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Antimicrobial, Cytotoxicity and Antioxidant Activity of *Tinospora crispa*

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Abstract: *Tinospora crispa* (family: Menispermaceae) has been investigated for evaluation of the biological activities. The stem bark *T. crispa* of was extracted with methanol and the extract was partitioned with petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions. The chloroform, petroleum ether and methanolic extracts were subjected to antimicrobial screening against some gram-positive and gram-negative organisms by the disc diffusion method. In the brine shrimp lethality bioassay, the extracts of chloroform, petroleum ether and methanol were found to show LC_{50} of 11.5 µg/ml, 12.6 µg/ml and 12.0 µg/ml respectively. This indicated that the cytotoxicity exhibited by the chloroform, petroleum ether and methanol extract was very significant. These extractives were subjected to antioxidant screening- by DPPH free radical scavenging activity. In these cases, butylated hydroxytoluene (BHT) and ascorbic acid were used as antioxidant standard. By DPPH assay, it is found that the carbon tetrachloride soluble fraction of *T. crispa* showed strong antioxidant activity with the IC_{50} value 30 µg/ml. Besides petroleum ether and chloroform soluble fractions also showed free radical scavenging activity with the IC_{50} value 70 and 75 µg/ml, respectively.

Keywords: *T. crispa*, extracts, antioxidant activity, cytotoxicity, antimicrobial screening.

Introduction:

Tinospora crispa, is an indigenous climber plant that commonly grows wild in Asian countries including Malaysia known by various local names like 'akar patawali' and 'akar seruntum'[1], an infusion of the stems is consumed as vermifuge and decoction of the whole plant is used to treat cholera and diabetes among the Malay community. Its stem has been used by traditional folklore for various therapeutic purposes such as treatment for diabetes, hypertension, stimulation of appetite and protection from mosquito bites. Sometimes it was also used as an anti-parasitic agent in both man and domestic animals. [2-4] Despite its long usage among the traditional folklore, scientific data supporting its health claims and the biological assessment of this plant is not thoroughly studied. On the other hand, natural substances have been extensively studied especially during this decade as an alternative treatment or a preventive measure. This has led our interest to explore the biological properties of *T. crispa* in terms on its nutritional composition, the mineral and flavonoid content in the stem of the plant as well as its antioxidant ability *In vitro*.

Antioxidant compounds play an important role as a health-protecting factor. Antioxidants are substance that significantly prevents or delays the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions.[5] Oxygen-centered free radicals and other reactive oxygen species (ROS) have been associated with the beginning of many diseases and degenerative processes in ageing.[6] Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase and catalase or chemical compounds such as α-tocopherol, vitamin C (ascorbic acid), carotenoids, polyphenol compounds and glutathione.[7] However, these systems are frequently insufficient to totally prevent the damage, especially under the conditions of severe oxidative stress, resulting in diseases and accelerated ageing.[8] Natural products with antioxidant activity may be used to help the human body to reduce oxidative damage. Many herbs, fruits and vegetables have been investigated for their antioxidant activities in the last years.[9] Dietary sources have been recognized as safe and effective antioxidants in terms of their efficiency and non-toxicity.[10-11] The interest on the role of natural antioxidants as a tool to prevent aging and degenerative diseases process is growing[12] due to the toxicity of synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) at fairly high doses, which limits their therapeutic usage.[13-14]

On the basis of the folkloric use, this plant was selected for pharmacological testing with focus on cytotoxicity determined by the brine shrimp (*Artemia nauplii*) lethality bioassay.[15] The assay is considered as an useful tool for preliminary assessment of toxicity and it has been used for the detection of plant extract toxicity [16], heavy metals [17], pesticides [18] and cytotoxicity testing of dental materials.[19] It is also a useful tool for the isolation of bioactive compounds from plant extracts.[20] The method is attractive because it is simple, inexpensive and low toxin

amounts are sufficient to perform the test on the microwell scale. In the present study, we report herein on the cytotoxicity studies of crude extracts of *Tinospora crispa* plant.

According to Zaika scientific experiments on the antimicrobial properties of plants and their components have been documented in the late 19th century.[21] Naturally occurring microbial inhibitors have been isolated and identified from a wide variety of plants, including garlic, onion, fruits and spices. Other than the antimicrobial activity, the extract of these plants were also reported to possess other pharmacological activities, such as analgesic, sedative, anti-inflammatory, antidiabetic and antihypertensive to name a few. [22]

Methods and Materials:

Collection:

Plant sample of ***Tinospora crispa*** was collected from Mymensing in November 2008. They were sun dried for seven days. The plant materials were then sun dried. The dried stem was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Department of Pharmacy, University of Asia Pacific.

Extraction and Isolation:

About 500 gm of the powdered material was taken in clean, round bottomed flask (5 liters) and soaked in 2.5 liter of methanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 39°C with a Heidolph rotary evaporation. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained was 20 gm for methanol. A portion (5.0 g) of the concentrated methanol extract (ME) was fractioned with petroleum ether, chloroform and carbon tetrachloride by the modified Kupchan partitioning method.[23] Evaporation of solvents yielded petroleum ether (PE, 0.7 gm), chloroform (CF, 0.8 gm), carbon tetrachloride (CT, 0.5 gm) and aqueous soluble (AQ, 2.5 gm) materials.

Antimicrobial Screening:

The antimicrobial activity of the extractives was determined against the test organisms by the disc diffusion method.[24] Standard discs of doxycilline (30 µg/disc) and blank discs impregnated with solvents followed by evaporation were used as positive and negative control, respectively. Discs containing the test materials were placed onto Muller Hinton agar medium uniformly seeded with the test microorganisms. These plates were kept at low temperature (4ºC) about 2 hours to allow diffusion of the test materials. The plates were then incubated at 37ºC for 24 hours. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm.

Cytotoxicity Determination:

Brine shrimp lethality bioassay [15] technique was applied for the determination of cytotoxicity of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day *in vivo* assay. For this experiment, 4 mg of each of the methanol crude extract, chloroform and petroleum ether soluble fractions were dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution. Vincristine sulphate was used as positive control.

Antioxidant Activity Determination:

The antioxidant activity (free radical scavenging activity) of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method developed by Brand-Williams et al., 1995.[25] In this experiment, 2.0 mg of each of the extract was dissolved in methanol. Solution of varying concentrations such as 500, 250, 125, 62.50, 31.25, 15.62, 7.81, 3.91, 1.95, and 0.98 µg/ml were obtained by serial dilution technique. An aliquot of two ml of the extract in methanol was mixed with 3 ml of a DPPH-methanol solution (20 µg/ml) and was allowed to stand for 20 minutes for the reaction to occur. The absorbances were determined at 517 nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

$$\% \text{ inhibition} = [\, 1 - (ABS_{sample} / ABS_{control})] \times 100$$

Then % inhibitions were plotted against concentrations used and from the graph the IC_{50} was calculated by using tert-butyl-1-hydroxytoluene (BHT) as a positive control. The experiment was carried out in triplicate and the mean values were taken.

Results:

The antibacterial activity of different extracts of *T. crispa* was investigated at an initial dose of 400µgm/disc against gram positive bacteria, gram negative bacteria and few fungi ***(Table 1)***.

Table 1: Antimicrobial activity of the crude extracts of Tinospora crispa at 400µgm/disc

Test microorganisms	Diameter of zone of inhibition (mm)			
	CF	PESF	MEF	DOX
Gram Positive				
Bacillus cereus	8	0	0	50
Bacillus megaterium	8	0	0	50
Bacillus subtilis	7	0	0	46
Staphylococcus aureus	7	0	0	46
Sarcina lutea	9	0	0	50
Gram Negative				
Escherichia coli	7	0	0	49
Salmonella paratyphi	7	0	0	43
Salmonella typhi	9	0	0	51
Shigella boydii	7	0	0	50
Shigella dysenteriae	8	0	0	50
Vibrio mimicus	8	0	0	52

Vibrio parahemolyticus	7	0	0	50
Fungi				
Candida albicans	7	0	0	50
Aspergillus niger	7	0	0	50
Sacharomyces cerevacae	7	0	0	49

MEF: crude methanolic extract; CF: chloroform soluble fraction of the methanolic extract; PESF: petroleum ether soluble fraction of methanolic extract; DOX: Doxicilline (30 µg/disc).

Following the procedure of Meyer [15], the lethality of methanolic crude extract and its petroleum ether and chloroform soluble fraction to brine shrimp was determined after 24 hours of exposure. The LC_{50} were found to be 12.0, 12.6 and 11.5 µg/ml for methanol crude extract, petroleum ether and chloroform soluble materials, respectively ***(Table 2)***.

Table 2: Brine shrimp lethality bioassay of T. crispa extractives

Sample	LC_{50} (µg/ml)
VS	0.323
CHLF	11.5
PESF	12.6
MEF	12

VS: vincristine sulphate (std); MEF: crude methanolic extract; CHLF: chloroform soluble fraction of methanolic extract and PESF: petroleum ether soluble fraction of methanolic extract.

In the antioxidant assay, free radical scavenging activity of various fractions of the crude methanol extract was also evaluated. Figure 1 shows the antioxidant activity of the test samples. The IC_{50} values for the methanolic crude extract and its petroleum ether, carbon tetrachloride, chloroform and aqueous soluble partitionates were found to be 49, 70, 30, 75 and 70µg/ml, respectively ***(Table 3)***.

Figure 1: Free radical scavenging activity of different extracts of Tinospora crispa.

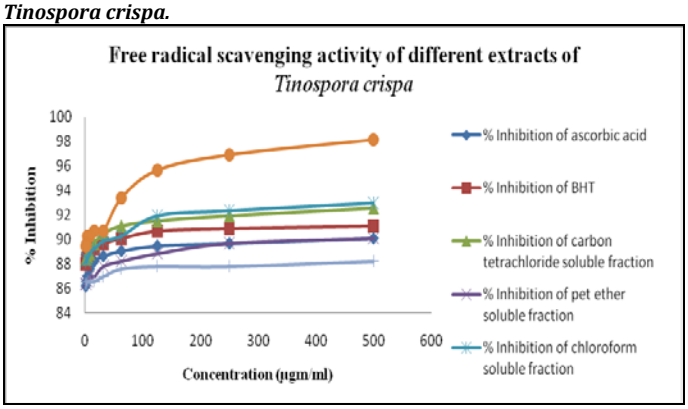

Table 3: Antioxidant activity of extractives of T. crispa

Sample	IC_{50} (µg/ml)
BHT	25
AA	15
PESF	70
CHLF	75
ME	49
AQ	70
CT	30

BHT: tert butyl-1-hydroxy toluene (standard); AA: Ascorbic Acid (standard); ME: crude methanolic extract; PESF: petroleum ether soluble fraction of methanolic extract; CT: carbon tetrachloride soluble fraction of methanolic extract; CHLF: chloroform soluble fraction of methanolic extract; AQ: aqueous soluble fraction of methanolic extract.

Discussion:

In this study chloroform soluble fraction of the methanolic extract of the plant showed significant activity against the tested organisms in case of antimicrobial screening. In comparison with the LC_{50} 0.323 µg/ml of the positive control (vincristine sulphate), the cytotoxicity exhibited by the chloroform extract (CHLF), petroleum ether extract (PESF) and methanol extract (MEF) are promising and further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compounds. The IC_{50} value exhibited by the standard (BHT) was 25µg/ml and another standard (Ascorbic Acid) was 15 µg/ml but the extracts of the *T. crispa* showed better results then the standards, which demonstrates that the *T. crispa* plant might be consider as a potential source of antioxidant.

Conclusion:

The study clearly indicates that the extracts possesses antioxidant, cytotoxic and few antimicrobial substances. These finding justify the traditional uses of this plant in the treatment of diabetes, cholera, hypertension and malarial fever. Further research is necessary for elucidating the active principles.

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