	2.55±0.71	2.82±0.65	3.01±0.88
Lymp (%)	71.62±9.67	74.83±6.71	76.40±11.11	76.96±10.18
Mono (%)	12.56±3.22	13.60±4.11	14.51±4.25	12.47±5.53
Gran (%)	18.47±7.70	16.73±7.34	15.00±8.17	17.14±6.25
RBC (10^6/mm^3)	8.51±0.79	8.29±0.27	8.54±0.43	8.30±0.44
Hb (g/dL)	15.24±1.19	15.56±0.31	15.56±0.46	15.40±0.42
Hct (%)	48.92±4.06	48.38±1.09	48.76±1.64	47.36±2.18
MCV (fL)	57.52±1.44	58.32±0.96	57.10±1.55	57.04±1.38
MCH (pg)	17.96±0.43	18.76±0.54	18.23±0.62	18.56±0.62
MCHC (g/dL)	31.18±0.52	32.18±0.86	31.93±0.54	32.54±0.79
RDW (%)	12.62±0.33	12.54±0.34	12.85±0.45	12.56±0.25
Platelet count (10^3/mm^3)	631.40±67.57	662.60±105.77	645.83±90.09	679.40±94.27
Female rats (*n*=5)				
WBC (10^3/mm^3)	2.54±0.94	2.46±0.92	2.33±0.52	2.59±0.79
Lymp (%)	73.33±10.48	72.86±8.64	74.20±9.85	70.77±7.47
Mono (%)	13.21±5.96	12.64±3.37	11.95±5.77	12.34±3.48
Gran (%)	18.30±8.66	15.51±6.51	16.46±7.21	16.25±6.28
RBC (10^6/mm^3)	8.09±0.20	8.05±0.458	8.18±0.32	8.15±0.28
Hb (g/dL)	15.05±0.52	14.90±0.70	15.33±0.35	15.31±0.48
Hct (%)	47.45±1.98	45.88±1.98	47.25±1.75	46.70±2.24
MCV (fL)	58.68±1.81	57.00±1.29	57.75±1.68	57.23±1.17
MCH (pg)	18.65±0.4	18.51±0.53	18.75±0.51	18.78±0.59
MCHC (g/dL)	31.84±0.51	32.47±0.92	32.47±0.90	32.85±1.14
RDW (%)	12.14±0.21	12.143±0.39	12.28±0.21	12.08±0.24
Platelet count (10^3/mm^3)	627.28±91.69	645.57±164.35	656.50±93.34	641.33±134.02

*WBC: White blood cell count; Lymp: Lymphocytes; Mono: Monocytes; Gran: Granulocytes; RBC: Red blood cell count; Hb: Hemoglobin; Hct: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red cell distribution width

DISCUSSION

The overwhelming interest in functional food has led to attention to ethnic herbal teas and herbal-based beverages that possess biofunctional characteristics with pleasing taste and functionality.[4] As shown in this study, a novel herbal-based functional tea made from Phy-Blica-D was acceptable to consumers, displayed notable *in vitro* antioxidant capacity and exerted no consistent treatment-related toxicological changes after repeated oral administrations over 28 days.

Consumer acceptability is an important issue for guiding the development of herbal-based teas that fit market demand. While containing the highest TPC and TFC and the greatest antioxidant property, Phy-Blica-O, a polyherbal infusion which conventionally has been consumed as a restorative drink, received the lowest mean score of overall acceptability and taste. Similar findings were obtained by studies by Castiglioni *et al*.,[20] Francisco and Resurreccion,[21] and Kobue-Lekalake *et al*.,[22] which showed infusions of *Camellia sinensis*, peanut skin and sorghum with high TPC and TFC caused an increase in bitter and astringent flavours and therefore, decreased their overall acceptability. Addition of sugar to beverages effectively increased consumer acceptability scores by up to 30%;[23] however, this increases the risk of obesity and non-communicable diseases associated with the consumption of sugar-sweetened beverages.[24] Therefore, our findings demonstrated that *P. emblica*-based herbal teas can be modified by either adding taste-modifying medicinal plants such as *G. glabra*, *S. torvum* and *A. marmelos* or reducing the content of *T. crispa*, which has a bitter taste, thereby improving the mean scores of taste and overall acceptability while not affecting the appearance and odor mean scores. The sensory attributes of the modified PeHT were lower than those of a ready-to-serve juice of *P. emblica*-containing added sugar[25] and fermented juice made from *A. marmelos* fruits,[26] but they were comparable with the commercially available tea, Lipton® and polyherbal teas made from *Cymbopogon citratus*, *Lippia multiflora,* and *Ganoderma lucidum*.[27]

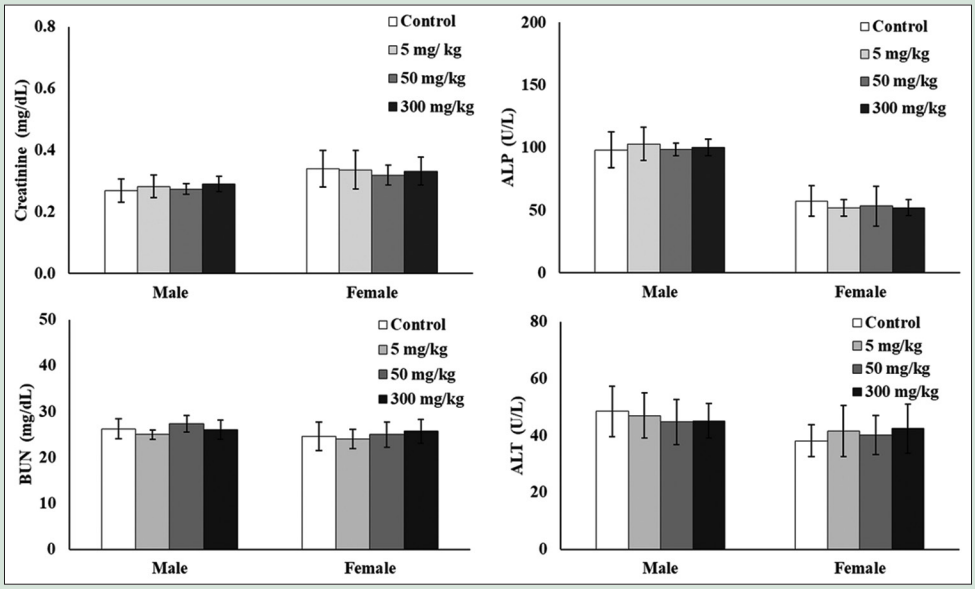

Figure 2: Serum biochemical values of male and female rats measured during the 28-day repeated oral dose toxicity study of Phy-Blica-D extract. Values are presented as mean ± standard deviation and *n* = 5. ALP: alkaline phosphatase; BUN: blood urea nitrogen; ALT: alanine aminotransferase

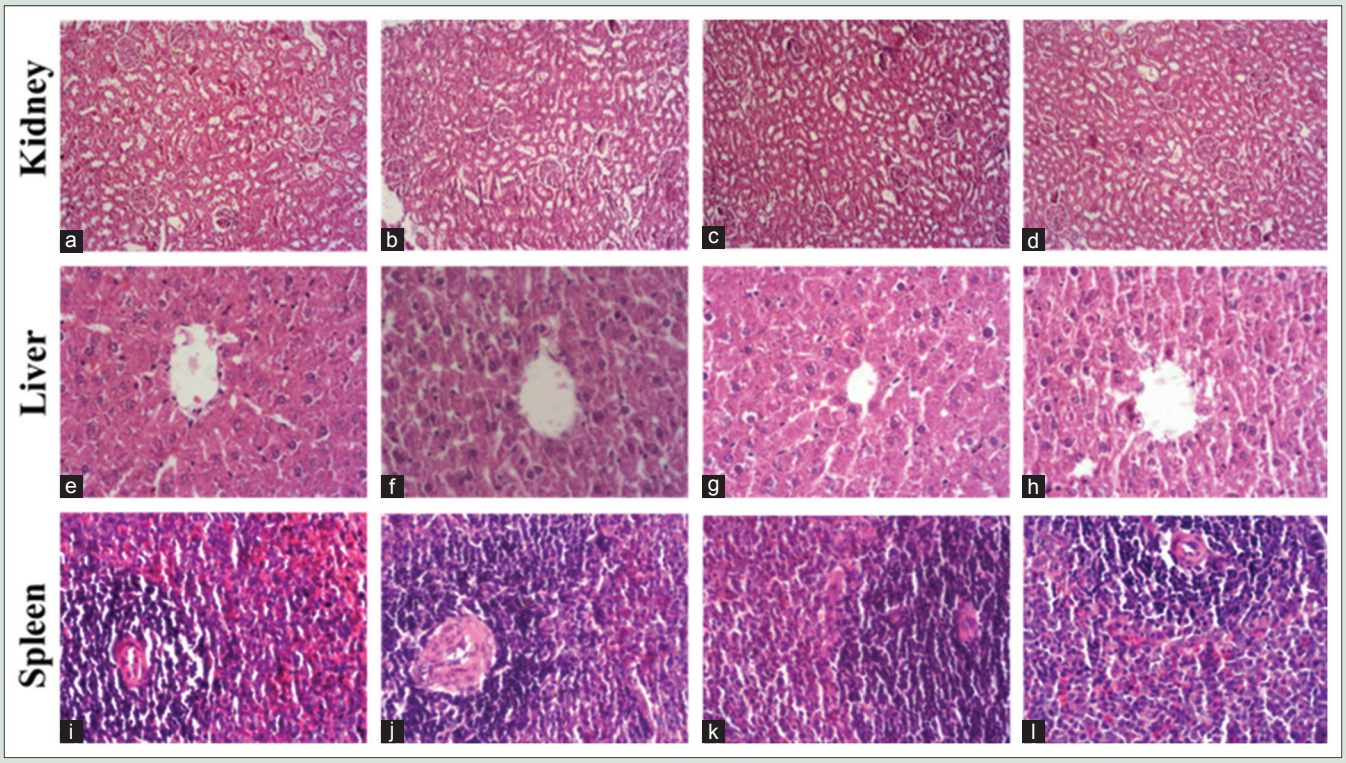

Figure 3: Photomicrographs of kidney (a-d) (×10), liver (e-h) (×40) and spleen (i-l) (×40) section stained with haematoxylin-eosin. Normal histology of kidney (a), liver (e) and spleen (l) tissue in control rat in subacute toxicity study of Phy-Blica-D extract sacrificed at the end of the study period. Photomicrograph of kidney, liver and spleen section of a rat treated with Phy-Blica-D extract at 5 mg/kg (b, f and j, respectively), 50 mg/kg (c, g and k, respectively) and 300 mg/kg (d, h and l, respectively)

Phy-Blica-D consists of *P. emblica*, *Terminalia arjuna*, *Terminalia bellirica*, *Cyperus rotundus*, *Maerua siamensis*, *T. crispa*, *Terminalia citrina*, *A. sativum*, *Piper retrofractum*, *Zingiber officinale*, *Alpinia galanga*, *G. glabra*, *S. torvum,* and *A. marmelos,* which exhibit notable biological activity as both primary and secondary antioxidants. Among these herbal components, the beneficial effects of *P. emblica*, *T. arjuna*, *T. bellirica*, *T. crispa*, *A. sativum,* and *Z. officinale* have been systematically proved in both animal models and human subjects including antioxidant, hypoglycemic, hypolipidemic, anti-inflammatory, and hepatoprotective effects.[9,28-33] Taste-modifying

medicinal plants, including *G. glabra*[34,35] and *A. marmelos*,[36] have been scientifically demonstrated to possess antioxidant capacity and their safety data have been confirmed. Based on the existing scientific evidences, these plants contain 6-galloylglucose,[37] fertaric acid,[38] naringerin,[39] glycyrrhizic acid,[40] and 6-gingerol,[41] which were found to possess antioxidant properties; therefore, these compounds should be noted as promising biological markers responsible for the antioxidant activity of Phy-Blica-D.

It was hypothesized that a traditional polyherbal formula should possess a high clinically effective dose and be safer than the utilization of a single medicinal plant, but a recent review revealed that there were no significant differences in the NOAEL and the human equivalent dose (HED) values between single herb and polyherbal formulations.[42] According to the OECD guidelines, repeated 28-day oral toxicity study of Phy-Blica-D showed no change in clinical, biochemical, or hematological parameters. The results of this subacute toxicity study revealed that the NOAEL of Phy-Blica-D extract is >300 mg/kg body weight/day or ~3.7 L/kg body weight/day in both sexes and there no target organs were affected. The calculation of HED value from the NOAEL values in this study[43] was found to be 48.39 mg/kg/day or ~600 mL/kg body weight/day. With the exception of *M. siamensis* and *P. retrofractum*, previous *in vivo* toxicological studies have revealed that LD_{50} of the herbal components varied from 1000 to 5000 mg/kg body weight. According to The Globally Harmonized System of Classification and Labelling of Chemicals, it should be noted that 10 out of 14 herbal ingredients of Phy-Blica-D, including *P. emblica,*[44] *T. arjuna,*[12] *T. bellirica,*[45] *C. rotundus,*[46] *T. crispa,*[9] *T. citrina,*[47] *A. sativum,*[11] *Z. officinale,*[48] *A. galanga,*[13] and *S. torvum*[49] were classified under category-5, with the LD_{50} ranging from >2000 to 5000 mg/kg body weight. The results of this study not only provide scientific evidence for the safety of PeHT but also importantly provide the dose range of Phy-Blica-D for subsequent experiments in rats, such as study of hypoglycemic effect, hypolipidemic effect and the ability of Phy-Blica-D to attenuate *in vivo* oxidative stress in non-communicable diseases.

CONCLUSION

Our novel *P. emblica*-based herbal tea Phy-Blica-D developed from a traditional revitalization formula had significantly high consumer acceptance scores and showed promising antioxidant capacity. Importantly, sub-acute oral administration of Phy-Blica-D at doses of up to 300 mg/kg body weight/day (~ equal to 3.7 L infusion/kg body weight/day) for 28 days was revealed to be generally safe in both sexes of rats. However, further information on subchronic toxicity, *in vivo* antioxidant capacity and in particular, reduction of oxidative stress-related-non-communicable diseases, as well as clinical investigations are required to confirm the safety and effectiveness of Phy-Blica-D in humans as an herbal-based functional beverage or for its development as a functional ingredient.

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Conflicts of interest

There are no conflicts of interest.

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