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ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF *Tinospora crispa (putarwali)* STEMS METHANOLIC EXTRACT

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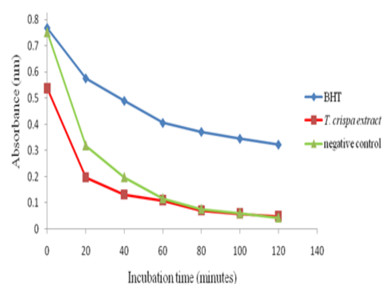
Graphical abstract



Abstract

Apart from being the primary source of food to other living things, plants also have medicinal value to treat various kinds of diseases. In recent years, it has been proposed that the extract from plants may be used as natural antioxidants which can help to prevent the generation of carcinogens in human body. In addition, plants also have antimicrobial agents to inhibit the growth of pathogenic microbes. This study was intended to investigate the antioxidant properties and antimicrobial activity of methanolic extract of *Tinospora crispa* stems extracted using soxhlet extraction method. The antimicrobial properties of *T. crispa* stems extract were tested using disc diffusion method against *Staphylococcus aureus ATCC 25923, Bacillus cereus ATCC 11778, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Candida albicans IMR C S23/11 A and Saccharomyces cerevisiae IMR S 617/068*. The antioxidant properties of the extract were investigated by using Total Phenolics Content (TPC), Total Flavonoids Content (TFC), DPPH free radical scavenging and β–carotene bleaching assays. The TPC value was 6.12 g GAE/100 g of dried extract while the TFC value was 55.58 g QE/100 g of dried extract. The IC_{50} of DPPH scavenging assay for the extract and ascorbic acid were 0.21 and 0.04 mg/mL, respectively. The average percentage of β–carotene bleaching assay was 38.3 % as compared to BHT, which was 45.1%. The disc diffusion method showed no inhibition zone against all the strains of microorganisms at all concentrations of the extracts (0.5, 1.0, 2.5 and 5.0 mg/disc).

Keywords: *Tinospora crispa*, antioxidants, antimicrobial

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1.0 INTRODUCTION

Infectious diseases remain the main cause of death and disability that affecting millions of people worldwide. The occurrence of antibiotic resistance microorganisms is due to indiscriminate use of antibiotics [1]. Medicinal plants have been used since ages to treat various ailments. This is due to the

presence of the secondary metabolites such as tannins, alkaloids, terpenoids, quinones, flavonoids and polyphenols [2, 3]. Antimicrobial agents from plants believed to have fewer side effects compared to the synthetic antimicrobials [4, 5]. Thus, wide range of research has been demonstrated on plants to discover antimicrobial agents and antioxidant agents for food preservation [6].

T. crispa is one of the medicinal plants that believed to have antioxidant antimicrobial properties in its secondary metabolites. It is a small herb which grows in tropical parts of Asia, such as Malaysia, Thailand, Indonesia, India, Philippines, China and Vietnam, which have tropical and subtropical climates. *T. crispa* also known as *Menispermum crispum, M. rumphii* and *M. tuberculata* [7]. *T. crispa* locally known as *makhabuhai* (Philippines), *wab kab hoi yai* (Thailand), *vasanavalli* (Sanskrit), *andawali* (Indonesia), *da yen ruan jin teng* (China) and *kattukkodi* (Tamil) [8]. In Malaysia, it is called *putarwali, akar patawali, akar seruntum* and *batang wali* [9]. *T. crispa* has been widely used by local people as a treatment for intestinal worm infection, diabetes, and hypertension. In addition, it was also used to prevent mosquito bites and to stimulate appetites. The Malay community used the infusion of *T. crispa* stems as a vermifuge to expel intestinal worms, while a decoction of the whole plant is used to treat cholera and diabetes [9]. Other than that, *T. crispa* also used for the treatment of skin diseases, anti-periodic in fever, anti-malarial, tonic, oral hypoglycemic agent and as a wound cleansing agent for rheumatic wounds [7].

To the best of our knowledge, no researches have been made on the investigation of antioxidant properties and antimicrobial activity of *T. crispa* stems methanolic extract. Therefore, this study was intended to investigate the antioxidant properties also antimicrobial activity of methanolic extract of *T. crispa* stems extracted using soxhlet extraction method. The *in vitro* antimicrobial activity of *T. crispa* stems methanolic extract were tested against two selected Gram–positive bacteria (*Staphylococcus aureus ATCC 25923* and *Bacillus cereus ATCC 11778*), two Gram–negative bacteria (*Escherichia coli ATCC 25922* and *Pseudomonas aeruginosa ATCC 27853*) and two fungi (*Candida albicans* IMR C S23/11 A and *Saccharomyces cerevisiae IMR S 617/068*).

2.0 EXPERIMENTAL

2.1 Sample Collection And Extraction

The stems of *T. crispa* were collected from Kampung Muka Bukit, Machang, Kelantan, on September 2013. *T. crispa* stems were washed thoroughly with tap water to remove dirt and soil. After completely dried using oven drying at 40 °C, it was powdered and kept in closed container and stored at 4 °C until further used. Powdered *T. crispa* (34.3 g) was extracted using Soxhlet extractor with methanol as the extracting solvent. The extract was then concentrated to dryness under vacuum using rotary evaporator. The concentrated crude extract (8.3 g, 24.2 % w/w) was stored at 4 °C until further used.

2.2 Antioxidant Testing

2.2.1 Total Phenolic Content

Determination of the total phenolic content in *T. crispa* stems extract was done by using the Folin-Ciocalteu method according to Almey *et al*. [10]. In a test tube, 100 µL of sample or standard stock solution, 0.75 mL of Folin-Ciocalteu reagent was added and mixed. The mixture was left for five minutes. After that, 0.75 mL of sodium carbonate was added into the test tube. After 90 minutes of incubation, the absorbance of the mixtures was measured at 725 nm. For calibration, the standard curve of gallic acid solution (0.02 to 0.10 mg/mL) was used and the test was done in triplicate. The results were expressed as gram of gallic acid equivalents (GAE) per 100 g of dried extract.

2.2.2 Total Flavonoid Content

Determination of the TFC in *T. crispa* stems extract was done according to the method described by Chew *et al*. [11]. In a test tube, 1.25 mL of deionized water was mixed with 0.25 mL of sample or standard stock solution. After that, 75 µL of $NaNO_2$ solution was added to the same test tube and the mixture was left to stand at room temperature for 6 minutes. Next, 150 µL of $AlCl_3$ solution was added to the same test tube and the mixture were allowed to stand for another 5 minutes. Then, 0.5 mL of NaOH (1M) was added followed by the addition of 275 µL of deionized water. The mixture was vortexed for 10 seconds and the absorbance of the mixtures was measured at 510 nm. For calibration, the standard curve of quercetin (0.05 to 0.50 mg/mL) was used. The test was done in triplicate, and the results were expressed as gram of quercetin equivalents (QE) per 100 g of dried extract.

2.2.3 DPPH Free Radical Scavenging Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of *T. crispa* extract was investigated based on the method described by Bhuiyan *et al*. [12]. Ascorbic acid was used as a positive control. Into a test tube, 3 mL of DPPH solution was added, followed by 100 µL of final concentrations of the positive control which were 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/mL and *T. crispa* extracts with final concentrations were 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/mL. After that, the mixtures were placed in the dark and left for 30 minutes at room temperature for incubation. The absorbance of the mixture was measured at 515 nm. Absolute methanol was used as a blank. The following formula was used to calculate the percentage

inhibition (I %) of DPPH scavenging activities of *T. crispa* extract:

$$I\,\% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100\%$$

2.2.4 β- Carotene Bleaching Assay

The total antioxidant activity of *T. crispa* stems extract and BHT as a positive control were measured according to the method of Ismail and Hong [13] with a slightly modification. An amount of 1 mL of β-carotene solution was mixed with 0.02 mL of linoleic acid and 0.20 mL of Tween 20. The chloroform was evaporated at 40 °C for 10 minutes using rotary evaporator. Next, 100 mL of distilled water was added into the mixture and shaken using a shaker for 30 minutes at 150 rpm to form an emulsion. Subsequently, 5 mL of the emulsion was added into a test tube containing 0.2 mL of the sample. The test tubes were immediately placed in a water bath at 50 °C. The absorbance of the mixture was recorded at 470 nm for every 20 minutes within 2 hours. Antioxidant activity (AA) was measured in terms of successful bleaching β–carotene. The following formula was used to calculate the antioxidant activity (AA) of the extract:

$$AA = [\ 1 - (A0 - At) / (A°0 - A°t)\ ] \times 100$$

AA= Antioxidant Activity
A0 = Measurement of absorbance at 0 minutes of time of the incubation for samples
A°0 = Measurement of absorbance at 0 minutes of time of the incubation for control
At= Measurement of absorbance at specific time in the samples or standards
A°t= Measurement of absorbance at specific time in the control
t = 2 hours

2.3 Antimicrobial Testing

2.3.1 Disc Diffusion Method

Disc diffusion method was carried out according to Shryock *et al*. [14]. The blank discs (6 mm in diameter) were filled with different concentration of extract which were 0.5, 1.0, 2.5 and 5.0 mg/disc.. For negative control, 10 µL of DMSO was filled into the blank disc (6 mm in diameter) while for positive control, standard antibiotic discs (30 µg for tetracycline or 100 unit of nystatin for bacteria and fungi, respectively) were used. After the discs completely dried, they were placed over the plates that have been spread with the microorganisms using sterile forceps. Each sample was done in triplicate. After that, the plates were incubated at 37 °C for 24 hours for bacteria-containing plate and at 30 °C for 48 hours for fungi-containing plate. The diameter of inhibition zone around each discs were measured. According to Bhalodia and Shukla [15], the sensitivities of the microorganism species to the plant extracts were determined by measuring the sizes of inhibitory zones (including the diameter of disc) on the agar surface around the discs.

2.4 Statistical Analysis

All the antioxidant tests were done in triplicate and statistical analysis were performed using Microsoft Excel.

3.0 RESULTS AND DISCUSSION

3.1 Total Phenolic Content

The equation obtained from the gallic acid standard curve was $y = 25.473x + 0.9249$; $r^2 = 0.954$. The TPC value obtained was 6.12 ± 0.92 GAE /100 g of dried extract. The TPC value of *T. crispa* stems extract from this study by using soxhlet extraction is slightly lower than the TPC value obtained from maceration technique with methanol as performed by Zulkefli *et al*. [16] (6.47 ± 0.16 GAE / 100 g). During phenol oxidation, the phosphomolybdic-phosphotungstic acid (Folin) reagent was reduced its colour from yellow to blue-colored complex. The product of the reduction of Folin-Ciocalteu reagent which is a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids were tungstene (W_8O_{23}) and molybdene (MO_8O_{23}). Sodium carbonate that was used in this test provided the alkaline condition and thus made this reaction to occur. The quantity of the phenolic compounds can be seen through the intensity of blue colour that produced [10]. In the present study, methanol was used as a solvent for the extraction of *T. crispa* stems. It is because; methanol had the capability to hinder the reaction of polyphenol oxidase which was the cause of phenolic oxidation. Beside different extraction method, the difference in the TPC value can also be attributed to the different parts of plant that are chosen to be tested, method of determination and also genotopic and environmental differences within species [17].

3.2 Total Flavonoid Content (TFC)

The TFC value presented in gram quercetin equivalents (QE)/100 gram of dried extract. The linear equation based on the quercetin standard curve is $y = 1.395x – 0.0261$ with $r^2 = 0.9904$. Based on the results, the TFC value of the extract was 55.58 g QE per 100 g extract. The TFC value of *T. crispa* extract was slightly high. Based on the principle of this method, acid stable complexes were formed when aluminum chloride reacted with either the C-3 or C-5 hydroxyl group of flavones and flavonols and also with C-4 keto group [18]. The formation of yellow colour intensity indicated the presence of flavonoid compound. The antioxidant properties of the flavonoid and TFC value

can be influenced by the different flavonoid structure in the plant species [19].

3.3 DPPH Free Radical Scavenging Activity

The IC_{50} of *T. crispa* stems extract and ascorbic acid that can scavenge 50% of DPPH free radical are given in Table 1. At the lowest tested concentration which was 0.0312 mg/ml, the ascorbic acid and the extract showed 39.90 % and 7.10 % of scavenging activity, respectively. Meanwhile, the IC_{50} values of the ascorbic acid and the extract was 0.04 mg/mL and 0.21 mg/mL, respectively. The result indicates that only a small amount of the positive control is required to inhibit 50% of the DPPH free radicals. The free DPPH radicals were dissolved in methanol and the methanol solution became violet in colour. The DPPH solution was decolorized by the radical scavenger presents in the *T. crispa* stems extract and ascorbic acid. It is because; in the presence of hydrogen-donating antioxidant, the stable DPPH radicals were reduced to diphenyl-pricrylhydrazine. The value of DPPH may be attributed to the phenolic and flavonoid contents of *T. crispa*.

Table 1 Scavenging activity (% of inhibition) of ascorbic acid (positive control) and *T. crispa* stems methanolic extract and their IC_{50} values.

Sample	IC_{50} (mg/mL)	% of inhibition at 0.0312 mg/mL
T. crispa extract	0.21	7.10
Ascorbic acid (positive control)	0.04	39.90

3.4 β–Carotene Bleaching

The degree of β-carotene bleaching was decreased in the presence of antioxidant. Figure 1 shows the antioxidant activities of *T. crispa* stems extract and positive control (BHT). Meanwhile, Figure 2 shows the graph of β–carotene bleaching of the extract, positive control (BHT) and negative control (β-carotene emulsion without sample). Based on the results showed in Table 2, in the first 20 minutes, the extract showed 52 % of β-carotene bleaching activity, while BHT showed 73 % of activity. After one hour of incubation, the percentage dropped to 38.3 % and 49 % for extract and standard, respectively. During the second hour, the percentage decreased to 31 % and 38 % for extract and standard, respectively.

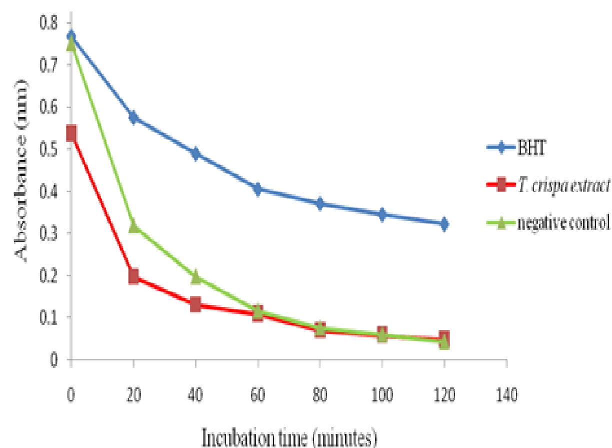

Figure 1 Antioxidant activity of *T. crispa* stems methanolic extract, positive control (BHT) and negative control (β-carotene emulsion without sample)

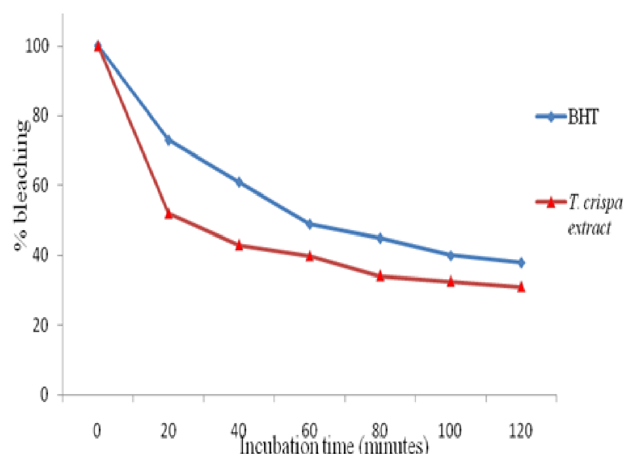

Figure 2 β-Carotene bleaching of *T. crispa* stems methanolic extract and positive control (BHT).

Table 2 Percentage inhibition of β-carotene bleaching within incubation time.

	Percent Inhibition (%)		
	Minute-20	**Minute-60**	**Minute-120**
BHT	73	49	38
T. crispa extract	52	38.3	31

The absorbance was decreased rapidly in the negative control and β-carotene was discolorized rapidly. Linoleic acid became a free radical with a hydrogen atom abstracted from one of its diallylic methylene groups. The highly unsaturated β-carotene molecules were then attacked by the radical that formed earlier. Through the oxidation process, the double bonds of β-carotene molecules will be lost [20]. Thus, the compound also loses its orange color. The absorbance measured of *T. crispa* stems extract retained for a longer time, which indicates the

presence of antioxidant properties. The free radicals formed in the system were neutralized by the antioxidative components in the BHT and *T. crispa* stems extract thus lead to the minimizing of the degree of β-carotene destruction. Based on the Figure 2, the average percentage of β–carotene bleaching of positive control, which is the BHT, was the highest, followed by the *T. crispa* stems extract. BHT is a known lipophilic antioxidant while ascorbic acid is one of the known polar hydrophilic antioxidant and its phenomenon has been defined in 'polar paradox theory' [21].

The polyphenol contents of *T. crispa* stems extract give influence to DPPH free radical scavenging activity and also β-carotene bleaching. According to Yim *et al*. [22], the β-carotene assay indicates the level of lipophilic compounds while the total phenolic content assay indicates the level of both hydrophilic and lipophilic compounds. Thus, the *T. crispa* stems extract could be lipophilic antioxidant and could be used for antioxidative preservative in emulsion-type because it's high level in β-carotene bleaching.

3.5 Disc Diffusion Method

T. crispa stems methanolic extract showed no inhibition zone against selected microorganisms at concentration 0.5, 1.0, 2.5 and 5.0 mg/disc. The results obtained from this study were similar to the findings from previous study conducted by Haque *et al*. [9]. The study showed methanol extract at concentration 0.4 mg/ disc did not have inhibition against *S. aureus*, *E. coli* and *C. albicans*. Another study conducted by Islam *et al*. [23] showed a little, but insignificant inhibition zone of methanol extract at concentration 0.4 mg/ disc against *S. aureus* (7.9 mm), *B. cereus* (4.6 mm), *E. coli* (2.8 mm) and *P. aeruginosa* (3.5 mm), *C. albicans* (5.5 mm) and *S. cerevisiae* (6.5 mm). Both of the previous studies used maceration technique meanwhile in the previous study, Soxhlet extractor was used to extract the compounds.

A number of factors can be attributed to the negative finding of the disc diffusion results. The active compounds in the crude extract only can possess their antimicrobial properties in a sufficient amount of concentration [3]. There is possibility that the active compound extracted through the soxhlet technique may not be sufficient enough to inhibit the growth of the microorganisms. Phenolic compounds are compounds which consist of various chemical structures and functional groups. They can be classified as simple phenols, phenolic acids, tocopherols, quinones, flavones, flavonoids, flavonols, stilbenes, ligans, tannins and coumarins [24, 25]. But, not all the classes of phenolic compounds have the antimicrobial properties. Thus, there is a possibility that the phenolic compounds in the extract did not have antimicrobial properties.

The other possibility could be all the selected microorganisms used in this study may have formed resistant to the methanolic extract of *T. crispa* stems. Each type of microorganisms has their own characteristics and ability to protect them, which make some of them resistant to certain antimicrobial compounds [26]. For the bacteria, the sensitivity towards the antimicrobial agents depends on the components that make up cell walls. The Gram–negative bacteria which have complicated components such as peptidoglycan, lipopolysaccharide, lipoprotein, phospholipid, and protein in their cell wall is more resistant compared to Gram–positive bacteria which only have two components such as peptidoglycan and teichoic acid in their cell wall [27].

4.0 CONCLUSION

T. crispa stems methanolic extract has a potential to be lipophilic antioxidant as it showed high activity in β-carotene bleaching assay. The polyphenol contents of *T. crispa* stems extract give influence to DPPH free radical scavenging activity and also β-carotene bleaching. However, the methanolic extract showed no inhibition zone against selected microorganisms at concentration 0.5, 1.0, 2.5 and 5.0 mg/disc.

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