CHAN MYAE NYEIN et al /J. Pharm. Sci. & Res. Vol. 9(10), 2017, 1750-1754

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Detection of Antioxidant and Cytotoxic Activites of *Tectona hamiltoniana* and *Terminalia chebula*

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Abstract

Six ethanolic extracts from two medicinal plants, *Tectona hamiltoniana* and *Terminalia chebula*, were detected for antioxidant activity and cytotoxicity against RD cell line (Human Rhabdomyosarcoma cell line). The antioxidant activities were measured by using three methods, DPPH free radical scavenging, reductive potential test and determination of the amount of total phenol. According to the results from three methods of antioxidant activity, *T.chebula* (Flowers) showed the most potent antioxidant activity. The cytotoxicity of six ethanolic extracts was determined by using Sulforhodamine B assay against RD cell line. The most cytotoxic extract was *T. hamiltoniana* (Leaves) (IC_{50} 9.62 µg/ml).

Key-words: RD cell, antioxidant, phenolic, reducing power, Cytotoxic

1. INTRODUCTION

The oxidative damage is considered to play a causative role in ageing and several degenerative diseases associated with it. [1] Antioxidants are naturally occurring or synthetic chemicals in foods that help to counter the detrimental effects of reaction oxygen species (ROS) and free radicals which causes degenerative human diseases such as cancer, heart diseases and cerebrovascular diseases. [2] Recently, natural foods and derived antioxidant such as vitamins and phenol phytochemicals have received growing attention. This is because they are known to function as chemo preventive agents against oxidative damage. [3]

Naturally occurring plant phenolics include several groups of compounds that have health promoting properties. Phenolics may act as antioxidants, thereby reducing the risk of atherosclerosis and coronary heart disease, which can be caused by oxidation of low-density lipoproteins. They may also protect against some forms of cancer [4]. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources.[5] There is therefore a parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants. [6][7]

Cancer is a notorious disease that now becomes the major cause of human mortality in the world. Almost half of the incidence and mortality happen in Asia, with lung and bronchus, breast, and colorectal cancers in women to be the most common fatal cancers.[8] Chemotherapeutic agents are the most effective treatments for cancer but these treatments have dangerous side effects. Regarding this dilemma, ongoing research on natural medicine sources in form of functional foods or nutraceuticals has been attracting many scientists. Phytochemicals containing antioxidant properties showed capacity to inhibit carcinogenesis. [9] Based on World Health Organization data, more than 80% of world inhabitants depend on using plant for their medicine and mangroves have been widely used for that purpose.[10][11]

In this study, the antioxidant activities of the ethanolic extracts of two plant samples were determined based on a DPPH (2-2-diphenyl-1-picrylhydrazyl) free radical scavenging assay, reductive potential power and the total phenolic content by Folin-Ciocalteau method. In addition, the effects on cell viability were accessed by Sulphorhodamine B assay.

2. EXPERIMENTAL

2.1 Plant Materials

All plant materials were collected from Kyaukse district, Mandalay region in Myanmar. Botanical identifications were carried in Pharmaceutical Research Department, Department of Research and Innovation, Yangon, Myanmar. All plant samples were air dried at room temperature in shade and made the powder with the mortar. The samples were separately extracted with 95% ethanol by using percolation method for one month. Then, they were filtered and the filtrates were concentrated by using rotary evaporator. The concentrated plant extracts were stored in refrigerator for testing. The selected plants were shown in Table 1.

2.2 Chemicals

Minimum Essential Medium Eagle (MEM), Gallic acid monohydrate, 1,1-di-phenyl-2-picrylhydrazyl (DPPH), Ferric cyanide, $FeCl_3$, NaH_2PO_4, Na_2HPO_4, Sodium bicarbonate, Folin-Ciocalteu's reagent, DMSO and Tris Base were obtained from HiMedia Co.Ltd, India. Fotal Bovine Serum, Trichloroacetic acid, Sulforhodamine B dye, Trysin EDTA were obtained from Sigma-aldrich Company. All chemicals and solvents were of analytical grade.

Table 1. Selected Myanmar Medicinal Plants

No	Botanical Name	Myanmar Name	Family Name	The Parts Used
1	*Tectona hamiltoniana*	Da-hat	*Verbenaceae*	Leaves, Flowers, Barks
2	*Terminalia chebula*	Phan-khar	*Combretaceae*	Leaves, Flowers, Fruits

2.3 DPPH Assay for Antioxidant Activity

The free radical-scavenging activity of the three extracts was determined by using the modified stable DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay in 96 micro-well flat plates [12]. Stock solutions of the extracts were prepared as 1 mg/ ml in methanol. Each well was filled in with 100 µl extract in methanol to get the final concentrations of the highest 100µg/ml to the lowest 5µg/m. Then, 100ml of the DPPH solution (0.2 mM) was added to each well. After keeping the plate in the darkness for 30 minutes, the absorbance of each well was read using SPECTRO^{star} Nano microplate reader at wavelength 517 nm. Control sample contained all the reagents except the extract. Percentage inhibition was calculated using the following formula while IC_{50} values were estimated from the % inhibition versus concentration plot. The data were presented as mean values ± standard deviation (n = 3).

% Inhibition = (A of Control-A of Sample)/(A of Control)×100

A graph of percentage inhibition of free radical activity was plotted against on centration of crude extract and concentration or 50% inhibition (IC_{50}) was obtained from the graph. The radical scavenging effect was examined and compared with ascorbic acid which was used as positive controls. The results were shown in table 2.

2.4 Reductive potential test

The reductive potential of the extracts was measured using a modification of the method described by Oyaizu.[13] A 250 µL sample containing different concentrations of plant extracts was mixed with 250 µL phosphate buffer (0.2 M, pH = 6.6) and 250 µL potassium ferricyanide (1%). The mixture was then incubated at 50°C for 20 min. Next, a portion (250 µL) of trichloroacetic acid (10%) was added to the mixture, which was subsequently centrifuged for 10 min at 3000 rpm. The upper layer of the solution (600 µL) was mixed with $FeCl_3$ (120 µL, 0.1%), and the absorbance was measured by using SPECTROstar Nano microplate reader at wavelength 700 nm. A higher absorbance of the reaction mixture indicated a greater reductive potential power.[14]

2.5 Total phenolic content (TPC) mesurements

The total amount of phenol compounds in the plant extracts was measured using a modification of the method described by Spanos and Wrolstad.[15] Briefly, 100 µL of the extracts were appropriately diluted to different concentrations. Each sample was then oxidized with 500 µL of freshly diluted Folin-Ciocalteu reagent, which is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenol and polyphenol antioxidants.[16] Folin-Ciocalteu reagent works by measuring the amount of the substance being tested that is required to inhibit the oxidation of the reagent.[17] This reaction was neutralized by adding 500 µL of 7.5% w/v sodium carbonate and then vortexing the samples for 20 sec. Next, the samples were incubated at 37°C for 1hour and the absorbance of the resulting blue color was measured by using SPECTROstar Nano microplate reader at wavelength 765 nm. [14] For each sample, three replicate assays were performed. The total phenolic content was calculated as gallic acid equivalent (GAE) by the following equation:

$$T = C \times V/M$$

T is the total phenolic content in mg g^{-1} of the extracts as GAE, C is the concentration of gallic acid established from the calibration curve in mg ml^{-1}, V is the volume of the extract solution in ml and M is the weight of the extract in g.[18] The results were shown in table 2.

2.6 Cytotoxicity testing by SRB assay

RD cell was grown RPMI 1640 medium with L glutamine supplemented with 10% fetal bovine serum, 1% antibiotics- antimycotic solution in a humidified atmosphere of a 5% CO_2 at 37°C. The monolayer cell culture was trypsinized and the cell count was adjusted to 0.75- 0.8 x 10^5 cells/ml using medium containing 10% fetal calf serum. To each well of the 96 well microtitre plate, 100 µl of the diluted cell suspension (approximately 7,500 cells) was added. After 24 hours, when a partial monolayer was formed and 100µl of five different concentrations (250, 200, 150, 50, 5 µg/ml) of extracts were added to the cells in microtitre plates and doxorubicin is used as standard drug. The plates were then incubated at 37ºC for 48 hours in 5% CO_2 incubator and microscopic examination was carried out and observations recorded every 24 hours. After 48 hours, 50µl of 50% cold trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form an overall concentrations 10%. The plates were incubated at 4ºC for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air dried. The air dried plates were stained with 100µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100µl of 10mM Tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 545nm. The percentage growth inhibition was calculated using following formula,

$$\%\ \text{of Cell Growth} = \frac{[\text{mean ODsample} - \text{meanODday0}]}{[\text{mean ODneg control} - \text{mean ODday0}]} \times 100$$

The graph was plotted between drug (Sample) concentrations and percent cell growth to obtain IC_{50} values.

3. RESULTS AND DISCUSSION

Six plant extracts from two medicinal plants were screened for antioxidant activity by screening DPPH free radical scavenging, reductive potential test. The amount of total phenol was measured. The cytotoxic activities of six extracts were screened by Sulforhodamine B assay against RD cell line (human rhabdomyosarcoma cell line).

Table 2. The antioxidant activities determined by DPPH assay and total phenolic content determined by using Folin Calteau's reagent

No.	Plant Extracts	IC_{50} µg/ml (DPPH assay)	mgGAE/g of extract (Total phenolic content)
1.	*Tectona hamiltoniana* (Leaves)	47.29 ± 1.19	62.81±0.02
2.	*Tectona hamiltoniana* (Flowers)	42.07 ± 0.93	86.57±0.03
3.	*Tectona hamiltoniana* (Barks)	10.08 ± 0.97	163.78±0.03
4.	*Terminalia chebula* (Leaves)	14.41 ± 0.66	153.23±0.09
5.	*Terminalia chebula* (Flowers)	7.21 ± 0.96	372.81±0.17
6.	*Terminalia chebula* (Fruits)	7.48 ± 0.16	247.38±0.08
7.	Ascorbic acid	3.18 ± 0.19	

Table 3. The cytotoxic activities of two selected medicinal plants determined by SRB assay

No	Plant Extracts	IC_{50} µg/ml
1.	*T. hamiltoniana (*Leaves)	9.62 ± 2.43
2.	*T. hamiltoniana* (Flowers)	57.56 ± 2.6
3.	*T.hamiltoniana* (Barks)	52.54 ± 5.05
4.	*T. chebula* (Leaves)	15.87 ± 4.84
5.	*T. chebula* (Flowers)	43.57 ± 4.51
6.	*T. chebula* (Fruits)	67.67 ± 3.84

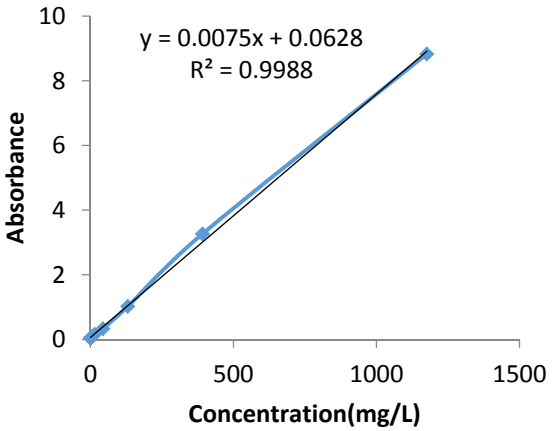

Figure 1. Standard calibration curve of gallic acid at concentrations of 0, 1.7, 5, 15, 44, 131, 392 and 1176 mg•L^{-1}. Spectrophotometric detection was at 765nm.

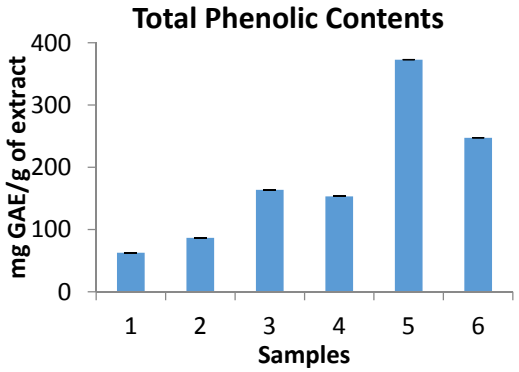

Figure 2. Total phenolic content of extracts from two plants species determined by the Folin-Ciocalteu assay and calculated as GAE in mg•g^{-1} extract based on dry weight. Results were the average of triplicates ± SD.

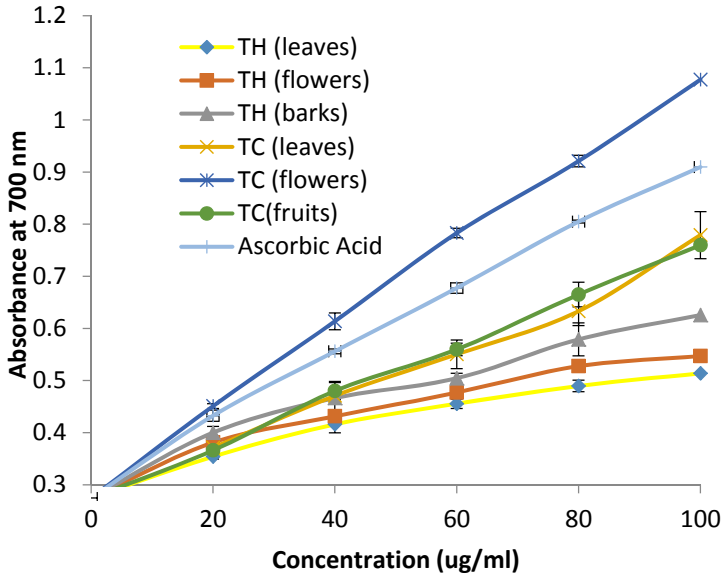

Figure 3. The Fe (III) reductive potential power of solvent extracts. Spectrometric detection was at 760 nm.

Antioxidant activities of plant extracts:

The antioxidant activities of the ethanolic extracts of two plants were measured by DPPH free radical scavenging assay and reductive potential tests. The DPPH results and the amounts of total phenolic content were shown in Table 1. DPPH assay were carried out by using five different concentrations (100µg/ml, 80µg/ml, 60µg/ml, 40µg/ml, 20µg/ml). After that 50% inhibitory concentrations were calculated compared with the standard, ascorbic acid. *T.chebula*(Flowers) (IC_{50} 7.21 µg/ml) showed the most potent antioxidant activity compared to standard, ascorbic acid (IC_{50} 3.18 µg/ml). According to the results obtained, the order of antioxidant activities was *T.chebula* (Flowers)> *T.chebula* (Fruits) > *T. hamiltoniana* (Barks) > *T. chebula* (Leaves) > *T.hamiltoniana* (Flowers) > *T. hamiltoniana*(Leaves). The DPPH tests revealed that there is a positive correlation between phenolic content and free radical scavenging activity.

The results of reductive potential tests were shown in Figure 3. *T.chebula*(Flowers) showed the greater reductive potential power. *T. hamiltoniana* (Leaves) showed the lowest reductive potential. So we can assume that there is correlation between DPPH assay and reductive potential tests.

Ethanolic extracts from the plant samples were standardized for their phenolic contents. The calibration curve showed linearity for gallic acid in the range of 0 - 1176 mgL^{-1}, with a correlation coefficient (R^2) of 0.9988 (Figure 1). The results of total phenolic contents of plant extracts were shown as the gallic acid equivalence (GAE) in Figure 2.

It is well known that there is a strong relationship between total phenol content and antioxidant activity, as phenols possess strong scavenging ability for free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may directly contribute to their antioxidant action [19, 20, 21].

The high phenolic content of *T. chebula* (Flowers) (372.81mg GAE/g) shows the linear correlation between phenolic content and IC_{50} value of antioxidant activity (7.21µg/ml). Among six extracts, *T. chebula* (Flowers) showed the most potent free radical scavenging activity, highest reductive power and high total phenolic content clustered as antioxidant compounds. We can assume that these three methods, DPPH free radical scavenging assay, reductive power test and determination of total phenolic content have the linear correlation for screening of antioxidant activity.

SRB assay

The cytotoxic activities of plant extracts were measured by using Sulforhodamine B assay against RD cell line (Human Rhabdomyosarcoma cell line). Table 2 shows IC_{50} value of plant extracts. The criteria of cytotoxicity activity for the crude extracts, as established by the American National Cancer Institute (NCI) is an $IC_{50} < 30$ µg/ml in the preliminary assay [22]. According to the data, *T. hamiltoniana* (Leaves) (IC_{50} 9.62 µg/ml) and *T. chebula* (Leaves) (IC_{50} 15.87 µg/ml) showed the potent cytotoxic activity.

4. CONCLUSION

The result obtained in this research indicated that three extracts of *T. chebula* showed potent antioxidant activity and *T. chebula*(Flowers) is the most potent. In Myanmar, *T. chebula*(Fruits) is used as food and it will be great benefit to contribute knowledge.

T. hamiltoniana (Leaves) and T. chebula (Leaves) showed cytotoxicity against RD cell line and should be tested with different human cancer cell lines. *T. hamiltoniana* (Dahat teak) is a local endemic species confined to Myanmar, and endangered species. Therefore, Moreover, cultivation and preservation of these promising plants are needed.

The presence and quantities of bioactive compounds in plants are influenced by several factors including seasons, weather condition, environment, plant part used, intra species variations and plant age, among other factors [23]. Therefore, evaluation of plant samples collected at various seasons should also be carried out to compare the levels of antioxidant activities and cytotoxicity.

ACKNOWLEDGEMENTS

This work was financially supported by the Biotechnology Research Department, Ministry of Education, Myanmar. The authors are grateful to Pharmaceutical Research Department, Ministry of Education, Myanmar for helping with the sample collection and solvent extraction and National Health Laboratory, Ministry of Health and Sports, Myanmar for providing the RD cell line.

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