Original Article

Determination of total antioxidant activity in three types of local vegetables shoots and the cytotoxic effect of their ethanolic extracts against different cancer cell lines

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Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infections and degenerative diseases. Literature shows that the antioxidant activity is high on herbal and vegetable plants. Realizing the fact, this research was carried out to determine total antioxidant activity and the potential anticancer properties in three types of selected local vegetable shoots such as *Diplazium esculentum* (paku shoot), *Manihot utillissima* (tapioca shoot) and *Sauropous androgynus* (cekur manis). The research was also done to determine the effect of boiling, on total antioxidant activity whereby samples of fresh shoots are compared with samples of boiled shoots. In every case, antioxidant activity is compared to alpha-tocopherol and two methods of extraction used are the organic and the aqueous methods. Besides that, two research methods used were the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) with absorbance of 500nm and 532nm respectively. Oneway ANOVA test at $P < 0.05$ determines significant differences between various samples. In the cytotoxic study, the ethanolic extract and several cell lines i.e. breast cancer (MDA-MB-231 and MCF-7), colon cancer (Caco-2), liver cancer (HepG2) and normal liver (Chang liver) were used. The IC_{50}-value was determined by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The antioxidant study found that all the samples in both aqueous and organic extraction were significantly different. The total antioxidant activity values of aqueous extract in descending order are as follows : *M. utilissima* (fresh) > *D. esculentum* (fresh) > *S.androgynus* (fresh) > *M.utilissima* (boiled) > *D. esculentum* (boiled) > *S.androgynus* (boiled). It also was found that *S.androgynus* shoots ethanolic extract was able to inhibit the viability of the breast cancer cell lines, MDA-MB-231 with the IC_{50} value of 53.33 µg/ml. However, *S.androgynus* shoots and *D. esculentum* shoots ethanolic extracts did not inhibit the viability of MDA-MB-231 cell line. While, the tapioca shoot ethanolic extract was able to inhibit the viability of MCF-7 cell line with the IC_{50} value of 52.49 µg/ml. *S.androgynus* shoots and *D.esculentum* shoots ethanolic extracts did not give an IC_{50} value against the MCF-7 cell line. *S.androgynus*, tapioca and *D.esculentum* shoots ethanolic extracts did not show cytotoxic effect against the Caco-2 and HepG2. There was no IC_{50}-value from any sample against Chang Liver cell line. In conclusion, the antioxidant activity of both fresh and boiled samples were higher than alpha-tocopherol, although fresh vegetable shoots were found to be higher in antioxidant activity compared to boiled shoots. This study also suggested that *S.androgynus* shoots and tapioca shoots have potential as an anticancer agent against certain breast tumours.

Key words: antioxidant vegetables, shoots, paku shoot *Diplazium esculentum,* **tapioca *Manihot utillissima*, cekur manis** *Sauropous androgynus,* **cytotoxic, cancer, breast, colon, liver, Malaysia, tropics**

Introduction

The presence of free radicals in the body causes cell and tissue damage. This sort of damage is known as oxidative damage.[1] The most effective way to eliminate free radicals is with the help of antioxidant nutrients such as ascorbic acid (vitamin C), alpha-tocopherol (vitamin E) and beta-carotene (vitamin A) which can be found in vast amounts in fruits and vegetables. Studies carried out by the researchers have shown that low consumption of vegetables is associated with an increased risk of cancer.[2] *Sauropus androgynus*, *Manihot utilissima* and *Diplazium esculentum* are well known local vegetables which have been found to have antioxidant activity.[3] This antioxidant activity may have some benefit in preventing cancer.

Manihot utillissima, also known as cassava or tapioca shoots, originates from South America.[4] Today, this plant can be found in almost every tropical country. Its tuber roots play a major role in food industry.[5] According to Terra (1991)[6], there are two types of cassava shoots such as green twig and black twig. Both are popular in Malaysia. Another popular vegetable shoot among Malays is *Diplazium esculentum*. This plant can attain an

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average height of 0.5 to 2.5 meters and is eaten as 'ulam' or green edible leaves, usually consumed with hot sauce. This practice of eating the 'ulam' with sauce is also known as 'krawoo'.[7]

Souropus androgynus is known as 'katoo'. It is planted vastly in the Jawa mountain and is also found in India. The plant has small green leaves with yellow flowers that bloom occasionally. Lee (1989)[8] states that *Souropus androgynus* can be useful as a dye in food colouring. Eating too much of this vegetable can lead to muscle cramps and aches.

An in vitro study was designed to determine the cytotoxic effect of the shoots' ethanolic extracts against the proliferation of breast cancer cell lines (MDA-MB-231, the non-oestrogen dependent; MCF-7, the oestrogen dependent), colon cancer (Caco-2), liver cancer (HepG2) and transformed liver cell line (Chang Liver) as comparison.

This study was carried out to determine the total antioxidant activity and the anticancer properties of three types of selected vegetables shoots such as *Diplazium esculentum*, *Manihot utillissima* and *Souropus androgynus*. These are traditional Malay foods consumed mostly in rural areas of the country. The research was also done to determine the effect of boiling on total antioxidant activity, whereby samples of fresh shoots were compared to samples of boiled shoots.

Materials and Methods

Diplazium esculentum, Manihot utillissima and Souropus androgynus were purchased at Serdang market. The total antioxidant activity of the vegetable shoots was tested using two assay methods: ferric thiocyanate (FTC) and thiobarbituric acid (TBA). Both the organic and aqueous extraction methods were used. The FTC method was used to measure the amount of peroxide at the beginning of the lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ion. The ferric ion will then unite with ammonium thiocyanate and produce ferric thiocyanate. The substance is red in colour. The thicker the colour, the higher the absorbance. Whereas the TBA method measures bodies present after peroxide oxidation. Both FTC and TBA methods were used to determine total antioxidant activity. The total antioxidant activity is measured by dividing the absorbance value of the control on the seventh day (absorbance of the control is at maximum) with the absorbance of a certain sample in that particular day.

Total antioxidant activity =

$$\frac{\text{Absorbance of control on day maximum}}{\text{Absorbance of sample on the same day}} \times 100$$

Organic extraction[9]

Twenty grams of dried sample was soaked in 200ml hexane for 8 hours. Then, the sample was filtered and the hexane was discarded. The sample residue was soaked for a second time in 100ml hexane for 20 minutes while stirring. Then the hexane was filtered and discarded. After that, 15g of the sample residue was taken and soaked in 100ml methanol for 8 hours and filtered. The sample residue was again soaked in 100ml methanol for 8 hours. The sample was filtered and the second filtrate was kept with the first filtrate. The filtrate was then evaporated using the rotary evaporator. Finally, the methanol extract was ready to use.

Aqueous extraction[10]

One gram of dried sample was soaked in 20ml 0.45% salt solution and put into the water bath (40^0C) for 20 minutes. The solution was then centrifuged at 3000rpm for 30 minutes. The supernatant was collected and kept in -20^0C until the experiment commenced.

Antioxidative assays

a) Ferric Thiocyanate (FTC) method

The method of Mitsuda *et al.*, (1967)[11] and Osawa and Namiki (1981)[12] were slighty modified by Kikuzaki and Nakatani (1993).[13] A mixture of 4mg plant extract for each sample in 4ml absolute ethanol, 4.1 ml of 2.52% linoleic acid in absolute alcohol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in a vial with a screw cap and then placed in an oven at 40^0C in the dark. To 0.1 ml of this solution was added 9.7ml of 7.5% ethanol and 0.1ml ammonium thiocyanate. Precisely 3 minutes after an addition of 0.1ml of 0.02M ferrous chloride in 3.5% hydrochloride acid to the reaction mixture, the absorbance was measured at 500 nm every 24 hours until the absorbance of the control reached maximum.

b) Thiobarbituric acid (TBA) method

The method of Ottolenghi (1959)[14] was referred. An amount of 2ml of 20% trichloroacetic acid (TCA) aqueous and 2ml thiobarbiturate acid (TBA) aqueous solutions were added into 1ml of sample solution, prepared and incubated as above. The mixture was placed in a boiling water bath for 10 minutes. After cooling, it was centrifuged at 3000 rpm for 20 minutes and the absorbance of the supernatant was measured at 532 nm.

Cytotoxicity assay

An amount of 100g each of fresh vegetable shoots were homogenised and soaked in ethanol overnight. Filtration was done by using the Sinta glass filter and the filtrates were evaporated with a rotary evaporator. The dried residue was resuspended in DMSO to in vitro cyto-toxicity. HepG2 and Chang Liver were obtained from American Type Culture Collection (ATCC). The cells were cultured in Minimum Essentials Medium with Earle's salt supplemented with 10% of fetal calf serum, 100 IU/ml of penicillin and 100 µg/ml of streptomycin (Sigma) using 25-cm^2 flasks (Nunc, Denmark), in a CO_2 incubator at 37°C. The viability of cells was determined with trypan blue. Exponentially growing cells were harvested, counted and diluted with medium, yielding a concentration of $1x10^5$ sel/ml. From this cell suspension, 100 µl were pipetted into 96-well microtiter plates (Nunc, Denmark). The diluted range of extract was added to each well and the final concentrations of the test extracts were 5, 10, 20, 40, 60, 80 and 100 µg/ml. Cytotoxicity of the *S.androgynuss, M. utilissima,* and *D.esculentum* shoots extracts were determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay at incubation period of 72 hours and recorded as the

drug concentration causing 50% growth inhibition of the tumour cells (IC_{50} value).

Statistical Analysis

All the estimations were carried out in six replicates and the data were subjected to statistical analysis of ANOVA and student's t-test. A value of $P < 0.05$ was considered to be significant.

Results

Study of antioxidant activities in three types of selected vegetable shoots

Figure 1 showed the total antioxidant activity of the FTC method compared to the TBA method in three types of selected vegetable shoots. Results were also obtained for the antioxidant activity of the aqueous and organic extracts. The influence of the boiling process on antioxidant activity is shown in Figure 1. Oneway ANOVA test at $P<0.05$ determines significant differences between various samples. It was found that all the samples in both aqueous and organic extracts were significantly different. The total antioxidant activity values of aqueous extract in descending order are as follows: *M.utilissima* (fresh) > *D.esculentum* (fresh) > *S.androgynus* (fresh) > *M.utilissima* (boiled) > *D.esculentum* (boiled) > *S.androgynus* (boiled); and for the organic extract, *S.androgynus*(fresh) > *D.esculentum* (fresh) > *M.utilissima* (fresh) > *M.utilissima* (boiled) > *D.esculentum* (boiled) > *S.androgynus* (boiled).

Study of anticancer properties in three types of selected vegetable shoots

From this study, it was found that *S.androgynus* shoots ethanolic extract was able to inhibit proliferation of the breast cancer cell lines, MDA-MB-231 with the IC_{50} value of 53.33 µg/ml (Fig.2). However, *S.androgynus* shoots and *D.esculentum* shoots ethanolic extracts did not inhibit the proliferation of MDA-MB-231 cell line. While the *M.utilissima* ethanolic extract was able to inhibit the proli-feration of MCF-7 cell line with the IC_{50} value of 52.49 µg/ml (Fig.3). *S.androgynus* and *D.esculentum* shoots ethanolic extracts did not give IC_{50} value against MCF-7 cell line. *S.androgynus*, *M.utilissima* and *D.esculentum* shoots ethanolic extracts did not show cytotoxic effect against the Caco-2 and HepG2. There was no IC_{50} value obtained from all the samples against Chang Liver cell line at the tested concentration.

Discussion

Figure 1 showed the total antioxidant activity of the FTC method compared to the TBA method in which the activity of antioxidant for FTC method is higher than the TBA method. This may indicate that the amount of peroxide in the initial stage of lipid peroxidation is greater than the amount of peroxide in the secondary stage. Furthermore, the secondary product such as malon-aldehyde is not stable for a period of time. It will turn into alcohol and acid which cannot be detected by spectro-photometer (Ottolenghi, 1959).[14]

The result obtained also showed that the aqueous extract exert greater antioxidant activity compared to the organic extract. This may be because most of the active compounds in the leafy part the vegetables may dissolve in the water instead of the organic solvent. In addition,

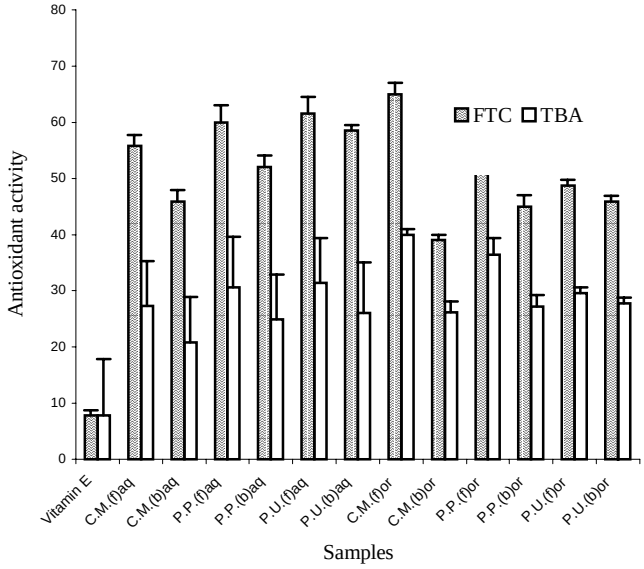

Figure 1. A comparison between total antioxidant activity using the FTC method and the TBA method of aqueous (aq) and organic (or) extracts from fresh (f) and boiled (b) samples. *CM=S.androgynus,PU=M.utilissima,PP=D.esculentum*

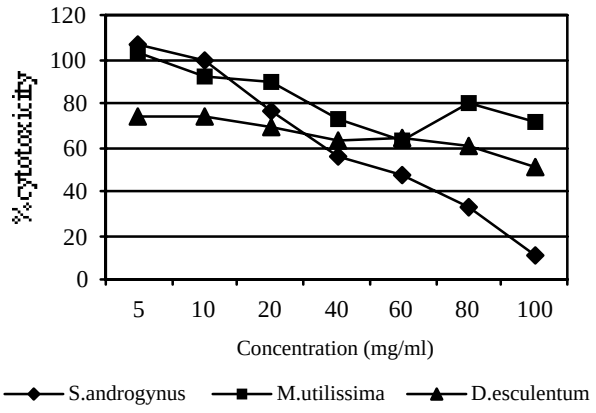

Figure 2. Cytotoxic effect of *S.androgynus*, *M.utilissima* and *D.esculentum* ethanolic extracts against MDA-MB-231 cell lines. IC_{50} = 53.33 µg/ml

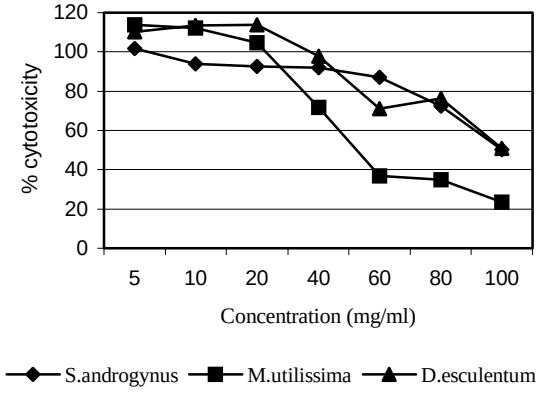

Figure 3. Cytotoxic effect of *S.androgynus*, *M.utilissima* and *D.esculentum* ethanolic extracts against MCF-7 cell lines. IC_{50} = 52.49 µg/ml

referring to the polarity, it is found that water is more polar than the organic solvent.

As predicted, all the vegetable shoot samples showed a higher total antioxidant activity compared to alpha-tocopherol. The experiment also measured the influence of boiling on antioxidant activity. Fresh vegetable shoots were found to have a higher antioxidant activity than boiled shoots. According to Winston (1999)[15], the leafy part of the vegetables contain the active component which consist of the flavonoid, terpenoid, lignan, sulphide, polyphenol, carotenoid, caumarin, saponin, curcumin and sterol. So, boiling the leaves may deteriorate the active compounds stated above. Furthermore, the vitamins which may act as antioxidants can also be diminished in the boiling water.

The potential role of antioxidant vitamins such as vitamin C and E, β-carotene and proanthocyanidins, antioxidant minerals such as zinc and selenium, and antioxdant enzymes such as glutathione, superoxide dismutase and catalase, have been extensively studied in the prevention of numerous degenerative diseases including tumour growth and carcinogenesis (Halliwell *et al.*, 1992).[16]

This study showed that *S.androgynus* and *M.utilissima* shoots were selectively cytotoxic against breast cancer cell lines (MDA-MB-231 and MCF-7). The selectivity was studied by using the transformed liver cell line, whereas no IC_{50}-values were obtained.

Conclusion

The antioxidant activity of fresh and boiled shoot samples was higher than alpha-tocopherol. However, fresh vegetable shoots were found to have a higher antioxidant activity than the boiled shoots. This study also found that *S.androgynus* and tapioca shoots may have potential as anticancer agents against certain breast tumours. On the other hand *S.androgynus*, tapioca and *D.esculentum* shoots did not appear to have potential as anticancer agents for colon and liver cancers.

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