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Original Article

Comparative study of herbal plants on the phenolic and flavonoid content, antioxidant activities and toxicity on cells and zebrafish embryo

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ABSTRACT

Natural antioxidants derived from plants have shown a tremendous inhibitory effect on free radicals in actively metabolizing cells. Overproduction of free radicals increases the risk factor of chronic diseases associated with diabetes, cancer, arthritis and cardiovascular disease. *Andrographis paniculata, Cinnamon zeylanicum, Curcuma xanthorrhiza, Eugenia polyantha* and *Orthosiphon stamineus* are ethnomedicinal plants used in the Asian region to treat various illnesses from a common fever to metabolic disease. In this study, we have quantified the total phenolic (TPC) and flavonoid content (TFC) in these plants and its inhibitory effect on 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radicals as well as the cytotoxicity effect on cell lines proliferation and zebrafish embryogenesis. Results showed that *Cinnamon zeylanicum* and *E. polyantha* have the highest phenolic and flavonoid content. Furthermore, both herbs significantly inhibited the formation of DPPH and ABTS free radicals. Meanwhile, *O. stamineus* exhibited minimum cytotoxicity and embryotoxicity on tested models. Good correlation between IC50 of 3T3-L1 cells and LC50 embyrotoxicity was also found. This study revealed the potent activity of antioxidant against free radical and the toxicology levels of the tested herbal plants.

1. Introduction

Reactive oxygen species (ROS); superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) are byproduct molecules generated during aerobic cell metabolism. The presence of these molecules is crucial as they modulate diverse physiological signaling and homeostasis. In turn, overproduction of ROS results in oxidative stress and contributes to the development of cancer, diabetes, aging, inflammation, cardiovascular disease and neurodegenerative diseases.[1–3] Synthetic and naturally derived antioxidants exhibit tremendous protective responses against ROS through free radicals scavenging, metals chelating, quenching of single and breaking the autoxidative chain reaction and restore the 'redox homeostasis' state to its original level.[4–9] Evidences showed that natural antioxidants deliver better effectiveness as compared to synthetic antioxidants. A study of vitamin E on human plasma and tissues revealed that naturally derived vitamin E gives twice higher absorption rate as compared to synthetic vitamin E.[10] In addition, dietary supplement from fruits, vegetables, cereals, beverages, spices and herbs not only offer targeted beneficiary antioxidant compounds, but a whole range of antioxidants, vitamins and phytochemicals. *Andrographis paniculata* (AP), *Cinnamon zeylanicum* (CZ), *Curcuma xanthorrhiza* (CX), *Eugenia polyantha* (EP) *and Orthosiphon stamineus* (OS) are commonly used in daily culinary as well as traditional medicinal preparations in South Asian region. Rich with aromatic flavors, pharmacological studies discover that these herbs contained a variety of potent phytochemicals that help to treat various illnesses.

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Even though most herbs and spices are generally recognized as safe, but adverse events sometimes occur after consuming herbal products. In 2013, statistical evaluation by Malaysian Adverse Drug Reaction Advisory Committee (MADRAC), National Pharmaceutical Control Bureau and Ministry of Health showed that 11,473 cases of adverse drug reactions were reported and 0.2% were caused by herbal products.[11] In most countries, safety and toxicology evaluation are not a mandatory requirement for herbal based product registration. In addition, lack of policies focusing on herbal product production contributes to low quality, ineffective and potentially hazardous consumption. 'Natural' term has misled the consumers' perceptions especially on the possible adverse effects that might arise from inappropriate usage of herbal medications. Adverse effect events may cause from several factors including the side effects of active compounds, contamination or substitution with toxic herbs, heavy metal contamination and herbs-drugs interactions.[12] These factors responsible for causing liver, kidney and lung failure, high blood pressure, heart attack and stroke have been reported.[12–16]

Current safety and toxicology testing for preclinical studies comprise of *in vitro* and *in vivo* models. Typically, these tests are time consuming, expensive and required a large number of animals. Potentially, zebrafish (*Danio rerio*), an emerging model in the early drug discovery and toxicological screening offers several advantages on physiological, biological and molecular alteration.[17–19] Its low maintenance, high fertility, *ex utero* development and transparent eggs offer clear visualization in all stages of organogenesis monitoring. Fish embryo acute toxicity test (FET) is designed by the OECD as a guideline to evaluate the embryotoxicity effects of certain compounds on the early 96 h of developmental stages of embryos.[20] The use of zebrafish is not only limited to toxicity screening. Over the past years, scientists have developed transgenic zebrafish through gene alteration and targeted mutation for specific diseases such as cardiovascular, neurogenesis, digestive system, muscles, cancer, immunology, diabetes and inflammation.[21–26]

In the present study, the water extracts of AP, CX, CZ, EP and OS were examined for its total phenolic and flavonoid content as well as their antioxidant inhibitory effect on DPPH and ABTS radicals. Furthermore, the toxicity effects of these herbs were evaluated using 3T3-L1, 1.1B4 and WRL-68 cell lines and compared with zebrafish embryotoxicity assay.

2. Materials and methods

2.1. Materials

0.2 N Follin-Ciocaltue reagent, sodium carbonate (Na_2CO_3), gallic acid, sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulphate ($K_2O_8S_2$), were purchased from Sigma Aldrich (St. Louis, MO, USA). Ascorbic acid and methanol was purchased from Qrëc™ (Selangor, Malaysia). Mouse 3T3-L1 pre-adipocyte and normal liver cells WRL-68 were purchased from American Type Culture Collection, ATCC, Manassas, USA and pancreatic beta cells 1.1B4 from Public Health England, Salisbury, UK. DMEM, RPMI, fetal bovine serum (FBS), fetal calf serum (FCS) and penicillin strep (PS) were purchased from Gibco, Life Technologies (Rockville, MD, USA). Adult, wild type, zebrafish were obtained from commercial supplier, Jurassic Fish, Johor, Malaysia.

2.2. Herbs and extraction

A. paniculata (AP) (leaves), *Cinnamon zeylanicum* (CZ) (bark), *C. xanthorrhiza* (CX) (rhizome), *E. polyantha* (EP) (leaves) *and O. stamineus* (OS) (whole plant) were purchased from NatureMedic Supply (Terengganu, Malaysia) and taxonomically confirmed by Prof Dr. Fadzilah Adibah Abdul Majid. All specimens were deposited at Tissue Culture Engineering Research Group, Universiti Teknologi Malaysia under vouchers no AP-TCERG-2013, CZ-TCERG-2013, CX-TCERG-2013, EP-TCERG-2013 and OS-TCERG-2013. Raw materials were ground into coarse form and extracted with filtered water by the ratio 1.5: 10 for 3 h at 60 °C. Spray drying were carried out in Institute of Bioproduct Development (IBD) (Johor, Malaysia). Extracts were stored at 4 °C until future purposes.

2.3. Biomarker fingerprinting and chromatography technique

2.3.1. FT–IR detection

Samples were subjected to IR spectroscopic study using Perkin Elmer FT–IR spectrophotometer by employing standard potassium bromide (KBr) pellet technique. Samples were ground with KBr in the ration of 1:100. Mixture was placed in the mold and pressed. The pellet was scanned over the wavelength ranged from 4000–370 cm^{-1}. FTIR spectrums were expressed as percent transmission (%T).

2.3.2. GS–MS analysis

Extracts were analyzed by gas chromatography–mass spectrometry (GC–MS) (Shimadzu QP2010 Ultra) equipped with a BP5MS capillary column (30 m length, 0.25 mm in diameter, 0.25 mm film thickness). Analyses were carried out using a programmed temperature from 50 °C to 300 °C and helium as a carrier gas. Compound identification was established based on the comparison of the GC retention factors with standards and the comparison of the mass spectra with the Wiley 138 library data of the GC–MS system.

2.3.3. Selected biomarkers identification and quantification

One hundred mg of AP, CX, CZ, EP and OS were dissolved in 25 ml of methanol, vortexed for 5 min to ensure dissolution and filtered through a 0.45 μm nylon filter. Standard stock solutions of biomarkers were prepared by dissolving 1 mg of biomarkers in 1 ml of methanol and sonicated for 5 min. Quantification of andrographolide, curcumin, catechin, gallic acid and rosmarinic acid were determined by HPLC fingerprinting detection; Waters 2690 Alliance Separation Module with LiChrospher® 100 RP-18 endcapped, Merck column cartridge (250 × 4.6 mm, 5 μm). The chromatograms were monitored at wavelengths 340 nm (rosmarinic acid), 272 nm (gallic acid), 223 nm (andrographolide), 280 nm (cathechin) and 420 nm (curcumin).

2.4. Total phenolic content

Quantification of total phenolic content (TPC) was carried out by Folin–Ciocalteu method[27] with slight modifications. Briefly, 100 μl of extract (1 mg/ml diluted in distilled water) was mixed with 100 μl of 0.2 N Folin–Ciocalteu reagents. After 5 min, 80 μl of 7.5% sodium carbonate (Na_2CO_3) solution was added and incubated for 2 h at RT. The absorbance was measured at 750 nm against the blank. The calibration curve was prepared using the standard gallic acid solution. The total phenolic content was expressed in mg of Gallic acid equivalent (GAE/100 g of sample).

2.5. Total flavonoid content

Total flavonoid content (TFC) was determined using aluminum chloride colorimetric method[28] with slight modifications. Briefly, 200 μl of extract was mixed with 12 μl $NaNO_2$ and 12 μl $AlCl_3$. After 5 min incubation at RT, 80 μl of NaOH was added and re-incubated

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