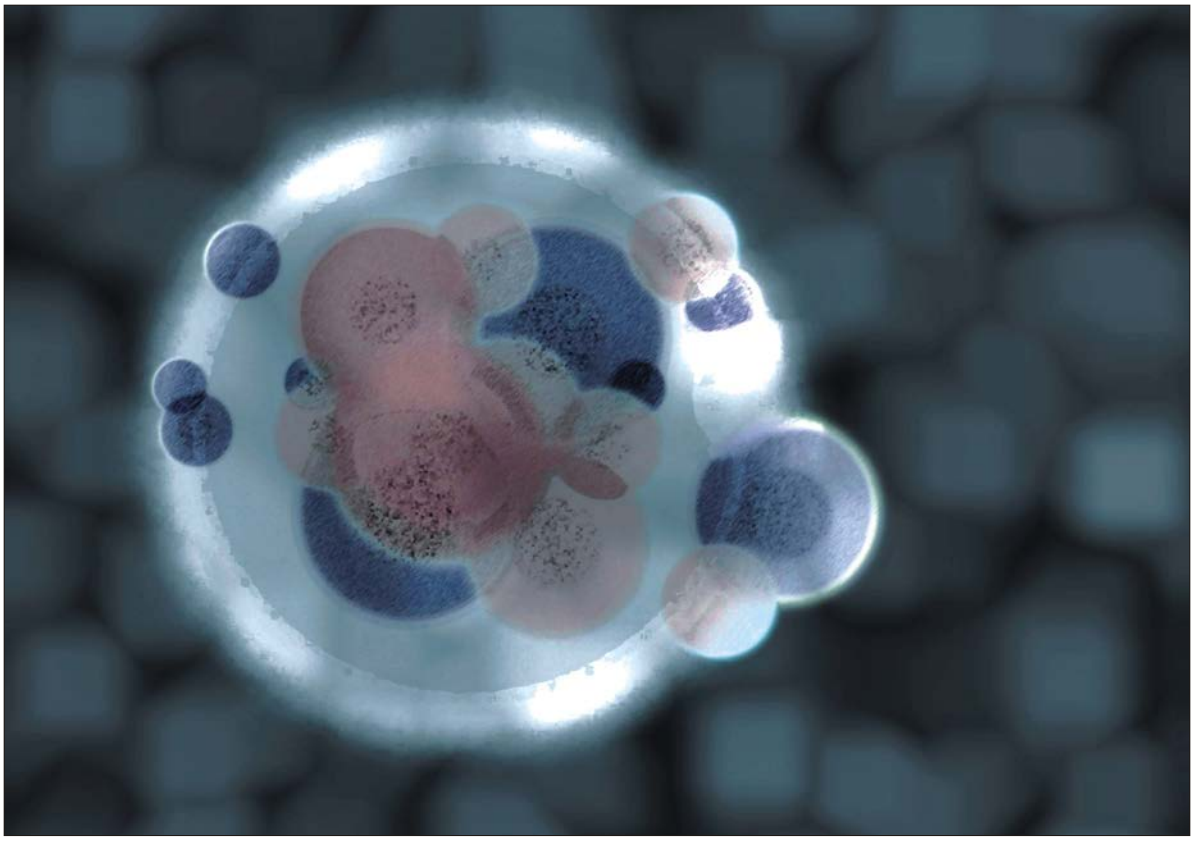

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 6 (6): 1005-1010, 2006
ISSN 1727-3048

Chemical Constituents, Antioxidant Activity and Cytotoxic Effects of Essential Oil from *Strobilanthes crispus* and *Lawsonia inermis*

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Abstract: The aimed of this study is to extract the essential oil from *Strobilanthes crispus* (Acanthaceae) and *Lawsonia inermis* Linn (Lythraceae) and to investigate the chemical constituents, antioxidant activity and cytotoxicity effects of these oils on several cancer cell lines and normal cell lines. From GC-MS analysis revealed that the presence of at least 28 components for *Strobilanthes crispus* and 23 components in *Lawsonia inermis*. The total antioxidant activity from (FTC) and (TBA) methods showed that the essential oils of both plants have higher antioxidant activity compared to α-tocopherol. The essential oil from *Lawsonia inermis* displayed the strongest cytotoxic effect on liver cancer cell lines (HepG2) with IC_{50} (concentration that inhibit 50% of cell proliferation) value of 24 μg mL^{-1}. However, the essential oil from *Strobilanthes crispus* did not give any cytotoxic value against all the cell lines tested. No cytotoxic effects of both oils in normal cell lines. The essential oils from both plants can be used as nutraceutical supplement to increase antioxidant needed in body to enhance defence systems, especially towards the incidence of degenerative diseases. The essential oil from *Lawsonia inermis* can be used as supplement in cancer patients especially in liver cancer patients.

Key words: *Strobilanthes crispus*, *Lawsonia inermis*, chemical constituents, antioxidant activity, cytotoxic effects

INTRODUCTION

The use of natural products together with their therapeutic properties is as ancient as human civilisation and, for a long time, mineral, plant and animal products were the main sources of drugs (Hernandez-Ceruelos *et al*., 2002). In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Schwartsmann *et al*., 2002). However, the potential use of plants as a source of new drugs is still poorly explored. Of the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and an even smaller percentage has been properly studied in terms of their pharmacological properties (Rates, 2001).

Strobilanthes crispus ZII 109 (L) Bremek or *Saricocalix crispus* ZII 109 (L) Bremek (Acanthaceae) plant is native to countries from Madagascar to Indonesia (Sunarto, 1977) and was first authored by Thomas Anderson (1832-1870) who classified the plant under Spermatophyta (Flowering plants and Gymnosperma) (Brummit and Powell, 1992).

Strobilanthes (cone-head) was named from the combination of strobilos which means a pine-cone and anthos which means flower (Plowden, 1968). *Crispus* means to be phyllostachyus or spike-like leaf ('phyllo' means leaf and 'stachyus' means spike) (Jackson, 1960). The conjunction of the names leads to the meaningful definition of the plant physical. It is commonly known as daun picah beling in Jakarta or enyoh kilo, kecibeling or kejibeling in Java (Sunarto, 1977).

This bush-like plant can be found on riverbanks or abandoned fields while some Javanese use this plant as fence. The leaves are oblong-lanceolate rather obtuse and shallowly crenate-crispate (Baker and Bakhuizen, 1965). The top surface of the leaves is darker green in colour and less rough as compared to underside (Sunarto, 1977). The leaves covered with short hairs whereas the flowers are short, dense and panicled spikes (Backer and Backhuizen, 1965). This plant has many cystoliths of calcium carbonate and an infusion is mildly alkaline (Perry and Metzger, 1980).

A study in Indonesia found that an infusion of the dried leaves of *S. crispus* has been used as antidiabetic, diuretic, antilytic and laxative (Sunarto, 1977). A recent

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study indicated that the water extract of *S. crispus* contained compounds with very high binding affinity to protein molecules that bind the active site of reverse transcriptase. It inhibits the proliferation of retrovirus; an agent in viral disease such as Acquired Immune Deficiency Syndrome (AIDS) and Adult T-cell Leukemia (Kusumoto *et al*., 1992). Recent study also indicated that *Strobilanthes crispus* leaf extracts have high antioxidant activity due to its phytochemical especially mineral contents, antioxidant vitamins as well as catechin (Ismail *et al*., 2000).

On the other hand, *Lawsonia inermis* Linn (Henna) is a plant, which grows wild in abandoned areas (Muhammad and Mustafa, 1994) and commonly known as 'inai' in Sumatra and Malaysia or 'Pachar kuku' in Java. This plant is a known worldwide as a cosmestic agent used to stain hair, skin and nails (Hanna *et al*., 1998). In India and Pakistan, henna is widely used by both men and women for coloring of the nails, fingers, hands and hair. However, it is not only relevant to cosmetics. Henna also was reported to have anti tuberculostatic activity (Sharma, 1990). Henna extracts possess hypotensive, intestinal antispasmodic and uterine sedative effects (Shihata *et al*., 1978). Alcoholic extracts of henna leaves showed mild anti bacterial activity against *Micrococcus pyrogenes* var *Aureus* and *Eschericia coli* (Kritikar and Basu, 1981). The leaves also used in the manufacture of perfumed oils and as a tanning agent (Uphof, 1968). The leaves are used in wounds, ulcers, cough, bronchitis, lumbago, rheumatalgia, inflammations, diarrhoea, dysentry, leucoderma, scabies, boils, anaemia, haemorrhages, fever, falling of hair and greyness of hair (Burkhill, 1966; Vaidyaratnam, 1995). Poultices of the leaf are said to remedy various types of tumours (Hartwell, 1971).

In Malaysia, the leaf decoction is used after childbirth and for beri-beri, rheumatism, skin disorders, stomach disorders and venereal disease. In Indonesia, leaves are used for jaundice, leprosy and scurfy affections (Perry, 1980). Mixed with the poisonous *Plumbago*, it is said to be an abortifacient. A tea of the leaves is said to be taken to prevent obesity and an ointment made of very young fruit has been suggested for the treatment of itch. Elsewhere, the plant is used for amebiasis and headache (Leung, 1980). Cambodians drink a root decoction as a diuretic. In Arabic medicine, the bark decoction is used for jaundice and nervous symptoms (Kritikar and Basu, 1981).

The purpose of this study is to extracts essential oil from *Strobilanthes crispus* and *Lawsonia inermis* and determine the chemical constituents, antioxidant activity of potential anti-tumour properties of both oils.

MATERIALS AND METHODS

Plants materials: The leaves of *Strobilanthes crispus* and *Lawsonia inermis* were harvested from the herbal garden of the Faculty of Medicine and Health Sciences, UPM. The herbarium voucher specimens were identified and deposited by Mr. Ahmed Zainuddin from the Department of Botany, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The voucher number of *Strobilanthes crispus* and *Lawsonia inermis* are AZ-6803 and AZ-6804, respectively. The leaves were separated from the stalks thoroughly washed with tap water and rinsed with distilled water. Fresh leaves that had been cleaned were used for development of essential oil.

Extraction of essential oils: The fresh leaves were hydrodistilled at 100°C for 6 h in a modified Dean and Stark apparatus to obtain a yellowish volatile oil. The oily layer (after removal of excess water) was further dried by anhydrous sodium sulphate. The aqueous layer from the distillate was extracted with hexane. The hexane layer was dehydrated with anhydrous sodium sulphate. The yields were calculated based on dry weight of the plant materials. The oils were subjected to spectroscopic determination by using Gas Chromatography-Mass Spectrophotemotry (GC-MS analysis). The oils were also subjected to determine the antioxidant activities and cytotoxic effect.

Gas Chromatography-Mass spectroscopy (GC-MS analysis): The GC equipment used was a Hawlett-Packard (HP) s ystem, model HP 4880 equipped with a HP5MS (5% phenyl methylsiloxane) capillary column of dimension 30.0 m × 250 × 0.25 μm. helium was used as a carrier gas. The GC oven temperature was programmed from 50-250°C at rate of 5°C min^{-1}. Mass Spectroscopy was taken at 70 eV. The library search was carried out using Wiley 275 and PMW 70×2 mass spectral database. The resultant mass spectra, which was compared directly with those from published and minimum quality is 80% merge.

Determination of antioxidant activity. Ferric Thiocyanate (FTC) method: FTC method (Kikuzaki and Nakatani, 1993) was used to determine the amount of peroxide at the initial stage of lipid peroxidation. The peroxide reacts with ferrous chloride to form a reddish ferric chloride pigment. In this method, the concentration of peroxide decreases as the antioxidant activity increases. In brief, a mixture of 4 mg weight sample in 4 mL absolute ethanol (Merck), 4.1 mL of 2.52% linoleic acid

(Sigma) in absolute ethanol, 8 mL of 0.05 M phosphate buffer (pH 7.0) and 3.9 mL of water was placed in a vial (⌀ = 38 mm, h = 75 mm) with a screw cap and then placed in an oven at 40°C in the dark. To 0.1 mL of this solution 9.7 mL of 75% ethanol and 0.1 mL 30% ammonium thiocyanate (Sigma) was added. Precisely 3 min after the addition of 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm at every 24 h until the absorbance of the control reached maximum. The control and standard were subjected to the same procedures as the sample except the control with which only the solvent was added and for the standard, 4 mg sample was replaced with 4 mg of vitamin E.

Thiobarbituric Acid (TBA) method: The method of Ottolenghi (1959) was used to determine the TBA values of the samples. The formation of malonaldehyde is the basis for the well-known TBA method used for evaluating the extent of lipid peroxidation. At low pH and high temperature (100°C), malonaldehyde binds TBA to form a red complex that can be measured at 532 nm. The increase of the amount of the red pigment formed correlates with the oxidative rancidity of the lipid. Two milliliter 20% trichloroacetic acid and 2 mL TBA aqueous solution were added to 1 mL of sample solution prepared as in the FTC procedure and incubated in a similar manner. The mixture was placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3,000 rpm for 20 min and the absorbance of the supernatant was measured at 532 nm. Antioxidant activity was based on the absorbance on the final day.

Cytotoxicity study

Culturing of cells: HepG2 (liver cancer), $CaCo_2$ (colon cancer), MDA-MB-231 (non-dependent hormone breast cancer), MCF-7 (hormone dependent breast cancer) and Chang Liver (normal) cell lines were obtained from American Type Culture Collection (ATCC, USA). The medium for HepG-2 and Chang liver were Minimum Essential Medium with Earle's salt (Gibco, USA) while $CaCo_2$, MDA-MB-231 and MCF-7 were grown by using Dulbecco's modified Eagle medium (Gibco, USA). The cells were cultured in their own medium supplemented with 10% of fetal calf serum, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} of streptomycin (Gibco, USA) using 25 cm^2 flasks (Nunc, Denmark), in a CO_2 incubator (Sanyo, Japan) at 37°C.

MTT assay (Roche Diagnostic, USA): The viability of cells was determined by staining with trypan blue. Exponentially growing cells were harvested and counted by using a haemocytometer. The specific medium for that particular cell line was used to dilute the cells to a concentration of 1×10^5 cells mL^{-1}. From this cell suspension, 100 μL was pipetted into a 96-well microtiter plate (Nunc, Denmark) and incubated for 24 h in a 5% CO_2 incubator (Sanyo, Japan) at 37°C. Sample extracts in two range of doses were added into the plate. The first range of the dose were 0.1, 0.3, 3, 10 and 30 μg mL^{-1}, meanwhile the second range were 5, 10, 20, 40, 60, 80 and 100 μg mL^{-1}. After adding the samples extract, new medium were added to make up the final volume of 200 μL in each well. The plate was incubated in a 5% CO_2 incubator (Sanyo, Japan) at 37°C for 96 h. Then, 20 μL of MTT reagent (Roche, USA) was added into each well. This plate was incubated again for 4 h in CO_2 incubator (Sanyo, Japan) at 37°C. Subsequently, 100 μL of solubilization solution (Roche, USA) was added into each well. The cell was then left overnight in 37°C, CO_2 incubator. Finally, the absorbance was read with the ELISA reader (LX-800).

$$\text{Cytotoxicity (\%)} = \frac{\text{OD sample (mean)}}{\text{OD control (mean)}} \times 100\%$$

OD = Optical Density

DATA ANALYSIS

Antioxidant activities and cytotoxicity studies were carried out in sixplicate and data were subjected to analysis of variance by using SPSS software

RESULTS

Hydrodistillation of the leaves of *Strobilanthes crispus* and *Lawsonia inermis* yielded 0.43% v/w and 0.82 v/w of oil, respectively. The GC-MS analysis revealed the presence of at least 28 components for *Strobilanthes crispus*. The main component of *Strobilanthes crispus* oil was phytol (46.01%) and other components of the oil were alpha cadinol (3.47%), tau-murolol (2.49%), ledol (1.81%) and eugenol (1.08%) as shown in Table 1. The leaves oil of *Lawsonia inermis* contained at least 23 components and the percentage composition is listed in Table 2. The major components were heptadecane (23.48%), tetradecane (16.77%), hexadecane (14.88%) and phytol (10.30%).

The total antioxidant activity results from FTC and TBA methods showed that the essential oil of both plants have higher antioxidant activity compared to α-tocopherol. Essential oil from *Lawsonia inermis* showed higher antioxidant properties compared to *Strobilanthes crispus* (Fig. 1 and 2).

Table 1: Chemical constituents of essential oil from *Strobilanthes crispus* leaf

Peak	Retention time	Substance	(%)
1	10.830	2,3-dihydrobenzofuran	1.68
2	16.216	Megastigmatrienone	1.21
3	16.865	unknown	1.73
4	17.108	Alpha-cadinol	3.47
5	17.267	Tau-murolol	2.49
6	17.342	unknown	1.21
7	17.392	Ledol	1.81
8	17.525	1-Naphtalenol	1.97
9	17.992	Eugenol	1.08
10	19.133	2-Undecanone	5.84
11	19.308	Phenol	3.07
12	20.042	2-hexyl,1- decanol	3.69
13	20.283	Isophytol	0.96
14	20.800	Nonadecanoic acid	2.10
15	20.918	9,17-Octadecadienal	2.34
16	22.000	Hexyl octyl ether	1.77
17	22.082	Phytol	46.01
18	22.443	Tetradecanal	0.87
19	22.873	2,6,10-trimethyl pentadecane	0.93
20	23.839	Eicosane	1.10
21	24.071	13-tetradece-11-yn-1-ol	1.02
22	24.768	Heptadecane	1.26
23	25.691	Tridecyl iodide	1.70
24	26.243	Di-n-octyl phtalate	2.62
25	26.687	Tetratetracontane	1.45
26	27.804	Octacosane	1.88
27	30.614	Pentadecane	3.00
28	34.590	Heptacosane	1.73

Table 2: Chemical constituents of essential oil from *Lawsonia inermis* leaf

Peak	Retention time	Substance	(%)
1	10.85	Dodecane	0.34
2	13.93	Tridecane	7.77
3	16.58	1-(1,5-dimethyl-4-hexenyl)--4-methyl-benzene	0.70
4	16.83	Tetradecane	16.77
5	18.56	2-methyl-decane	0.27
6	18.75	3-methyl-hexadecane	0.28
7	19.56	Heptadecane	23.48
8	21.16	2-methyl dodecane	0.26
9	21.36	Pentadecane	0.21
10	22.12	Hexadecane	14.88
11	24.52	Octadecane	0.55
12	25.63	Hexahydropseudoionone	0.31
13	26.83	Eicosane	0.51
14	27.39	2-hexyl-1-decanol	0.27
15	27.91	Isophytol	0.37
16	28.49	Dibutyl phthalate	1.68
17	31.00	Heptafluorobutyric-acid, n-tetradecyl ester	0.31
18	31.43	Phytol	10.30
19	39.47	Isooctyl phtalate	5.34
20	45.12	8-heptyl pentadecane	1.70
21	46.61	Dotriacontane	1.00
22	48.03	Octacosane	1.01
23	49.44	7-hexyl-eicosane	0.49

The essential oils of henna or *Lawsonia inermis* displayed the strongest cytotoxic effect on liver cancer cell line (HepG2) with IC_{50}-value of 24 μg mL^{-1} (Fig. 3). However, the essential oil of *Strobilanthes crispus* did not give any cytotoxic value against all the cell lines tested (not shown).

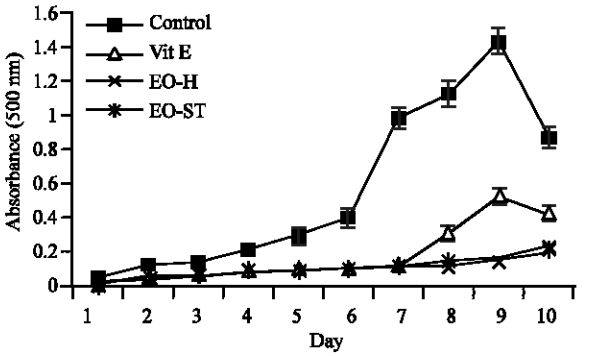

Fig. 1: Absorbance value of samples at 0.02% concentration using (FTC) method. Vit E: Vitamin E (α-tocopherol), EO-H: Essential oil of *Lawsonia inermis* EO-ST: Essential oil of *Strobilanthes crispus*

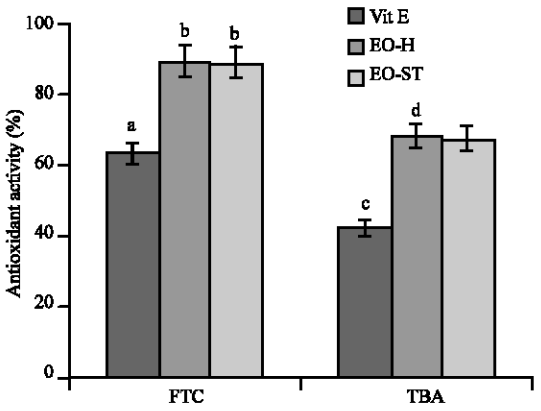

Fig. 2: Antioxidant activity of samples at 0.02% concentration using (FTC) and (TBA) methods. a-d: Mean with different alphabets were significant different ($p<0.05$). Vit E: Vitamin E (α-tocopherol), EO-H: Essential oil of *Lawsonia inermis*, EO-ST: Essential oil of *Strobilanthes crispus*

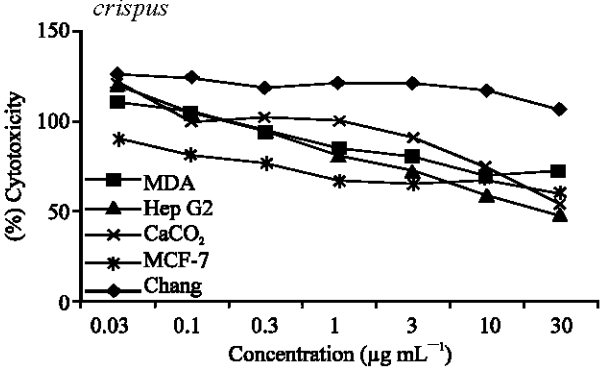

Fig. 3: The effect of *Lawsonia inermis* essential oil on different human cell lines. IC_{50} of 24 μg mL^{-1} was obtained for HepG2 cell lines

DISCUSSION

The history of intensive research associating nutritional elements with chronic diseases is almost 40 years old. In the mean time, much progress has also been made in the relationship between nutrients and cancer. It has been claimed that several nutrients, such as carotenoid, tocopherol and ascorbic acid derivatives and non-nutrients, the so called 'phytochemicals', in plants, would reduce the incidence of various cancer (Weisburger and Chung, 2002). Polyphenolic in green tea, for example, are known to possess antimutagenic and anticancer activity. On the other hand, black tea, which has been known to contain catechin, significantly inhibited leukemia and liver cancer (Dufresne and Famworth, 2001). Many other plants with specific nutrients and phytochemicals have been reported to have anticancer properties.

The present study elaborates on the potential use of two types of plants, *Strobilanthes crispus* and *Lawsonia inermis*, as a source of antioxidant supplement and anticancer agents. Both plants are used widely in the traditional medicinal preparations in this part of the world. Although they have been used widely in the treatment of various ailments, scientific data on these plants, especially on their essential oils and biological activities are still lacking.

The essential oils obtained by hydrodistillation of fresh leaves of *Strobilanthes crispus* showed that phytol is its major component with 46.01%, whereas *Lawsonia inermis* contains 10.30% of phytol. Phytol is an acyclic diterpene alcohol, colourless with high boiling oil. It was found as the phytyl ester in chlorophyll. Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Many terpenes are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes or ketones (*terpenoids*) are also found. Aoyama *et al.* (1988) reported that terpenes show a synergistic effect in antioxidation with other antioxidants.

In addition, the antioxidant activity of henna essential oil was also attributed to hexahydropseudoionone or hexahydrofarnesyl acetone due to its isoprene structure. Terao (1989) found that compounds with basic structure of isoprene, including β-carotene or carotenoids such as canthaxanthin and astaxanthin, exhibited inhibitory effect on the oxidation of methyl linoleate. Similar findings were also observed with the essential oils of *Terminallia catappa* leaves by Wang *et al.* (2000). This might be the reason for essential oil of henna leaves to show better antioxidant activity. Furthermore, various chemical structures of terpenes have been reported useful as chemopreventive agents, showing a reduction in mammary carcinogenesis, when the 7,12-dimethylbenz[a]anthracene-induced rat mammary carcinogenesis model was used. Terpenes were also found to have a potent protective effect on D-galactosamine/lipopolysaccharide-induced acute liver injury in mice.

The potential anti-tumour properties of both oils were determined using MTT-based cytotoxicity assay and the selectivity effects of both oils towards several cancer cell lines (MDA-MB-231, MCF-7, HepG2 and $CaCO_2$) as well as normal cell lines (Chang liver). This assay based on the reduction of soluble tetrazolium salt, by mitochondrial dehydrogenase activity of viable tumour cells, into an insoluble coloured formazan products, which can be measured spectrophotometrically after dissolution. Under the experimental conditions of this study, the enzyme activity and the number of formazan formed were proportionally to the number of cells. Reduction in the number of cells by particular agent (cytotoxicity) can generally be explained by cell killing and/or inhibition of cell proliferation. The IC_{50} value (concentration that inhibit 50% of cell lines) was used as a parameter for cytotoxicity (Smith *et al.*, 1995).

Henna or *Lawsonia inermis* has been proven scientifically to be effective in reducing severity of chemical hepatocarcinogenesis in rats by maintaining the tumour marker enzymes toward normal level (Rosnah *et al.*, 1995). The potential anticancer activity of henna was strengthen by this study especially the IC_{50} (concentration that inhibit 50% of cell proliferation) of this plant oil is 24 μg mL^{-1}, which is considered as active. This result showed similarities between *in vitro* and *in vivo* experiments in term of anticarcinogenic and henna can be potentially promoted as anticancer supplement.

The result of this study shown a good correlation between the cytotoxic effect of the oils and its antioxidant activity. Antioxidants are known to alleviate oxidative stress, which is generally perceived as one of the major factor causes for the accumulation of mutations in genome. Antioxidant are believed to provide protection against cancer (Ames, 1983).

In conclusion, *Strobilanthes crispus* and *Lawsonia inermis* oils were both found to have the potential to be used as nutraceutical suplement due to its antioxidant activity as well as to be a potential supplement as prevention in degenerative diseases. The essential oil of *Lawsonia. inermis* also show promising effect on liver cancer cell lines and its use could be commercialized as chemotherapeutic supplements due to its high antioxidant activity and inhibit liver cancer cell lines *in vitro*.

ACKNOWLEDGMENTS

This study was funded by Intensifying Research Priority Areas (IRPA) Grant No. 06-02-04-0050 from the Ministry of Science, Technology and Environment (MOSTE), Malaysia.

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