Research Article

Inhibition of lipid peroxidation, cyclooxygenase enzyme and human tumor cell proliferation by compounds in herbal water

Yunbao Liu[1], Vanisree Mulabagal[1], Camille S. Bowen-Forbes[1], Rejanish Aviayan[2] and Muraleedharan G. Nair[1]

[1] Bioactive Natural Products and Phytoceuticals, Departments of Horticulture and National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan, USA
[2] Veliyamparambath, Kuzhoor, Kerala, India

A powdered mixture of dried herbs, "Panamrutham", is sold in India for the preparation of "herbal drinking water". The hot water extract of this herbal mixture gave lipid peroxidation (LPO), cyclooxygenase (COX-1 and -2) enzyme and human tumor cell proliferation inhibitory activities between 25 and 250 μg/mL. The bioassay-guided purification of the water extract afforded a novel compound (1), along with phenolics (2, 4, 6, and 7) and sesquiterpenoids (3 and 5). The isolates were evaluated for LPO, COX-1 and -2 enzyme and human tumor cell proliferation inhibitory activities. At 25 μg/mL, compounds 1 – 7 inhibited LPO by 22 – 73% and COX-1 and -2 enzymes by 3 – 14% and 14 – 74%, respectively. Compounds 5 and 6 at 25 μg/mL showed growth inhibition of colon, gastric, lung, breast and central nervous system human tumor cell lines by 60 and 67, 43 and 60, 24 and 64, 34 and 65, 6 and 27%, respectively. Compounds 2, 4 and 7 displayed weak or moderate growth inhibition of colon, gastric and breast human tumor cell lines. This is the first report on the LPO inhibitory activities of compounds 1 and 3 – 7 and the COX and tumor cell proliferation inhibitory activities of compounds 1, 3 – 5 and 7.

Keywords: Antioxidant / Antiinflammatory / Anticancer / Heterodimer / Protosappanin

Received: December 1, 2008; revised: February 4, 2009; accepted: February 4, 2009

1 Introduction

In recent years, more and more people have resorted to herbal medicine to maintain good health and vitality. Herbal medicine is still the mainstay of about 75 – 80% of the world's population, mainly in the developing countries for primary healthcare. Some herbal medicines used are powdered mixtures of several plants and contain complex mixtures of bioactive compounds which differ from conventional supplements and Pharma drugs [1, 2]. The herbal mixture, in most cases, is generally prepared as a decoction by boiling the prescribed dose with water for a prolonged period of time. The decanted supernatant will then be consumed in portions until the dose is completed. The bioactive compounds present in the herbal plant mixture may or may not be present intact in the boiled water extract because the potential exists for new products resulting from rearrangement and conversion due to prolonged heating. Therefore, it is necessary to characterize the bioactive molecules in the decoction rather than the bioactive compounds in the herbal mixture.

"Panamrutham", a powdered mixture of the dried plants Khadira (*Acacia catechu*), Useera (*Vetiveria zizanioides*), Padmaka (*Prunus cerasoides*), Lavanga (*Syzygium aromaticum*), Rakta Chandana (*Pterocarpus santalinus*), Sariba (*Hemidesmus indicus*), Vilwa (*Crataeva religiosa*) and Ela (*Elettaria cardamomum*), is one of the popular herbal products sold in Southern India. The compounds reported from the constituent plants belong to different classes including triterpenoids, sesquiterpenoids, flavonoids, steroidal glycosides, lignans, coumarins, stilbenes, phenolics and essential oils [3 – 11]. These natural antioxidants are important ingredients that facilitate the prevention of the oxidative deterioration in *in vivo* and *in vitro* systems. Herbal medicines containing these antioxidants are expected to have preventative activity against oxidative-stress related diseases. Anecdotal

Correspondence: Professor Muraleedharan G. Nair, Bioactive Natural Products and Phytoceuticals, 173 National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan, USA
E-mail: nairm@msu.edu
Fax: +1-517-432-2310

claims on the product package suggest that its consumption improves digestion and blood purification (see Supporting Information). It is used for the exclusive preparation of a herbal water as an everyday drink. Therefore, in our study "Panamrutham" was boiled with water, filtered and the supernatant was lyophilized. The aim of this study was to isolate the putative bioactive compounds present in the drink by using bioassay-guided fractionation and purification methods. In this paper, we report the isolation and characterization of seven compounds including a novel compound (1) present in the "Panamruthum" drink and the quantification of compound 1 in both cold and boiled water preparations. Also, we report the LPO, COX and tumor cell proliferation inhibitory activities of the isolated compounds.

2 Materials and methods

2.1 General experimental

All solvents used for isolation and purification were of ACS reagent grade (Aldrich Chemical Co., Inc., Milwaukee, WI). ^{1}H NMR spectra were recorded on a 500 MHz Varian VRX instruments. ^{13}C NMR spectra were recorded at 125 MHz on the same instruments. The COX-1 enzyme was prepared from ram seminal vesicles purchased from Oxford Biomedical Research, Inc. (Oxford, MI) and the COX-2 enzyme was prepared from insect cells cloned with human PGHS-2 enzyme. Arachidonic acid was purchased from Oxford Biomedical Research, Inc. (Oxford, MI). The nonsteroidal anti-inflammatory drug Aspirin, used in COX inhibitory assay, was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Celeberex™ capsules and Vioxx® tablets were physician's professional samples provided by Dr. Subash Gupta, Sparrow Pain Center, Sparrow Hospital, MI. Positive controls used in LPO assay, *t*-butyl hydroquinone (TBHQ), butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT), were purchased from Sigma–Aldrich Chemical Company. Human tumor cell lines MCF-7 (breast), SF-268 (central nervous system, CNS) and NCI-H460 (lung) were purchased from the National Cancer Insitute (NCI, Bethesda, MD). HCT-116 (colon) and AGS (gastric) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). All cell lines were maintained in the bioactive natural products and phytoceuticals laboratory at Michigan State University.

2.2 Plant material

The commercial product "Panamrutham" (sealed packets of 50 g) was purchased from Vaidyaratnam Oushadhasala, Thaikkattussery, Ollur, Thrissur, Kerala, India in February 2007 and stored at room temperature in the bioactive natural products and phytoceuticals laboratory at Michigan State University until the preparation of herbal water for analysis. This product is a mixture of dried plant materials Khadira (*Acacia catechu*), Useera (*Vetiveria zizanioides*), Padmaka (*Prunus cerasoides*), Lavanga (*Syzygium aromaticum*), Rakta Chandana (*Pterocarpus santalinus*), Sariba (*Hemidesmus indicus*), Vilwa (*Crataeva religiosa*), and Ela (*Elettaria cardamomum*). The percentage of each plant material in the mixture is a trade secret of the manufacturer.

2.3 Extraction and isolation

The plant mixture "Panamrutham" (140 g) was boiled with water (6 L) for 30 min, cooled to room temperature, centrifuged for 5 min and lyophilized to yield a red powder (10.9 g). An aliquot of this red powder (10 g) was stirred with MeOH (2 × 10 mL) and centrifuged for 10 min to yield the MeOH-soluble fraction (7.11 g). The residue was insoluble in water and methanol and kept aside with the assumption that it was the residual plant material. A portion of the MeOH extract (2.5 g) was fractionated by silica gel MPLC and eluted using $CHCl_3$/MeOH gradient systems. $CHCl_3$/MeOH (4:1) yielded fractions A (22 mL), B (30 mL), C (105 mL), D (50 mL), E (60 mL), F (170 mL) and G (75 mL); $CHCl_3$/ MeOH (2:1) gave fraction H (500 mL) and 100% MeOH gave fractions I (300 mL) and J (200 mL). Based on TLC evaluation, fractions A, D, G, H, I and J did not show compounds of interest after developing the plate (viewed under UV and sprayed with acid) and hence kept aside. Fraction B (200 mg) was subjected to MPLC and eluted with $CHCl_3$/MeOH (10:1) to afford three fractions B_1 (70 mg), B_2 (30 mg), and B_3 (90 mg). Fractions B_1–B_3 were further purified with PTLC to obtain compounds 2 (19 mg), 3 (20 mg), and 5 (40 mg), respectively. Fraction C (180 mg) was subjected to medium pressure MPLC and eluted with $CHCl_3$/MeOH (8:1) to yield fractions C_1 (62 mg), C_2 (58 mg), C_3 (10 mg), C_4 (10 mg), C_5 (20 mg), and C_6 (20 mg). The two major fractions (C_1 and C_2) were further purified by preparative PTLC ($CHCl_3$/MeOH, 30:1) to obtain compounds 7 (21 mg) and 4 (5.5 mg), respectively. Fraction E (50 mg) was purified by C-18 preparative HPLC using MeOH/H_2O (50:50) as the mobile phase at a flow rate of 4 mL/min to yield compound 1 (11 mg). Fraction F (71 mg) was subjected to MPLC by using of $CHCl_3$/MeOH (10:1) as the solvent system to afford compound 6 (15 mg).

2.4 Spectral data

Compound 1: White powder; mp 188–189°C; UV (MeOH) λ_{max} 254, 280 nm; IR (NaCl) ν_{max} 3202, 1610, 1497, 1439, 1260, 1024, 821 cm^{-1}; ^{1}H NMR (500 MHz, in DMSO-d_6): δ 6.91 (2H, each 1H, d, J = 8.0 Hz, H-1/1′), 6.53 (1H, brd, J = 8.0 Hz, H-2), 6.48 (1H, brd, J = 8.0 Hz, H-2′), 6.46 (1H, brs, H-4), 6.38 (1H, brs, H-4′), 4.24, 3.37 (each 1H, d, J = 12.0 Hz, H-6), 3.95, 3.74 (each 1H, d,

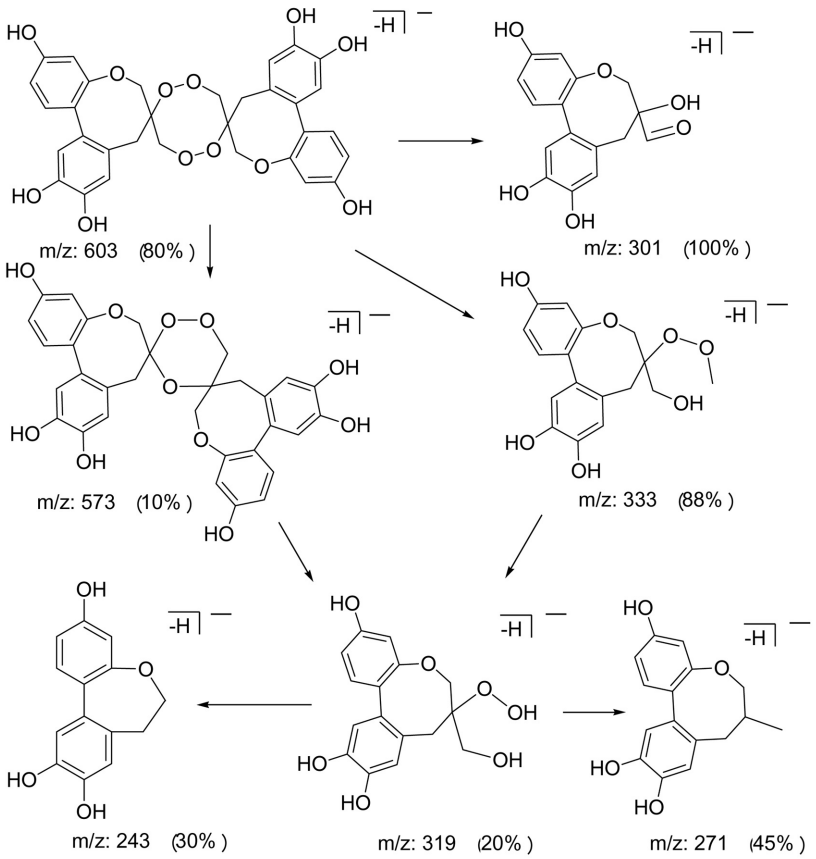


Figure 1. Proposed negative ion ESIMS fragmentation of compound 1.

J = 12.0 Hz, H-6′), 2.54, 2.43 (each 1H, d, *J* = 13.0 Hz, H-8), 2.35 (2H, brs, H-8′), 6.58 (1H, s, H-9), 6.54 (1H, s, H-9′), 6.66 (1H, s, H-12), 6.62(1H, s, H-12′), 3.34, 3.25 (each 1H, d, *J* = 12.5 Hz, H-13), 3.31, 3.16 (each 1H, d, *J* = 11.0 Hz, H-13′); ^{13}C NMR (125 MHz, in DMSO-d_6): δ 131.9/131.0 (C-1/1′), 111.0/110.2 (C-2/2′), 157.7/157.7 (C-3/3′), 107.9/107.0 (C-4/4′), 159.2/157.9 (C-4a/4′a), 76.2/74.7 (C-6/6′), 71.4/71.2 (C-7/7′), 42.0/39.5 (C-8/8′), 123.7/121.9 (C-8a/8′a), 116.7/116.2 (C-9/9′), 143.7/143.6 (C-10/10′), 143.7/143.6 (C-11/11′), 119.4/118.4 (C-12/12′), 130.0/129.8 (C-12a/12′a), 126.7/126.2 (C-12b/12′b), 67.2/64.3 (C-13/13′); LR-ESIMS (negative ion,% intensity) gave a molecular ion at *m/z* 603 (80, $[M-H]^-$) and fragments at *m/z* 399 (40), 333 (88), 319 (20), 301(100), 271 (45) and 243 (50); LR-ESIMS (positive ion,% intensity) showed a molecular ion at *m/z* 627 (20, $[M + Na]^+$), 363 (35), 357 (100), 261 (35) and 217 (40); HR-ESIMS *m/z* 603.1493 $[M-H]^-$ (calcd. for $C_{32}H_{27}O_{12}$, 603.1503). We have proposed fragmentation patterns with relative intensity of compound 1 under negative and positive ion ESI mass spectral conditions (Fig. 1 and 2).

Compound 2: liquid; ^{1}H NMR (300 MHz, CD_3OD) δ 3.23 (2H, d, *J* = 7.2 Hz, α-H_2), 3.81 (3H, s, OCH_3), 5.00 (2H, m, γ-H_2), 5.89–9.98 (1H, m, β-H), 6.60 (1H, dd, *J* = 7.8, 2.8H-6), 6.69 (1H, d, *J* = 7.8 Hz, H-6), 6.72 (1H, d, *J* = 2.8 Hz, H-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 148.1 (C-1), 141.8 (C-2), 139.4 (β-C), 132.4 (C-4), 121.9 (C-6), 116.1 (γ-C), 115.4 (C-3), 113.2 (C-5), 56.3 (OCH_3), 40.8 (α-C). Based on the spectral data, compound 2 was identified as eugenol [2-methoxy-4-(2-propenyl) phenol] [12].

Compound 3: Pale yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.85 (1H, m, H-3), 1.20 (3H, s, 15-Me), 1.17 (3H, s, 12-Me), 1.16 (3H, s, 13-Me), 0.86 (3H, s, 14-Me); ^{13}C NMR (125 MHz, $CDCl_3$) δ 54.4 (C-1), 199.3 (C-2), 126.8 (C-3), 163.3 (C-4), 47.4 (C-5), 24.2 (C-6), 49.6 (C-7), 22.4 (C-8), 40.0 (C-9), 37.5 (C-10), 72.7 (C-11), 27.5 (C-12), 27.9 (C-13), 16.7 (C-14), 22.0 (C-15). Based on the spectral data, compound 3 was identified as isopterocarpolone [13].

Compound 4: Yellow powder; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.03 (1H, d, *J* = 8.5Hz, H-1), 6.67 (1H, dd, *J* = 8.5, 2.5 Hz, H-2), 6.64 (1H, d, *J* = 2.5 Hz, H-4), 6.23 (1H, s, H-9), 6.61 (1H, s, H-12), 2.01 (2H, s, H-6), 2.24 (2H,s, H-8); ^{13}C NMR (125 MHz, DMSO) δ 131 (C-1), 113 (C-2), 159 (C-3), 109 (C-4), 78.9 (C-5), 206 (C-6), 49.4 (C-7), 125.1 (C-8), 117 (C-9), 145 (C-10), 145 (C-11), 117 (C-12), 131 (C-13), 127.2 (C-14); ESI- *m/z*: 271 $[M-H]^-$. Based on the spectral data, compound 4 was identified as protosappanin [14].

Compound 5: White amorphous powder; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.72 (3H, s, 14-Me), 1.23 (6H, s, 12, 13-Me), 4.85, 4.59 (each 1H, m, 15-H), 3.90 (1H, m, 2-H), 2.68 (3-H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 50.6 (C-1), 67.6

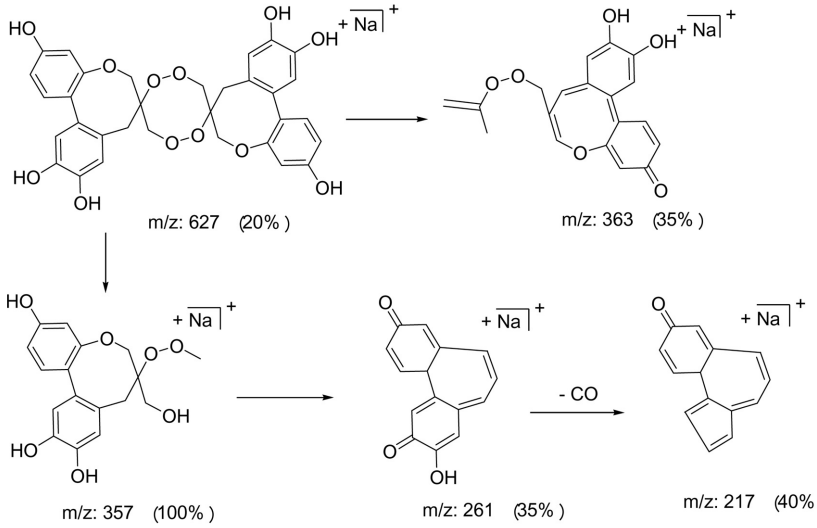


Figure 2. Proposed positive ion ESIMS fragmentation of compound 1.

(C-2), 46.2 (C-3), 147.8 (C-4), 49.2 (C-5), 40.6 (C-6), 49.1 (C-7), 24.5 (C-8), 21.8 (C-9), 35.0 (C-10), 72.8 (C-11), 27.1 (C-12), 27.0 (C-13), 17.1 (C-14), 107.8 (C-15). Based on the data, compound 5 was identified as pterocarpol [15].

Compound 6: Pale yellow solid; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.39 (2H, d, J = 7.0 Hz, H-2, 6), 7.13 (1H, d, J = 16.5 Hz, H-7), 6.91 (1H, d, J = 16.5 Hz, H-8), 6.74 (2H, d, J = 7.0 Hz, H-3, 5), 6.69 (2H, d, J = 2.5 Hz, H-2, 6), 6.35 (1H, t, 2.5 Hz, H-4), 3.75 (6H, s, OMe-3, 5). ^{13}C NMR (CD_3OD, 125 MHz) δ 162.5 (C-3, 5), 158.5 (C-4), 141.3 (C-1), 130.2 (C-4), 129.1 (C-7), 128.9 (C-2, 6), 126.8 (C-8), 115.5 (C-3, 5), 105.2 (C-2, 6), 100.3 (C-4), 55.7 (CH_3O-3, 5). Based on the spectral data, compound 6 was identified as pterostilbene [16, 17].

Compound 7: Reddish-yellow crystals; ^{1}H NMR (CD_3OD, 500 M Hz): δ 7.18 (1H, d, J = 8.5 Hz, H-1), 6.70 (1H, s, H-11), 6.59 (1H, s, H-8), 6.45 (1H, dd, J = 8.5, 2.0 Hz, H-2), 6.28 (1H, d, J = 2.0 Hz), 3.92(1H, d, J = 11 Hz, H-6α), 3.68 (1H, d, J = 11 Hz, H-6β), 3.96 (1H, s, H-12), 3.01 (1H, d, J = 15.5 Hz, H-7α), 2.77 (1H, d, J = 15.5 Hz, H-7β). ^{13}C NMR (CD_3OD, 500 MHz) δ 157.6 (C-4a), 155.4 (C-3), 145.3 (C-9 and 10), 137.1 (C-11a), 131.8 (C-1), 130.9 (C-7a), 115.2 (C-1a), 112.5 (C-1a), 112.1 (C-11), 109.5 (C-2), 103.9 (C-4), 77.7 (C-6a), 70.4 (C-6), 50.6 (C-12), 42.5 (C-7); ESI-MS m/z: 285 $[M-H]^-$. Based on the spectral data, compound 7 was identified as brazilin [18].

2.5 Lipid peroxidation inhibitory assay

Compounds 1 – 7 and the hot water extract were tested for LPO inhibitory activity by using Large Unilamellar Vesicles (LUVs) according to the previously published method [19]. Briefly, the LUVs were prepared by mixing the phospholipid, 1-stearolyl-2-linoleoyl-sn-glycerol-3-phosphocholine (Aventis Polar Lipids, Inc., Alabaster, AL) with a fluorescent probe 3-[p-(6-phenyl)-1, 3, 5-hexatrienyl] phenyl propionic acid (Molecucar Probes, Inc., Eugene, OR). The assay was conducted in a buffer consisted of HEPES (pH 8, 0.1 M, 100 uL), NaCl (1 M, 200 uL), N_2-sparged water (1.64 mL), test sample (20 uL) in DMSO and LUV (20 uL) suspension. The peroxidation was initiated by the addition of $FeCl_2$ (20 uL, 0.5 mM) solution and was monitored by observing the fluorescence at 0, 1, 3 and every 3 min thereafter up to 21 min using a Turner model 450 digital fluorometer (Brenstead Thermolyne, Dubuque, IA) at 384 nm. The relative fluorescence was determined by dividing the fluorescence value at a given time point by that at zero min. The antioxidant standards BHA, BHT and TBHQ were tested at 1 μg/mL.

2.6 Cyclooxygenase enzyme inhibitory assay

The COX-1 used in the assay was prepared from ram seminal vesicles (Oxford Biomedical Research Inc., Oxford, MI). COX-1 was isolated from microsomal preparations of ram seminal vesicles according to methods reported previously. A microsomal preparation of recombinant human COX-2, obtained from an insect cell lysate, was used as the source of COX-2 enzyme for the assay. [20, 21] COX enzyme inhibitory assays of the hot water extract and compounds 1 – 7 were performed in a micro oxygen chamber by monitoring the initial rate of oxygen uptake using an oxygen electrode (Instech Laboratories, Plymouth Meetings, PA) attached to a YSI model 5300 biological oxygen monitor (Yellow springs Instrument, Incl., Yellow Spring, OH) at 37°C. The assay was conducted according to the previously reported procedure [22]. The enzyme was diluted with Tris buffer (pH 7.0) to give a final concentration of 1.5 mg of protein/mL. Each assay mixture contained 10 μL of DMSO or test samples, 0.6 mL of 0.1 M Tris buffer (pH 7), 1 mM phenol and 85 μg hemoglobin. COX-1 or COX-2 enzyme

(10 µL) was added to the chamber and incubated for 2 min. The reaction was initiated by the addition of arachidonic acid (10 µL of a 1 mg/mL solution). Each sample was calculated for $n = 2$. The data were recorded using QuichLog for windows data acquisition and control software (Strawberry Tree, Inc., Sunnyvale, CA).

2.7 Tumor cell proliferation assay

The assays of the hot water extract and compounds 1 – 7 were performed according to the previously published method [23]. MCF-7 (breast), SF-268 (CNS), NCI-H460 (lung), HCT-116 (colon) and AGS (gastric) human tumor cells were cultured in RPMI-1640 medium containing penicillin-streptomycin (10 Us/mL for penicillin and 10 µg/mL for streptomycin) and 10% fetal bovine serum (FBS). The samples and standards were dissolved in DMSO and diluted to the desired concentration in media so that final concentration of DMSO did not exceed 0.2%. Aliquots of 100 µL of test compounds were added to each well containing the appropriate tumor cells and further incubated 48 h. After incubation, an aliquot (25 µL) of MTT solution (5 mg MTT dissolved in 1 mL of PBS solution) was added and the plated were further incubated for 3 h at 37°C after wrapping it with aluminum foil. The medium was removed from each well and cells treated with DMSO (200 µL). The plates were then shaken and OD was measured using a microplate reader at 570 nm. Adriamycin was used as positive control in this assay.

2.8 Quantification of compound 1 by HPLC

2.8.1 Preparation of samples for HPLC analysis

Cold water extract: 'Panamruthan' powder (10 g) was weighed and stirred with cold water (50 mL) for 12 h. An aliquot of the supernatant was filtered through a 0.2 µm filter disc and analyzed by HPLC. Boiled water extract: 'Panamruthan' powder (10 g) was weighed and boiled with water (50 mL) for 60 min. An aliquot of the supernatant was filtered through a 0.2 µm filter disc and analyzed by HPLC.

2.8.2 Quantitative analysis of compound 1

HPLC was performed on an Xterra C_{18} column (5 µm; 4.6 × 250 mm; Waters Associates, Milford, MA). The peaks were detected at 280 nm using a PDA detector (Waters Associates, Milford, MA). A gradient solvent system was used consisting of solvents A [water-acetic acid (99:1 v/v)] and B (ACN). The linear gradient began at 95% A and 5% B, reached at 65% A and 35% B in 60 min. In between injections, the column was equilibrated for 10 min. The flow rate was 0.3 mL/min. The injection volume for all samples was 20 µL. The pure compound 1, isolated from 'panamarutham' hot water extract, was dissolved and diluted with methanol to yield 1.0, 0.8, 0.4, 0.2, 0.1, 0.05 mg/mL concentrations, respectively, and analyzed in triplicate. The experiment was replicated. Calibration curves were obtained by plotting the average of the 6 mean peak areas of triplicate injections of each standard solution against concentrations.

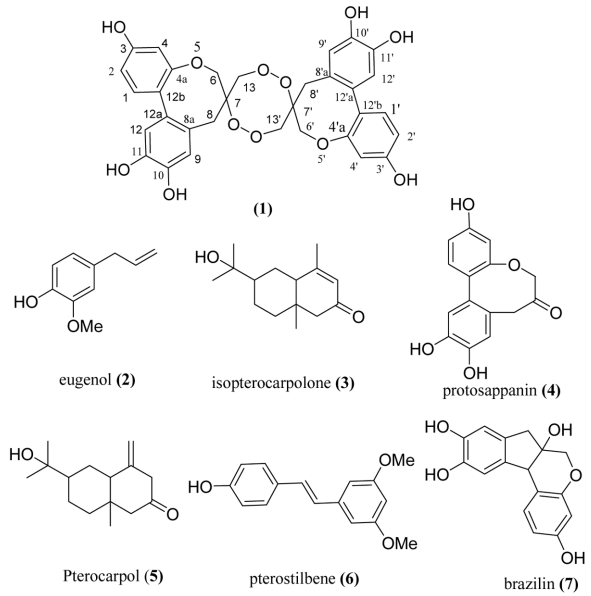
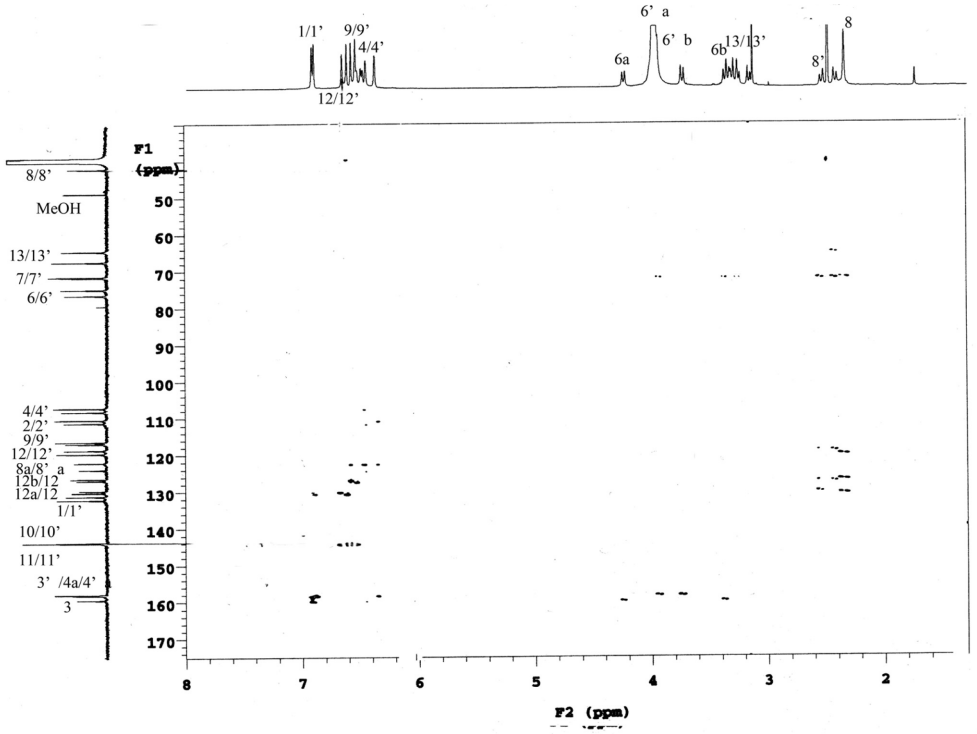


Figure 3. Structures of compound 1 – 7 isolated from the "Panamrutham" herbal drink.

3 Results and discussion

We used mechanism-based *in vitro* LPO and COX enzyme inhibitory assays along with tumor cells proliferation inhibitory activities to determine the potential efficacy of components in the herbal drink prepared by boiling "Panamrutham" with water. The instruction on the packet sold is to add 10 g of the mixture to 2 L of water and then boil. For the purpose of obtaining a sufficient amount of extractable material for chemical analysis, we prepared the extract by boiling 140 g (contents of three bags) with 6 L of water for 30 min. The lyophilized extract was a red powder which was kept refrigerated until the time of bioassays and chemical investigation.

The crude extract inhibited LPO by 96% at 25 µg/mL and COX-1 and -2 enzymes by 58 and 30%, respectively, at 250 µg/mL. At 100 µg/mL, it showed growth inhibition of colon, stomach, lung, breast and CNS human tumor cell lines by 69, 9, 48, 44, and 54%, respectively. The antioxidant, anti-inflammatory and antitumor activities demonstrated by the extract prompted us to perform a comprehensive chemical investigation of the bioactive principles present. Therefore, the extract was subjected to bioassay-guided purification using chromatographic methods including MPLC, HPLC and PTLC. Repeated purification of the bioactive fractions afforded a nevol compound, (1) along



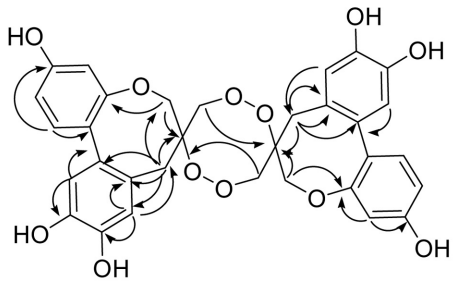


Figure 4. The significant HMBC correlations observed for compound 1.

with four phenolic compounds (2, 4, 6, and 7) and two sesquiterpenoids (3 and 5) (Fig. 3). The structures of all compounds were established by NMR and MS experiments.

Compound 1 was purified by preparative HPLC and obtained as a colorless powder. The molecular formula was determined as $C_{32}H_{28}O_{12}$ by HR-ESI-MS (negative ion), *m/z* 603.1493 $[M-H]^-$ (calcd 603.1503), with 19 degrees of unsaturation. The UV spectrum of 1 showed absorption bands at 254 and 280 nm. The IR spectrum displayed absorption bands at 3202, 1611, and 1497 cm^{-1}, which suggested the presence of a hydroxyl group and a benzyl ring. The ^{13}C NMR and DEPT spectra of compound 1 displayed 32 carbon resonances which included six methylenes, ten methines and sixteen quaternary carbons. The proton signals for two trisubstitued [6.91 (1H, d, $J = 8.0$ Hz), 6.53 (1H, brd, $J = 8.0$ Hz) and 6.46 (1H, brs)] and [6.91 (1H, d, $J = 8.0$ Hz), 6.48 (1H, brd, $J = 8.0$ Hz) and 6.38 (each, 1H, brs)] as well as two tetrasubstituted [6.66/6.58 (each 1H, s) and 6.62/6.54 (each 1H, s)] aromatic rings were evident in its ^1H NMR spectrum. It was further confirmed by the presence of carbon resonances assigned to two trisubstituted (131.9, 111.0, 157.7, 107.9, 159.2 and 126.7) and (131.0, 110.2, 157.7, 107.0, 157.9 and 126.2) as well as two tetrasubstituted (116.7, 143.7, 143.7, 119.4, 130.0 and 123.7) and (116.2, 143.6, 143.6, 118.4, 129.8 and 121.9) aromatic rings in the molecule. These data accounted for 16 of the 19 required degree of unsaturation, indicating the presence of three additional rings. The methylenes carbon signals at δ 76.2, 67.2 and 42.0, and the quaternary carbon singals at δ 71.4 were assigned to one ring located at 4a and 8a, which was further confirmed by HMBC correlations from H-6 [4.24 (1H, d, $J = 12.0$ Hz) to C-4a (159.2), C-7 (71.4), C-8 (42.0) and C-13 (67.2)]. Similarly, the methylenes carbon signals at δ 74.7, 64.3 and 39.5, and the quaternary carbon singals at δ 71.2 were assigned to another ring located at 4′a and 8′a. The ^1H NMR and ^{13}C NMR data were assigned

Table 1. ^{1}H NMR data of compound 1 (500 MHz, in DMSO-d_6).

No.	Protosappanin B and Isoprotosappanin B	No.	Compound 1	
1	7.00 (2H, d, *J* = 8.0 Hz)	1/1′	6.91 (1H, d, 8 Hz)	6.91 (1H, d, 8 Hz)
2	6.56 – 6.62(2H, dd, *J* = 8.0,2.5 Hz)	2/2′	6.53 (1H, brd, 8 Hz)	6.48 (1H, brd, 8 Hz)
4	6.48 – 6.56(2H, d, *J* = 2.5 Hz)	4/4′	6.46 (1H, brs)	6.38 (1H, brs)
6	3.16 – 4.35 (4H, each H *J* = 12 Hz, H-6)	6/6′	4.24, 3.37 (each 1H, d, 12 Hz)	3.95, 3.74 (each 1H, 12 Hz)
8	2.54 – 2.74 (4H, each 1H, *J* = 13 Hz)	8/8′	2.54, 2.43 (each 1H, 13 Hz)	2.35 (2H,brs)
9	6.71 – 6.84 (4H, s)	9/9′	6.58 (1H, s)	6.54 (1H, s)
12		12/12′	6.66 (1H, s)	6.62 (1H, s)
13	3.16 – 4.35 (2H, each H *J* = 11 Hz)	13/13′	3.34, 3.25 (each 1H, d, 12.5 Hz)	3.31, 3.16 (each 1H, d, 11 Hz)

Table 2. ^{13}C NMR data of compound 1 (125 MHz, in DMSO-d_6).

	Protosappanin B	Isoprotosappanin B	Compound 1			
No.	δ_C		No.	δ_C	No.	δ_C
1	134.2	1337.3	1	131.9	1′	131.0
2	112.8	112.1	2	111.0	2′	110.2
3	159.8[a)]	159.8[a)]	3	157.7[a)]	3′	157.7[a)]
4	109.9	109.1	4	107.9	4′	107.0
4a	159.8	160.2	4a	159.2	4′a	157.9
6	78.2	76.9	6	76.2	6′	74.7
7	73.7	73.2	7	71.4	7′	71.2
8	43.6	40.9	8	42.0	8′	39.5
8a	125.9	124.3	8a	123.7	8′a	121.9
9	118.4	118.3	9	116.7	9′	116.2
10	145.5[a)]	145.7	10	143.7[a)]	10′	143.6
11	145.5[a)]	145.5[a)]	11	143.7[a)]	11′	143.7[a)]
12	119.9	120.7	12	119.4	12′	118.4
12a	131.1	132.6	12a	130.0	12′	129.8
12b	129.4	128.3	12b	126.7	12′b	126.2
13	66.7	69.1	13	67.2	13′	64.3

a) Overlapping signals

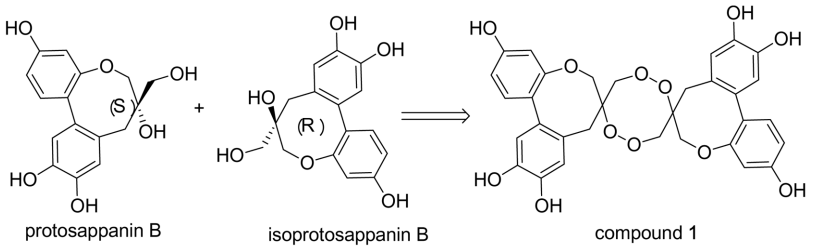


Figure 5. Possible biosynthesis pathway of compound 1 from protosappanin B and isoprotosappanin B.

according to HMQC and HMBC (^{1}H NMR data Table 1, ^{13}C NMR data Table 2 and HMBC correlations Fig. 4). The NMR data of 1 showed doubling of each signal as a pair and with remarkable similarity to those of protosappanin B and isoprotosappanin B (Fig. 5) [24]. This information along with the MS data indicated that compound 1 is a heterodimer of protosappanin B and isoprotosappanin B. The negative ion ESI-MS of 1 gave a molecular ion peak at *m*/*z* 603 $[M-H]^-$. The ion at *m*/*z* 301 $[M/2-H]^-$ was generated from the ion at *m*/*z* 603. Taking into account the MS fragments at *m*/*z* 333, 319 and 301, the presence of oxygen-oxygen bonds were implied in compound 1 (Fig. 1). The analysis of the positive ion ESI-MS fragments at *m*/*z* 627, 363, 357, 261 and 217 further confirmed the presence of oxygen-oxygen bonds in compound 1 (Fig. 2). Furthermore, The HMBC experiment showed the correlations from H-13 (3.34) to C-7′ (71.2) and from H-13′ (3.31) to C-7 (71.4). Thus, compound 1 was established as a novel heterodimer, named as 7 – 13′-dehydro-7′-13-dehydro-di (protosappanin B)-(isoprotosappanin B).

Compound 1 could possibly have formed as an artifact by the coupling of protosappanin B and isoprotosappanin B during the boiling of "Panamruthum" with water. In order to determine whether compound 1 was a natural product or an artifact, "Panamruthum" was also extracted separately with methanol and water at room temperature. A TLC com-

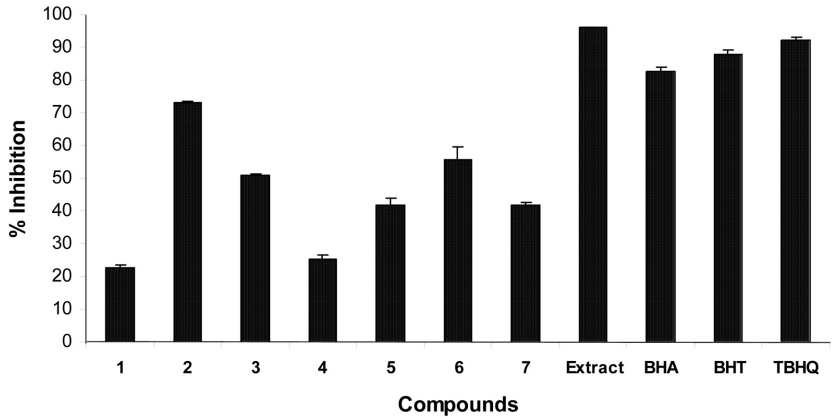


Figure 6. Lipid peroxidation inhibitory activities of compounds and the hot water extract of "Panamrutham". Commercial antioxidants BHA, BHT and TBHQ were tested at 1 μg/mL. Oxidation of lipid was initiated by the addition of Fe^{2+} ions. Compounds 1 – 7 and the extract were tested at 25 μg/mL. The vertical bars represent the SD of each data point ($n = 2$).

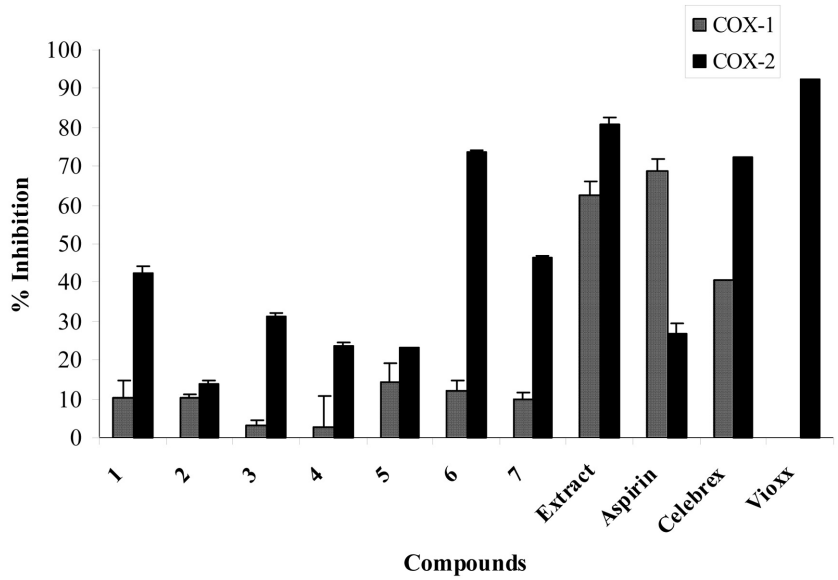


Figure 7. The COX-1 and COX-2 enzyme inhibitory activities of compounds 1 – 7 and the hot-water extract. Compounds 1 – 7 were tested at 25 μg/mL. Commercial NSAIDs Aspirin, Celebrex and Vioxx were used as positive control and tested at 180, 1 and 1 μg/mL concentrations, respectively. Vertical bars represent the SD of each data point ($n = 2$).

parison of the methanol, cold water and hot water extracts revealed the presence of compound 1 in all three extracts. Therefore, it was established that compound 1 is a natural product present in "Panamruthum" and was not produced during the course of extraction by boiling.

So far, a lot of peroxy compounds were isolated from natural products. The chemistry of mono- and polycyclic peroxides has attracted considerable recent attention since a significant number of peroxidic natural products with interesting pharmacological properties have been isolated [25, 26]. In our experiment, $NaBH_4$ was used to reduce compound 1 to get the monomers, but all attempts were unsuccessful. Even refluxing compound 1 with $NaBH_4$ for 24 h did not yield the monomers. Interestingly, we were able to isolate the parent compound intact from the reaction mixture. This confirms the fact that peroxy compounds are stable and occur freely in plants. It was reported that 4-bond correlations can usually be observed in concentrated samples of conjugated systems [27 – 29]. Although they are weak, the unusual 4-bond HMBC correlation signals were observed in compound 1.

All isolated compounds were further analyzed for LPO, COX-1 and -2 enzymes and human tumor cell proliferation inhibitory activities. The lipid peroxidation inhibitory effect of compounds 1 – 7 is shown in Fig. 6. At 25 μg/mL, Compounds 1 – 7 inhibited Fe^{2+} catalyzed LPO by 23, 73, 51, 25, 42, 56, and 42%, respectively. The commercial antioxidants BHA, BHT and TBHQ assayed at 1 μg/mL gave 89, 87 and 98% of LPO inhibitory activities, respectively. Similarly, at 25 ppm, compounds 1 – 7 inhibited COX-1 and -2 enzymes by 10 and 42%; 10 and 14%; 3 and 31%; 3 and 24%; 14 and 24%; 12 and 74%; and 10 and 47%, respectively (Fig. 7). It is interesting to note that all of these isolates showed weak COX-1 enzyme inhibitory activity. Compound 6 showed the highest COX-2 enzyme inhibitory activity (74%) followed by compound 7 (47%). Compound 7, known as brazilin, had been used as a natural red pigment for histological staining [18].

The results of cancer cell proliferation inhibitory assay of compounds 1 – 7 are presented in Fig. 8. At 25 μg/mL, compounds 6 and 7 showed growth inhibition of colon, AGS, lung, breast and CNS human tumor cell lines by 60 and

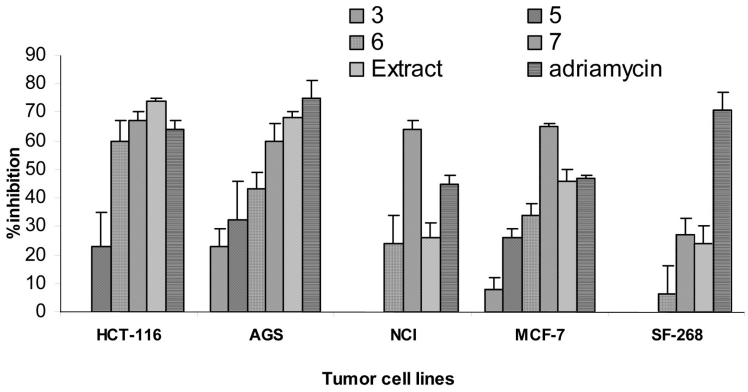


Figure 8. Cell proliferation inhibitory activities of compound 2, 3, 5, 6, 7, and the hot-water extract against human AGS (gastric), SF-268 (central nervous system, CNS), HCT-116 (colon) NCI-H460 (lung) and MCF-7 (breast) cancer cell lines. The compounds were tested at 25 μg/mL and the extract was tested at 100 μg/mL. DMSO (0.1%) supplemented with RPMI-1640 Medium and adriamycin (1.6 μg/mL) were used as solvent and positive controls, respectively. Compound 3 did not show the inhibitory activities against HCT-116, NCI-H460 and SF-268 cell lines. Compound 5 did not show the inhibitory activities against NCI-H460 and SF-268 cell lines.

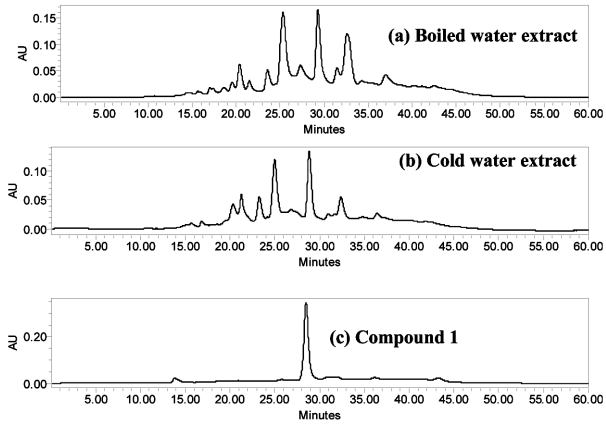


Figure 9. HPLC profiles of (a) boiled water extract; (b) cold water extract; (c) compound 1.

67%; 43 and 60%; 24 and 64%; 34 and 65%; 6 and 27%, respectively. Similarly, compounds 2 and 3 showed growth inhibition of colon and CNS cell lines by 38 and 9%; 18 and 4%, respectively. Compounds 3 and 5 displayed moderate growth inhibition of colon, AGS and breast human tumor cell lines whereas 1 and 2 showed no activity against any of the human tumor cell lines tested.

Although compounds 1 and 4 were the least active among the seven isolates tested, the novel dimer, 1, showed moderate COX-2 enzyme inhibitory activity. Compounds 6 and 7 showed activity against LPO, COX and cancer cell proliferation and hence may contribute to most of the biological effects of this "herbal drinking water". Based on the recommendation on the package, 10 g of the plant mixture yield 2 L of herbal drink. Based on our data and the recommended dose, the consumption of 1 L of drink per day will provide a daily dose of 0.389 g of total extract containing about 50 mg of a mixture of compounds 1 – 7. The concentration of compound 1 (R_t = 28.5 min) in both of cold and boiled water extracts was determined by HPLC (Fig. 9). The cold and boiled water preparation afforded 1.5 and 7.8 mg of compound 1, respectively, in 50 mL of herbal drink yielded from 10 g of herbal mixture. Although both cold and boiled water preparations contained compound 1, the boiled water preparation yielded higher amounts of compound 1.

One of the constituents in "Panamruthum", *P. santalinus*, is known for its possession of two major red pigments santalins A and B [30]. The red powder yielded from the lyophilized herbal drink most probably contained santalins A and B, but they were not isolated in this study and investigated for biological activities. Among the seven isolates, compound 2, eugenol, is a known essential oil and used in perfumes, flavoring agents, and as a local antiseptic and anesthetic [31]. In addition, it is reported to possess antioxidant [32] and tumor cell proliferation inhibitory activities [33, 34]. Similarly, recent reports indicate the antioxidant activities of compound 4 [35] and anticancer, antiinflammatory, antioxidant, and analgesic activities of compound 6 [36].

4 Concluding remarks

In conclusion, seven compounds were isolated from the "herbal drinking water" prepared by boiling the commercial product "Panamrutham". Among them, one is a novel compound and most showed significant inhibitions against LPO, COX enzymes, and the growth of stomach, lung, breast, colon, and CNS cancer cell lines. Our results do not provide supporting evidence to the claims such as "improve digestion, blood purification and natural health" printed on the product package. However, the bioassay results of the isolated major compounds from this "herbal drinking water" and the crude extract suggest that consumption of the drink may offer some health benefits.

The authors have declared no conflict of interest.

5 References

[1] Kamboj, V. P., Herbal medicine. *Curr. Sci.* 2000, *78*, 35 – 39.

[2] Aneja, V., Suthar, A., Verma, S., Kalkunte, S., Phyto-pharmacology of *Hemidesmus Indicus. Phcog. Rev.* 2008, *2*, 143 – 150.

[3] Seshadri, T. R., Polyphenols of *Pterocarpus* and *Dalbergia* woods. *Phytochemisty* 1972, *11*, 881 – 898.

[4] Jolly, C. I., Joy, J., Renjit, P., A review on chemical constituents, pharmacology and clinical effects of *Acacia catechu* (Linn filius) Wildenow. *Amala Res. Bull.* 2005, *25*, 152 – 155.

[5] Akhila, A., Rani, M., Chemical constituents and essential oil biogenesis in *Vetiveria zizanioides. J. Med. Arom. Plant Sci.* 2002, *20*, 73 – 109.

[6] Thapliyal, R. P., Bahuguna, R. P., Constituents of *Prunus cerasoides*. *Fitoterapia* 1993, *64*, 473.

[7] Singh, S. S., Srivastava, A., Saxena, R., Pandey, S. C., Sharma, V., Red sandal (*Pterocarpus santalinus*): chemistry, biological activities and uses – a review. *J. Med. Arom. Plant Sci.* 2005, *27*, 303 – 308.

[8] Austin, A., A review of Indian sarsaparilla, *Hemidesmus indicus* (L.) R. Br. *J. Biol. Sci.* 2008, *8*, 1 – 12.

[9] Gagandeep, M., Kalidhar, S. B., Chemical constituents of *Crataeva nurvala* (Buch-Ham) leaves. *Indian J. Pharm. Sci.* 2006, *68*, 804 – 806.

[10] Korikanthimathm, V. S., Prasath, D., Rao, G., Medicinal properties of *cardamom elettaria* cardamomum. *J. Med. Arom. Plant Sci.* 2001, *22/4A-23/1A*, 683 – 685.

[11] Chaieb, K., Hajlaoui, H., Zmantar, T., Kahla-Nakbi, A. B., *et al*., The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum* L. Myrtaceae): a short review. *Phytother. Res*. 2007, *21*, 501 – 506.

[12] Mitsuo, M., Masayoshi, H., Suppression of Chemical Mutagen-Induced SOS Response by Alkylphenols from Clove (*Syzygium aromaticum*) in the *Salmonella typhimurium* TA1535/pSK1002 *umu* Test. *J. Agric. Food Chem*. 2001, *49*, 4019 – 4025.

[13] Kitajima, J., Kamoshita, A., Ishikawa, T., Takano, A., *et al*., Glycosides of *Atractylodes lance*. *Chem. Pharm. Bull.* 2003, *51*, 673 – 678.

[14] Masuda, H., Ohtani, K., Mizutani, K., Ogawa, S., *et al*., Chemical study of *Haematoxylon campechianum*, a sweet principle and new dibenz[b,d]oxocin derivatives. *Chem. Pharm. Bull.* 1991, *39*, 1382 – 1384.

[15] Nasin, G., Piozzi, F., Pterocarpol and triterpenes from *Daemonorops draco*. *Phytochemistry.* 1981, *20*, 514 – 516.

[16] Dawidar, A. M., Jakupovic, J., Abdel-Mogib, M., Mashaly, I. A., Prenylstilbenes and prenylflavones from *Schoenus nigricans*. *Phytochemistry* 1994, *36*, 803 – 806.

[17] Belofsky, G., Percivill, D., Lewis, K., Tegos, G. P., Julie Ekart, J., Phenolic metabolites of *Dalea versicolor* that enhance antibiotic activity against model pathogenic bacteria. *J. Nat. Prod.* 2004, *67*, 481 – 484.

[18] Kjm, D. S., Baek, N., Oh, S. R., Jung, K. Y., *et al*., NMR assignment of brazilein. *Phytochemistry* 1997, *46*, 177 – 178.

[19] Reddy, M. K., Alexander-Lindo, R. L., Nair, M. G., Relative inhibition of lipid peroxidation, cyclooxygenase enzymes, and human tumor cell proliferation by natural food colors. *J. Agric. Food Chem*. 2005, *53*, 9268 – 9273.

[20] Laneuville, O., Breuer, D. K., DeWitt, D. L., Hla, T., *et al*., Differential inhibition of human prostaglandin endoperoxidase H synthase-1 and -2 by nonsteroidal anti-inflammatory drugs. *J. pharmacol. Exp. Ther.* 1994, *271*, 927 – 934.

[21] Meade, E. A., Smith, W. L., Dewitt, D. L., Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* 1993, *268*, 6610 – 6614.

[22] Jayaprakasam, B., Vanisree, M., Zhang, Y., Dewitt, D. L., Nair, M. G., Impact of alkyl esters of caffeic and ferullic acid on tumor cell proliferation, cyclooxygenase enzyme and lipid peroxidation. *J. Agric. Food Chem.* 2006, *54*, 5375 – 5381.

[23] Vareed, S. K., Reddy, M. K., Schutzki, R. E., Nair, M. G., Antrocyanins in *Cornus alternifolia*, *Cornus controversa*, *Cornus kousa* and *Cornus florida* fruits with health benifis. *Life Sci.* 2006, *78*, 777 – 784.

[24] Fu, L. C., Huang, X. A., Lai, Z. Y., Hu, Y. J., *et al*., A new 3-benzylchroman derivative from Sappan Lignum (*Caesalpinia sappan*). *Molecules* 2008, *13*, 1923 – 1930.

[25] Casteel, D. A., Peroxy Natural Products. *Natural Product Report* 1992, *9*, 289 – 312.

[26] Casteel, D. A., Peroxy Natural Products. *Natural Product Report* 1999, *16*, 55 – 73.

[27] Nguyen, M. T. T., Nguyen, S. A., Yasuhiro, T., Quan, L. T., Shigetoshi, K., Xanthine Oxidase Inhibitors from the Heartwood of Vietnamese *Caesalpinia sappan*. *Chem. Pharm. Bull*. 2005, *53*, 984.

[28] Karen, L. E., Kirk, R. G., Dennis, J. M., Lewis, K. P., *et al*., Myriastramides A – C, new modified cyclic peptides from the Philippines marine sponge *Myriastra clavosa*. *Tetrahedron* 2003, *59*, 10231 – 10238.

[29] Tetsuo, A., Kazuo, T. N., Yoshikazu T., Hiroshi, N., *et al*., Fluostatins A and B, New Inhibitors of Dipeptidyl Peptidase III, Produced by *Streptomyces* sp. TA-3391. *J. Antibiotics* 1998, *51*, 586.

[30] Arnone, A., Camarda, L., Merlini, L., Nasini, G., Structures of the red sandalwood pigments santalins A and B. *J. Chem. Soc. Perkin I*, 1975, *36*, 86 – 194.

[31] Jadhav, B. K., Khandelwal, K. R., Ketkar, A. R., Pisal, S. S., Formulation and evaluation of mucoadhesive tablets containing eugenol for the treatment of periodontal diseases. *Drug Dev. Ind. Pharm.* 2004, *30*, 195 – 203.

[32] Okada, N., Hirata, A., Murakami, Y., Shoji, M., *et al*., Induction of cytotoxicity and apoptosis and inhibition of cyclooxygenase-2 gene expression by eugenol-related compounds. *Antican. Res.* 2005, *25*, 3263 – 3269.

[33] Pratima, N. M., Larry, T., Malathy, P. V., Eduardo, P., *et al*., Inhibition of breast tumor growth and angiogenesis by a medicinal herb: Ocimum gratissimum. *Inter. J. Cancer.* 2007, *121*, 884 – 894.

[34] Yoshifumi, I. Eugenol: a new antidepressant. *Rec. Prog. Med. Plants*. 2008, *20*, 135 – 146.

[35] Hu, J., Yan, X., Wang, W., Wu, H., *et al*., Antioxidant activity in vitro of three constituents from *caesalpinia sappan* L. *Tsinghua. Sci. Technol.* 2008, *13*, 474 – 479.

[36] Connie, M. R., Jaime, A. Y., Yusuke, O., Karina, R. V., *et al*., Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. *Phytother. Res.* 2008, *22*, 169 – 179.

