UNIVERSITI PUTRA MALAYSIA

NUTRIENT COMPOSITION, ANTIOXIDANT AND ANTIPROLIFERATIVE PROPERTIES OF CLAUSENA EXCAVATA AND MURRAYA KOENIGII

WAN NOR I’ZZAH BT. WAN MOHAMAD ZAIN

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By

WAN NOR I'ZZAH BT. WAN MOHAMAD ZAIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Master of Science

November 2005
As I was writing this thesis, I realized that without my parents I would not be here today. It is with this thought that I dedicate this thesis to my parents,

Hj. Wan Mohamad Zain bin Wan Yaakob

Hjh. Nik Khairiah binti Nik Abdul Rahman

in remembrance of their loves, guidance and sacrifices.

May Allah bless both of you.
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : Associate Professor Asmah Rahmat, PhD
Faculty : Medical and Health Sciences

The proximate composition of Clausena excavata and Murraya koenigii leaves, together with the vitamin and mineral contents were investigated. Studies on the antioxidant and antiproliferative properties of the plant extracts and essential oils were also carried out. The proximate analysis showed that C. excavata leaves contained higher moisture, ash and crude fibre contents compared to M. koenigii. The contents of vitamins A, C and E in C. excavata were found to be 47.78 mg/100 g, 586.30 mg/100 g and 267.67 mg/100 g, while in M. koenigii the results were 1406.32 mg/100 g, 374.38 mg/100 g and 18.52 mg/100 g respectively. It seems that Murraya koenigii contained higher zinc (0.09 mg/100 g sample), copper (0.1 mg/100 g sample), sodium (0.4 mg/100 g sample) and potassium (0.91 mg/100 g sample) compared to Clausena excavata that showed 0.01 mg zinc and copper per 100 g sample, sodium (0.37 mg/100 g sample) and potassium (0.73 mg/100 g sample). Iron (0.32 mg/100 g sample), magnesium (0.96 mg/100 g sample) and calcium (5.46 mg/100 g sample) were found to be higher in C. excavata than
M. koenigii that possessed 0.14 mg iron per 100 g sample, 0.76 mg magnesium per 100 g sample and 5.28 mg calcium per 100 g sample.

The essential oils were obtained by hydrodistillation using fresh leaves and analysed using GC-MS spectrometry. The leaf oil of C. excavata was mainly made up of safrole (89.85%) while the leaf oil of M. koenigii was mainly made up of β-farnesene (42.85%). Other components that were present in appreciable amounts in M. koenigii oil were naphthalene (12.17%), α-caryophyllene (8.09%), caryophyllene (5.47%) and eudesmol (4.34%).

The methanol and water crude extracts together with the essential oils of C. excavata and M. koenigii leaves were investigated for their antioxidant capacities in two different assays, namely the β-carotene bleaching method and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. The methanol extract of M. koenigii showed the most potent antioxidant activity in β-carotene bleaching assay, giving a percentage of 86.13 %, while C. excavata showed 76.60 % in the assay. On the other hand, M. koenigii methanol extract showed weak effect in scavenging DPPH radical with an EC₅₀ value of 2.14 mg/ml, compared to the methanol extract of C. excavata which exhibited 0.89 mg/ml. The water extract of C. excavata showed higher antioxidant activity in both β-carotene bleaching method (76.02 %) and DPPH radical scavenging method (EC₅₀ value = 2.53 mg/ml) as compared to M. koenigii water extract which possessed 62.26 % antioxidant activity in β-
carotene bleaching method and 4.32 mg/ml EC$_{50}$ value in DPPH radical scavenging assay. Antioxidant activity of M. koenigii oil (91.01 %) was higher than C. excavata oil (66.3 %). Nevertheless, both of the essential oils did not present well in DPPH radical scavenging assay. The total phenolic content in the methanolic and water extracts of C. excavata and M. koenigii leaves, which were determined according to the Folin-Ciocalteu method, were expressed as gallic acid equivalents (GAEs); whereas the total phenolics in the methanolic extracts of both plants were higher than the water extracts. The methanolic extract of C. excavata had the highest phenolic content (103.33 mg of GAEs/g of sample extract) while M. koenigii methanol extract showed 63.92 mg of GAEs/g of sample extract. The total phenolic content of M. koenigii water extract possessed 53.62 mg of GAEs/g of sample extract while C. excavata exhibited 53.46 mg of GAEs/g of sample extract respectively.

HepG2 (hepatic cancer), MCF-7 (hormone-dependent breast cancer), MDA-MB-231 (non-hormone-dependent breast cancer), HeLa (cervical cancer) and CAOV3 (ovarian cancer) cell cultures were used to determine the antiproliferative activities of C. excavata and M. koenigii. The growth of viable cells was evaluated by using Microculture-tetrazolium (MTT) assay. Clausine-B, which was isolated from C. excavata was found to inhibit 50% of HeLa cancer cells’ proliferation at 22.90 $\mu$g/ml, followed by M. koenigii methanol extract (25.00 $\mu$g/ml), M. koenigii essential oil (31.10 $\mu$g/ml) and C. excavata methanol extract (34.51 $\mu$g/ml). The Clausena excavata methanol
extract, water extract and essential oil were found to cause 50% cell death of MCF-7 cancer cell line at concentrations of 36.50, 95.00 and 59.00 μg/ml respectively. Meanwhile, clausine-B and essential oil from M. koenigii were found to cause 50% cell death at 52.90 and 46.01 μg/ml respectively. For HepG-2 liver cancer cell line, the highest mean total IC₅₀ value could be seen in M. koenigii methanol extract which possessed 23.90 μg/ml. It was followed by clausine-B which was found to cause 50% cell death at a concentration of 28.94 μg/ml. The essential oil from M. koenigii and C. excavata methanol extract exhibited 48.00 and 53.03 μg/ml. Clausine-B and M. koenigii methanol extract were observed to inhibit the proliferation of MDA-MB-231 cell line at the concentrations of 21.50 and 37.98 μg/ml respectively. Three samples were found to cause 50% cell death of CAOV3 which is the ovarian cancer cell line. The samples are clausine-B (IC₅₀ = 27.00 μg/ml), M. koenigii methanol extract (IC₅₀ = 27.90 μg/ml) and C. excavata methanol extract (IC₅₀ = 79.00 μg/ml).

The findings of this study showed that the methanol extracts especially M. koenigii methanol extract have the great potential in antioxidant and antiproliferative activities. Clausine-B, was found to be active against all the cancer cell lines tested.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KOMPOSISI NUTRIEN, CIRI-CIRI ANTIOKSIDAN DAN ANTIPLIFERATIF CLAUSENA ECAVATA DAN MURRAYA KOENIGII

Oleh

WAN NOR I’ZZAH BT. WAN MOHAMAD ZAIN

November 2005

Pengerusi : Profesor Madya Asmah Rahmat, PhD
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Komposisi proksimat serta kandungan vitamin dan mineral bagi Clausena excavata dan Murraya koenigii dikaji. Kajian ke atas kandungan antioksidan dan antiproliferasi bagi ekstrak dan minyak pati bagi kedua-dua tumbuhan turut dilaksanakan. Analisis proksimat menunjukkan daun C. excavata mempunyai kandungan air, abu dan serat kasar yang lebih tinggi berbanding M. koenigii. Vitamin A, C dan E yang diperolehi bagi C. excavata adalah 47.78 mg/100 g, 586.30 mg/100 g dan 267.67 mg/100 g, manakala bagi M. koenigii keputusannya adalah 1406.32 mg/100 g, 374.38 mg/100 g dan 18.52 mg/100 g. Ia dapat dilihat bahawa Murraya koenigii mengandungi kandungan zink (0.09 mg/100 g sampel), kuprum (0.1 mg/100 g sampel), sodium (0.4 mg/100 g sampel) dan potasium (0.91 mg/100 g sampel) yang lebih tinggi berbanding dengan Clausena excavata yang menunjukkan 0.01 mg zink dan kuprum per 100 g sampel, sodium (0.37 mg/100 g sampel) dan potasium (0.73 mg/100 g sampel). Ferum (0.32 mg/100 g sampel), magnesium (0.96 mg/100 g sampel) dan kalsium (5.46 mg/100 g sampel)
didapati lebih tinggi di dalam C. excavata berbanding M. koenigii yang
menunjukkan 0.14 mg ferum per 100 g sampel, 0.76 mg magnesium per 100
g sampel dan 5.28 mg kalsium per 100 g sampel.

Minyak pati diperolehi melalui teknik penyulingan hidro yang
menggunakan daun segar dan dianalisis dengan menggunakan kaedah
spektrometri GC-MS. Kandungan utama minyak pati daripada C. excavata
adalah safrole (89.85%), manakala komponen utama bagi minyak pati
daripada M. koenigii ialah β-farnesene (42.85%). Komponen-komponen lain
yang diperolehi dalam amaun yang signifikan adalah naphthalene (12.17%),
α-caryophyllene (8.09%), caryophyllene (5.47%) serta eudesmol (4.34%).

Kapasiti antioksidan bagi ekstrak kasar metanol dan air serta minyak pati
daripada daun-daun C. excavata and M. koenigii dikaji melalui dua asai yang
berbeza iaitu kaedah penurunan β-karotena dan aktiviti penghapusan
radikal 1,1-difenil-2-pikrilhidrazil (DPPH). Ekstrak metanol M. koenigii
menunjukkan aktiviti antioksidan yang paling berkesan di dalam asai
penurunan β-karotena, dengan peratusan 86.13 %, manakala C. excavata
menunjukkan 76.60 % di dalam asai tersebut. Namun begitu, ekstrak
metanol M. koenigii adalah lemah di dalam penghapusan radikal DPPH
dengan nilai EC50 = 2.14 mg/ml, berbanding ekstrak metanol C. excavata
yang menunjukkan 0.89 mg/ml. Ekstrak air C. excavata mempamerkan
aktiviti antioksidan yang tinggi di dalam kedua-dua kaedah penurunan β-
karotena (76.02 %) serta penghapusan radikal DPPH (nilai EC\textsubscript{50} = 2.53 mg/ml) berbanding ekstrak air M. koenigii. Namun, aktiviti antioksidan minyak pati M. koenigii (91.01 %) adalah lebih tinggi jika dibandingkan dengan minyak pati C. excavata (66.3 %). Namun demikian, kedua-dua minyak pati terbabit tidak menunjukkan aktiviti yang berkesan di dalam asai penghapusan radikal DPPH. Jumlah kandungan fenolik bagi ekstrak metanol dan air daun C. excavata dan M. koenigii ditentukan melalui kaedah Folin-Ciocalteu, serta ditunjukkan dalam gallic acid equivalent (GAE); yang mana jumlah fenolik di dalam ekstrak metanol bagi kedua-dua tumbuhan adalah lebih tinggi berbanding ekstrak air. Ekstrak metanol C. excavata mengandungi jumlah fenolik yang paling tinggi (103.33 mg GAE/g ekstrak sampel) manakala ekstrak metanol menunjukkan 63.92 mg of GAE/g ekstrak sampel. Jumlah kandungan fenolik bagi ekstrak air M. koenigii memperlihatkan 53.62 mg GAE/g ekstrak sampel manakala C. excavata menunjukkan 53.46 mg GAE/g sampel ekstrak sampel.

Kultur sel HepG2 (kanser hepar), MCF-7 (kanser payudara bergantung hormon), MDA-MB-231 (kanser payudara tidak bergantung hormon), HeLa (kanser pangkal rahim) dan CAOV3 (kanser ovari) telah digunakan untuk menentukan aktiviti antiproliferasi C. excavata dan M. koenigii. Pertumbuhan sel hidup ditentukan melalui asai Mikrokultur-tetrazolium (MTT). Clausine-B, yang diasingkan C. excavata telah didapati merencat 50% proliferasi sel kanser HeLa pada kepekatan 22.90 µg/ml, diikuti dengan ekstrak metanol
M. koenigii (25.00 μg/ml), minyak pati M. koenigii (31.10 μg/ml) dan ekstrak metanol C. excavata (34.51 μg/ml). Ekstrak metanol, ekstrak air dan minyak pati Clausena excavata, telah dilihat menyebabkan kematian 50% sel bagi titisan sel kanser MCF-7 pada kepekatan-kepekatan berikut; 36.50, 95.00 dan 59.00 μg/ml. Manakala clausine-B dan minyak pati M. koenigii didapati menyebabkan kematian 50% sel pada kepekatan 52.90 dan 46.01 μg/ml. Bagi titisan sel kanser hepar HepG-2, jumlah purata nilai IC₅₀ dapat ditunjukkan di dalam ekstrak metanol M. koenigii yang mempamerkan 23.90 μg/ml. Ini diikuti dengan clausine-B yang didapati menyebabkan kematian 50% sel pada kepekatan 28.94 μg/ml. Minyak pati M. koenigii dan ekstrak metanol C. excavata memperlihatkan 48.00 dan 53.03 μg/ml. Clausine-B dan ekstrak metanol M. koenigii juga diperhatikan merencat proliferasi titisan sel kancer MDA-MB-231 pada kepekatan masing-masing 21.50 dan 37.98 μg/ml. Tiga sampel didapati menyebabkan kematian 50% sel CAOV3 yang mana merupakan titisan sel kancer ovari. Sampel-sampel terlibat adalah clausine-B (IC₅₀ = 27.00 μg/ml), ekstrak metanol M. koenigii (IC₅₀ = 27.90 μg/ml) dan ekstrak metanol C. excavata (IC₅₀ = 79.00 μg/ml).

Hasil penemuan kajian ini mendapati ekstrak metanol terutamanya ekstrak metanol M. koenigii mempunyai potensi yang besar di dalam aktiviti antioxidan dan aktiviti antiproliferasi. Manakala clausine-B didapati aktif ke atas kesemua titisan sel kancer yang diuji.
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Thank you.
I certify that an Examination Committee has met on 8 November 2006 to conduct the final examination of Wan Nor I'zzah Bt. Wan Mohamad Zain on her Master of Science thesis entitled “Nutrient Composition, Antioxidant and Antiproliferative Properties of Clausenia excavata and Murraya koenigi” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 07 FEB 2006
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

WAN NOR I'ZZAH BT. WAN MOHAMAD ZAIN

Date: 04 JAN 2006
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<td>Antioxidant activity of <em>C. excavata</em> and <em>M. koenigii</em> water extracts compared with BHT (BHT50), α-tocopherol (TOC50) and ascorbic acid (Ascorbic acid50) at 50 mg/l using β-carotene bleaching assay.</td>
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<td>4.7.</td>
<td>Antioxidant activity of <em>C. excavata</em> and <em>M. koenigii</em> essential oil compared with BHT (BHT50), α-tocopherol (TOC50) and ascorbic acid (Ascorbic acid50) at 50 mg/l using β-carotene bleaching assay.</td>
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</table>
Mean total antioxidant activity of *C. excavata* and *M. koenigii* methanol extracts, water extracts and essential oils and standards: BHT (BHT50), α-tocopherol (TOC50) and ascorbic acid (Ascorbic acid50). Superscripts of the same letter for each data were not significantly different at the level of p>0.05. Antioxidant activity was measured using β-carotene bleaching assay.

Scavenging activity of *C. excavata* (CE) and *M. koenigii* (MK) methanolic extracts, BHT, α-tocopherol (TOC) and ascorbic acid on DPPH radical. Values are expressed as mean ± standard deviation of six replicate measurements.

Scavenging activity of *C. excavata* (CE) and *M. koenigii* (MK) water extracts, BHT, α-tocopherol (TOC) and ascorbic acid on DPPH radical. Values are expressed as mean ± standard deviation of six replicate measurements.

Scavenging activity of *C. excavata* and *M. koenigii* essential oil, BHT, α-tocopherol (TOC) and ascorbic acid on DPPH radical. Values are expressed as mean ± standard deviation of six replicate measurements.

Phase contrast micrograph of antiproliferative activity of Clausine-B on HeLa cell line at 24, 48 and 72 hours incubation period (IC50 = 22.90 µg/ml).

A. Untreated HeLa cell line (control) after 24 hours incubation. x4. B. Treated HeLa cell line after 24 hours incubation. x4. C. Untreated HeLa cell line (control) after 48 hours incubation. x4. D. Treated HeLa cell line after 48 hours incubation. x4. E. Untreated HeLa cell line (control) after 72 hours incubation. x4. F. Treated HeLa cell line after 72 hours incubation. x4.

Scanning electron micrographs of the upper surfaces of fresh *C. excavata* leaves. A. (a) Thorny structure can be seen distributed in clusters on the upper surface, (b) New thorn originated from the surface. x25. B. Distance between one thorn to another thorn was equal forming a triangle. x30. C. Leaf cells surrounding thorny structures varied
in shape either square, angular or hexagonal, very closely packed and crowded. x100. D. (c) Thorns on the upper surface of C. excavata showing bulbous base, shaft, and swollen distal tip. x200.

4.14. Scanning electron micrographs showing numerous stomatas on the lower surfaces of fresh C. excavata leaves. A. (a) Thorns seen on the lower surface. (b) Certain parts of the surfaces appeared in flower-like shape. x100. B. Structure of the cells on the lower surface. x150. C. (c) Newly-developed thorn grew from the flower-like structure. x200. D. (d) The new thorn grew from this swollen base which originated from the flower-like structures. x500.

4.15. Scanning electron micrographs illustrate the upper surfaces of processed C. excavata leaves. A. Thorns were seen distributed in cluster on the upper surface. x500. B. Irregular lines on some parts of the upper surface. x800. C. Parallel striations on the upper surface. x1,000. D. Hairy cells structure on the upper surface. x1,500.

4.16. Scanning electron micrographs of the lower surfaces of processed C. excavata leaves. A. Curving thorn appeared at the middle of leaf lower surface; stomata could be seen closed to it. x500. B. Tapering thorn could be observed which was also located at the middle part of leaf lower surface. x550. C. Lower surface showing numerous scattering stomata. x600. D. Newly grown thorn could be found with opened stomata appeared adjacent to it. x900.

4.17. Scanning electron micrographs of the upper surfaces of fresh M. koenigii leaves. A. Middle part of M. koenigii leaf. x500. B. Structure of the upper surface. x500. C. (a) Flower-like structure located within irregular lines on M. koenigii upper surface. x400. Close-up view of the flower-like structure which was located within the irregular lines. x3,000.

4.18. Scanning electron micrographs illustrates the lower surfaces of fresh M. koenigii leaves. A. Middle part of M. koenigii lower surface. x100. B. Thorny
structure could be seen on the middle part. x500. C. Stomata located within irregular lines on rough surface of M. koenigii. x400. D. Close-up view of opened stomata which were located within the irregular lines. X2, 000.

4.19. Scanning electron micrographs illustrate the upper surfaces of processed M. koenigii leaves. A. (a) Flower-like structure could be found on the upper surface of M. koenigii. x300. B. Flower-like structure were seen on hairy surface. x500. B. Upper surface showing irregular lines structure. x1, 000. Parallel striation could be seen on the upper surface. x1, 500.

4.20. Scanning electron micrographs showing the lower surfaces of processed M. koenigii leaves. A. Lower surface of M. koenigii. x100. B. Stomata could be observed on rough surface. x300. C. Stomata located within irregular lines on rough surface of M. koenigii lower surface. x500. D. Close-up view of opened stomata which were located within the irregular lines. x1, 500.
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic Absorption Spectrophotometry</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
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<tr>
<td>ATCC</td>
<td>American Type of Culture Collection</td>
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<tr>
<td>BHT</td>
<td>butylated hyroxyltoluene</td>
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<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
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<tr>
<td>DPPH</td>
<td>α, α-diphenyl-β-picrylhydrazyl</td>
</tr>
<tr>
<td>ECCC</td>
<td>European Collection of Cell Culture</td>
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<tr>
<td>EDX</td>
<td>Energy Dispersive X-ray</td>
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<td>GAE</td>
<td>Gallic Acid Equivalent</td>
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<td>GC-MS</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
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<tr>
<td>VPSEM</td>
<td>Variable Pressure Scanning Electron Microscope</td>
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