NUTRITIONAL, PHYTOCHEMICAL, ANTIOXIDANT AND ANTICANCER PROPERTIES OF *Eurycoma longifolia* Jack AND *Clinacanthus nutans* Lind. HERBAL TEAS

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I hereby declare that the material in this thesis is my own except for quotations, citations, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Camellia sinensis tea is popularly consumed due to its nutraceutical and pharmaceutical values. Its consumption triggers the attention towards other medicinal plants in search for more promising drinks called herbal teas. However, many processing factors influence the tea and herbal tea quality by affecting their nutrient and bioactive compounds. The aim of this study was to investigate the effect of fermentation, drying techniques, water temperature and steeping time towards the nutritional and phytochemical content as well as the antioxidant and anticancer potentials of herbal teas developed from leaves of *Eurycoma longifolia* Jack and *Clinacanthus nutans* Lind. Two local commercial products of *C. sinensis* (BOH green tea and SABAH black tea) were used as comparison throughout the study. The plants were processed into unfermented and fermented leaves before freeze dried and microwave-oven dried. Their infusions were prepared by steeping the dried leaves in (i) different water temperature (50, 60, 80 and 100 °C) for 10 min and (ii) 100°C water for different steeping time (1, 2, 5, 10, 15 and 20 min). The first part of the study was to evaluate the physical appearance and proximate compositions in the dried leaves, as well as the colour, pH value, mineral and vitamin C content in their infusions. For the evaluation of phytochemical, antioxidant and anticancer properties, their infusions were tested. All data were analysed using GraphPad Prism version 5.01 and expressed as means ± standard deviation (S.D.) of five replicate analyses in five independent experiments. One-way analysis of variance (ANOVA) followed by Tukey's multiple range test was carried out to determine the significance between means. The statistical significant level was set at $P \leq 0.05$. In the nutritional content determination, the results showed that commercial teas of *C. sinensis* and herbal teas of *E. longifolia* and *C. nutans* were physically different in their dried leaf colour and surface morphology, as well as the infusion colour and pH value. Proximate composition showed that *C. nutans* dried leaves contained the highest percentage of ash, moisture and crude protein; while *E. longifolia* dried leaves contained the highest crude fibre. Crude lipid in dried leaves was relatively similar between *E. longifolia* and *C. nutans*. Higher total carbohydrates were observed in *E. longifolia* dried leaves compared to that of *C. nutans*. High contents of elements were detected in dried leaves of *E. longifolia* and *C. nutans*, but low in their respective infusions. Infusions of *C. nutans* showed promising Ca, K, Mg and Na sources as these elements were significantly higher compared to the commercial tea infusions. Vitamin C also was detected in both *E. longifolia* and *C. nutans* infusions. In the phytochemical content studies, the highest TPC was recorded in unfermented freeze dried leaf of *E. longifolia* infusion (635.36 ± 57.16 mg TAE/L) when steeping the leaves in 100 °C boiling water for 20 min, while the highest FC was recorded in unfermented freeze dried leaf of *E. longifolia* infusion (168.17 ± 2.89 mg CE/L) when the leaves was steeped in 100°C boiling water for 10 min. At least 5 individual phenolics were detected in *E. longifolia* infusions (gallic acid, chlorogenic acid, vanillic acid, ECG and EGCG), whilst, 8 individual phenolics were detected in *C. nutans* infusions (gallic acid, chlorogenic acid, *p*-coumaric acid, caffeic acid, ferulic acid, EC, ECG and EGCG). The effects of fermentation and drying techniques towards those phenolic compounds were varied, depending on their chemical structures. In the antioxidant activity determination, *E. longifolia* infusions possessed higher FRAP

values compared to those of *C. nutans*. While in ABTS and DPPH assays, similar strength of free radical scavenging activity was observed for *E. longifolia* infusions and the commercial teas. The unfermented freeze dried leaf of *E. longifolia* infusion had the highest antioxidant activity in all three assays when steeping the leaves in 100°C boiling water for 10 min or longer. The phenolic content was strongly correlated with the antioxidant activity of the commercial tea and herbal tea infusions. In the anticancer activity evaluation of herbal extracts, the unfermented freeze dried leaf of *E. longifolia* extract was the most potent anticancer source due to its high cytotoxicity effect towards the non-hormone dependent breast cancer cell line MDA-MB-231, hormone dependent breast cancer cell line MCF-7 and colon cancer cell line HT-29 (IC_{50} =45.0 ± 3.5 µg/ml, 69.3 ± 17.2 µg/ml and 97.9 ± 1.8 µg/ml, respectively) in dose-dependent manners. The protective effect against hydrogen peroxide-induced damage towards normal cells NIH-3T3 and synergistic effect with drug tamoxifen against the breast cancer cell lines were also displayed. There was a presence of orange colour or green-orange colour which indicates the apoptotic cells when stained with acridine orange/propodium iodide. The typical characteristics of apoptotic cell including shrinkage of cell, bleb of membrane, condensed chromatin and fragmented nuclear were observed under high magnification of fluorescence microscope. Multiple DNA bands were formed on MDA-MB-231 and MCF-7 treated cell lines; while single DNA band formed on HT-29 treated cell line. In cell death mode analysis, the cells were arrested at G2/M for both HT-29 and MCF-7 cell lines; while observed at S-phase for MDA-MB-231 cell line. The number of apoptotic cell of MCF-7 and MDA-MB-231 were increased from 24h to 48h but slightly decreased at 72h of treatment. Treated HT-29 cells have showed gradual increase number of apoptotic cells from 24 h to 72 h of treatment. In possible anticancer mechanism analysis, HT-29 demonstrates the down-regulation of Bcl-2 and up-regulation of Bax expressions with p53 independent activity. The treated HT-29 cells also displayed the exclusion of caspase 3 and 8 activation and release of cytochrome c. The up-regulation of Bax showed in increase manner from 24 h to 48 h of treatment for MDA-MB-231 cells with down-regulation of Bcl-2 expression in same period. This expression also might responsible for the expression of cytochrome c and caspase 3 which caused the apoptosis cell death. The expression of caspase 8 and cytochrome c were detected in the treated MCF-7 cells, with up-regulations of Bax and p53 and also down-regulation of Bcl-2 expression. Those various mechanisms of action were displayed by cancer cells when treated with the extract have confirming the apoptotic cell death and the involvement of Bcl-2 family regulation. In short, among the herbal teas of *E. longifolia* and *C. nutans*, the unfermented freeze dried leaf of *E. longifolia* infusion displayed good nutritional values with the highest total phenolic and flavonoid contents as well as the most effective antioxidant and anticancer sources. As a conclusion, the fermentation, drying technique, water temperature and steeping time clearly did affect those properties in commercial teas and herbal teas tested. However, the effect was differently as it might depend on the nature or chemical structure of nutrient constituents and the phytochemicals that responsible for the antioxidant and anticancer properties of herbal teas; hence create a difficult decision to determine the exact best way of leaves processing and infusion preparation.

ABSTRAK

SIFAT-SIFAT NUTRISI, FITOKIMIA, ANTIOKSIDA DAN ANTIKANSER BAGI TEH-TEH HERBA *Eurycoma longifolia* Jack DAN *Clinacanthus nutans* Lind.

Teh camellia sinensis digunakan secara meluas kerana nilai nutraseutikal dan farmaseutikalnya. Penggunaannya mencetuskan perhatian terhadap tumbuh-tumbuhan ubatan lain untuk mencari minuman yang lebih sihat yang dipanggil teh herba. Walau bagaimanapun, banyak faktor pemprosesan mempengaruhi kualiti teh dan teh herba dengan mempengaruhi nutrisi dan sebatian-sebatian bioaktif. Tujuan kajian ini adalah untuk mengkaji kesan fermentasi, teknik pengeringan, suhu air dan masa rendaman terhadap kandungan nutrisi dan fitokimia serta antioksida dan antikanser potensi bagi teh herba yang dihasilkan daripada daun Eurycoma longifolia Jack. dan Clinacanthus nutans Lind. Dua produk komersial tempatan teh C. sinensis (BOH teh hijau dan SABAH teh hitam) digunakan sebagai perbandingan sepanjang kajian. Tumbuh-tumbuhan diproses menjadi daun tanpa difermentasi dan difermentasi sebelum dikeringkan secara beku dan oven ketuhar. Rendaman-rendaman disediakan dengan merendamkan daun kering dalam (i) suhu air yang berbeza (50, 60, 80 dan 100 °C) selama 10 minit dan (ii) air 100°C dalam masa redaman yang berbeza (1, 2, 5, 10 , 15 dan 20 min). Bahagian pertama kajian ini adalah untuk menilai penampilan fizikal dan komposisi proksimat dalam daun kering, serta warna, nilai pH, kandungan mineral dan vitamin C dalam rendaman-rendaman. Dalam penilaian fitokimia, antioksida dan antikanser, rendaman-rendaman teh telah diuji. Semua data dianalisis dengan menggunakan GraphPad Prism versi 5.01 dan dinyatakan sebagai min ± sisihan piawai (S.D.) daripada lima replika analisis dalam lima eksperimen berbeza. Analisis satu hala varians (ANOVA) diikuti oleh ujian kepelbagaian Tukey dijalankan untuk menentukan keberkesanan antara min. Tahap statistik yang berkesan telah ditetapkan pada $P \leq 0.05$. Bagi penentuan kandungan nutrisi, keputusan menunjukkan bahawa teh komersial C. sinensis dan teh herba E. longifolia dan C. nutans adalah berbeza secara fizikal bagi warna dan morfologi permukaan daun kering, serta warna dan nilai pH rendaman. Komposisi proksimat menunjukkan daun kering C. nutans mengandungi peratusan tertinggi abu, kelembapan dan protein mentah, manakala daun kering E. longifolia mengandungi serat kasar tertinggi. Lipid mentah dalam daun kering agak sama antara E. longifolia dan C. nutans. Jumlah karbohidrat yang lebih tinggi diperhatikan dalam daun kering E. longifolia berbanding C. nutans. Kandungan unsur-unsur yang tinggi dikesan dalam daun kering E. longifolia dan C. nutans, tetapi rendah dalam rendaman masing-masing. Rendaman-rendaman C. nutans boleh menjadi sumber yang terbaik bagi Ca, K, Mg dan Na kerana unsur-unsur ini jauh lebih tinggi berbanding dengan rendaman teh komersial. Vitamin C juga dikesan dalam rendaman-rendaman E. longifolia dan C. nutans. Dalam kajian kandungan fitokimia, TPC tertinggi direkodkan dalam rendaman daun kering beku E. longifolia (635.36 ± 57.16 mg TAE / L) apabila merendamkan daun dalam 100 °C air mendidih selama 20 minit, manakala FC tertinggi direkodkan dalam rendaman daun kering beku E. longifolia yang tidak difermentasi (168.17 ± 2. 89mg CE / L) apabila merendamkan daun dalam air mendidih 100°C selama 10 minit. Sekurang-kurangnya 5 jenis fenolik

dikesan dalam rendaman-rendaman E. longifolia (asid gallic, asid chlorogenic, asid vanillik, ECG dan EGCG), manakala 8 jenis fenolik dikesan dalam rendaman-rendaman C. nutans (asid gallic, asid chlorogenic, asid p-coumaric , asid kafein, asid ferulik, EC, ECG dan EGCG). Kesan fermentasi dan teknik pengeringan terhadap sebatian fenolik itu berbeza-beza, bergantung kepada struktur kimia mereka. Dalam penentuan aktiviti antioksida, rendaman-rendaman E. longifolia mempunyai nilai-nilai FRAP yang lebih tinggi berbanding dengan C. nutans. Bagaimana pun, dalam ujian ABTS dan DPPH, kekuatan yang sama terhadap aktiviti pembasmian radikal bebas diperhatikan bagi rendaman-rendaman E. longifolia dan teh komersial. Rendaman daripada daun kering beku E. longifolia yang tidak difermentasi mempunyai aktiviti antioksida yang tertinggi dalam ketiga-tiga ujian ini, apabila daun direndamkan dalam air mendidih 100°C selama 10 minit atau lebih lama. Kandungan fenolik sangat berkaitan dengan aktiviti antioksida teh komersial dan teh herba. Dalam penilaian aktiviti antikanser ekstrak teh herba, ekstrak daun kering beku E. longifolia yang tidak difermentasi adalah sumber antikanser yang paling berkesan disebabkan oleh kesan toksik yang tinggi terhadap sel kanser payudara yang tidak bergantung kepada hormon (MDA-MB-231), sel kanser payudara yang bergantung kepada hormon (MCF-7) dan sel kanser kolon (HT-29) (masing-masing IC_{50} = 45.0 ± 3.5 µg / ml, 69.3 ± 17.2 µg / ml dan 97.9 ± 1.8 µg / ml). Kesan perlindungan terhadap kerosakan disebabkan hidrogen peroksida keatas sel-sel normal NIH-3T3 dan kesan sinergistik dengan ubat tamoxifen terhadap sel-sel kanser payudara juga dipaparkan. Terdapat sel-sel yang berwarna oren atau warna hijau-oren yang menunjukkan sel-sel apoptotik apabila diwarnakan dengan "acridine orange/propodium iodide". Ciri-ciri khas bagi sel apoptosis termasuk pengecutan sel, membran mengunjur, kromatin berganda dan pemecahan nuklear diperhatikan di bawah kuasa pembesaran tinggi mikroskop pendarfluor. Jaringan DNA yang berganda terbentuk bagi sel kanser MDA-MB-231 dan MCF-7; manakala jaringan DNA yang tunggal terbentuk bagi sel kanser HT-29 yang dirawat. Dalam analisis mod sel mati, sel-sel terkumpul di fasa G2/M untuk sel kanser HT-29 dan MCF-7; manakala diperhatikan pada fasa S bagi sel kanser MDA-MB-231. Jumlah sel apoptotik bagi MCF-7 dan MDA-MB-231 meningkat dari 24 h hingga 48 h tetapi menurun sedikit pada 72 h tempoh rawatan. Sel kanser HT-29 yang dirawat telah menunjukkan peningkatan jumlah sel apoptosis secara beransur-ansur dari 24 h hingga 72h tempoh rawatan. Dalam analisis mekanisme antikanser yang berkemungkinan, sel kanser HT-29 menunjukkan penurunan ekspresi Bcl-2 dan peningkatan ekspresi Bax tanpa bergantung terhadap ekspresi p53. Sel HT-29 yang dirawat juga menunjukkan tiada pengaktifan caspase 3 dan 8 dan juga pembebasan cytochrome c. Ekspresi Bax meningkat dari 24 h hingga 48 h tempoh rawatan untuk sel kanser MDA-MB-231 dengan penurunan ekspresi Bcl-2 dalam tempoh yang sama. Ekspresi ini juga mungkin bertanggungjawab untuk ekspresi cytochrome c dan caspase 3 yang menyebabkan kematian sel secara apoptosis. Ekspresi caspase 8 dan cytochrome c telah dikesan dalam sel kanser MCF-7 yang dirawat, dengan regulasi-naik Bax dan p53 dan juga regulasi-turun Bcl-2. Mekanisme tindakan yang ditunjukkan oleh sel-sel kanser apabila dirawat dengan ekstrak telah mengesahkan kematian sel secara apoptosis dan penglibatan protein regulasi keluarga Bcl-2. Pendek kata, antara teh herba E. longifolia dan C. nutans, rendaman bagi daun kering beku E. longifolia yang tidak difermentasi menunjukkan nilai nutrisi yang baik dengan jumlah kandungan fenolik dan flavonoid tertinggi serta sumber antioksida dan antikanser yang paling berkesan.

Sebagai kesimpulan, fermentasi, teknik pengeringan, suhu air dan tempoh rendaman jelas memberi kesan terhadap sifat-sifat dalam teh komersial dan teh herba yang diuji. Walau bagaimanapun, kesan itu berbeza kerana ia bergantung kepada sifat atau struktur kimia unsur-unsur nutrisi dan fitokimia yang bertanggungjawab terhadap sifat antioksida dan antikanser teh herba. Oleh itu, keputusan sukar dibuat untuk menentukan satu cara yang tepat dan terbaik untuk memproses daun dan penyediaan rendaman teh.

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