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FREE RADICAL SCAVENGING ACTIVITY OF THE *Tinospora cordifolia* STEM METHANOLIC FRACTIONS

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ABSTRACT

Free radicals injure the cells and damage the DNA that may cause diseases such as cancer, which has been found to cause 22% of all non-communicable diseases in 2012. Antioxidants take part in the prevention of cellular damage caused by oxidative stress. Plants with components such as alkaloids and flavonoids have been proven to exhibit antioxidant properties. A study on the anti-oxidant activity of the methanolic fractions of *Tinospora cordifolia* (Makabuhay) was performed by determining the free radical scavenging activity by DPPH assay. Different concentrations of 1.25 μg/μL, 0.625 μg/μL, 0.3125 μg/μL, 0.156 μg/μL, and 0.0781 μg/μL of the different fractions namely: the hexane fraction (F1), the water-methanol fraction (F2), the dichloromethane fraction (F3), the water-butanol fraction (F4), and the water fraction (F5) were prepared by serial dilutions from 1mg/mL stock solution of each fraction. Quercetin, vitamins C and E were used as standard for the TFC determination and DPPH assay. F3 showed the highest amount of TFC ranging from 340.03 ± 5.76, while the lowest amount (6.36 ± 0.58) was observed in F5. For the DPPH method, the % inhibition is directly proportional with the concentration of each fraction.

F2 showed potential anti-oxidant activity with an IC_{50} of 0.861 µg/µL ($CI_{95\%}$: 31.11 to 4582, R^2 = 0.988), as compared to that of ascorbic acid (IC_{50} 0.209 µg/µL; $CI_{95\%}$: 0.19 to 0.23, R^2 = 0.934) and alpha-tocopherol (IC_{50} 0.267 µg/µL; $CI_{95\%}$: 0.25 to 0.29, R^2 = 0.976), which are well-known anti-oxidants. In conclusion, F2 exhibited the highest antioxidant property among the tested fractions.

Keywords: Anti-oxidant, *Tinospora cordifolia*, 2,2-diphenyl-1-picrilhydrazyl (DPPH), Total Flavonoid Content

INTRODUCTION

Reactive oxygen species [ROS] also known as active oxygen species, which consist of free radicals like hydroxyl radical, superoxide ions, activated oxygen and other forms as well as hydrogen peroxide (H2O2), a non free-radical species [1,2]. These molecules serve as aggravating agents in cellular injury as well as degenerative and aging processes such as cancers, inflammations, coronary heart diseases and neurodegenerative disorders [1,3]. Hydroxyl radical, being the most reactive among these molecules, causes lipid peroxidation which contributes to DNA fragmentation and mutation [2,4].

Natural antioxidants found in plants and drugs formulated with these agents gained the interest of many health practitioners because of their association with health benefits particularly cancer prevention. Flavonoid and phenolic compounds which are naturally produced in plants are proven to have biological effects like antibacterial, anti-cancer and anti-oxidant activities. Dietary intake of foods rich in antioxidants like fruits and vegetables is related to the decreasing incidence of degenerative diseases [1,2,6]. These paved the way to the screening of plants and its extracts with potential anti-oxidant activity.

Tinospora cordifolia (Menispermaceae) is popularly known as Makabuhay and is found abundantly in the country. Extracts of this plant have been shown to possess many therapeutic properties including anti-inflammatory, antispasmodic, antitumor, antipyretic, anti-amoebic, anthelmintic and anti-atherogenic effects [7,8]. Conventionally, decoction of crude drugs and other preparation containing secondary metabolites have been accepted as therapy due to the usual beliefs that they are safe and present insignificant side effects [9]. Furthermore, in the study performed by Onkar, Bangar and Karodi (2012), the results revealed potent scavenging activity of *T. cordifolia* when compared with a standard, which is the Butylated hydroxytoluene. Thus, the findings indicated promising anti-oxidant activity of crude extracts.

The researchers aimed to investigate the presence of flavonoids and alkaloids in the methanolic fractions of *Tinospora cordifolia* stems by Thin layer chromatography and determine whether these fractions possess anti-oxidant properties.

The study aims to identify the concentration of the fractions which exhibit the highest amount of flavonoids that will provide the most anti-oxidant activity through free radical scavenging.

RESEARCH AND METHODS

All the chemicals and solvents used were of analytical grade. Methanol, DCM, hexane, butanol, ferric chloride, potassium ferricyanide, Dragendorff's reagent were purchased from LESO, University of Santo Tomas and Merck Phalillpines while quercetin, Vitamins C and E and DPPH (1, 1,-diphenyl-2-picryl-hydrazyl) were donated by UST RCNAS Pharmacology Lab.

Collection and Preparation of Sample

Fresh samples of *Tinospora cordifolia* were collected from Angat, Bulacan, Philippines in April 2014. The collected specimen was authenticated by the Research Cluster for the Natural and Applied Sciences of the Tomas Aquinas Research Complex, University of Santo Tomas, Manila, Philippines. The plant sample was cut into matchstick-sized pieces and was air-dried. The dried samples were ground using the Wiley Mill. The powdered sample was then subjected to percolation until exhaustion using 95% methanol for 24 hours to extract the polar compounds and the residue was extracted with dichloromethane for 24 hours to extract non-polar compounds. The extracts obtained were kept in a clean, dried, well-sealed amber glass bottle for protection from sunlight and were then further extracted into fractions using the Kupchan's solvent partitioning scheme with some modifications as described by Corpuz *et al.* (2012). The filtrate, the crude dichloromethane (DCM) extract, was partitioned with hexane, methanol, and water with 2:1:1 ratio to obtain the hexane fraction (F1) and water-methanol fraction (F2). The crude methanolic extract, on the other hand, was partitioned with DCM and water with 1:1 ratio to produce DCM fraction (F3) and water fraction while the water fraction was further partitioned with water and n-butanol in a 1:1 ratio to produce the water-butanol fraction (F4) and the water fraction (F5). The five fractions were subjected to different tests to identify the fraction with the highest antioxidant activity and their total flavonoid content.

Thin-Layer Chromatographic (TLC) Analysis of the Five fractions

Extracts (Berberine standard, F1-F5 fractions from fractionation) were applied on different pre-coated TLC plates by placing 5 spots of each of the fractions. Each plate must have one spot of Berberine and two spots of two different fractions. Each spot was allowed to dry before applying the next. The spots were made sure to be as small as possible to prevent overlapping.

In separate beakers, developing chambers were prepared by placing different solvents shown in Table 1. These were covered and were left to stand for 15 minutes to allow them to equilibrate.

The plates were carefully placed in the developing chamber, allowing the solvent system to rise up to 1 cm from the upper end. Once the spots are visible, the retardation factor, also known as the R_f value, of each spot is determined by dividing the distance traveled by the solute with the distance traveled by the mobile phase using the initial spotting site as reference. These values depend on the mobile phase used wherein the highest affinity of each fraction was considered.

The following equation was used to determine the retardation factor:

$$Rf\ value = \frac{distance\ of\ solute}{distance\ of\ solvent}$$

The TLC plates of each of the fractions with the best profiles were then subjected under ultra-violet (UV) light, in order to view the different constituents present for each of the fractions. They were also sprayed with Dragendorff's reagent to detect the presence of alkaloids and a mixture of 1% ferric chloride and 1% potassium ferricyanide for flavonoids. Their *Rf* values were then computed and are shown on Tables 1,2 and 3 [12].

Preparation of the sample

The extract stock solution was prepared by weighing 1 mg of each extract and diluting in 1 mL of absolute ethanol. The freshly prepared stock solutions were then diluted to 1.25, 0.625, 0.3125, 0.156, and 0.0781 μg/μL. All samples, standards and blanks were placed in their assigned wells in a 96-well microplate. A UV-Vis spectrophotometer microplate reader was used to read their corresponding absorbances. All tests were performed in quadruplet.

Determination of total flavonoid content (TFC)

The total flavonoid content was determined using the methods described by Corpuz *et al.*

(2012), where Quercetin was used as the standard. Each well contained 100 µL of each concentration for all the fractions and the standard. About 100 µL of 2% $AlCl_3$ was added to all the wells excluding the wells that contained the 200 µL blank. The plates were then incubated for an hour at room temperature and the absorbance was read at 420 nm.

The total flavonoid content was calculated based on the equation that was obtained from the Quercetin standard curve:

$$X = (A_o \text{ x } M_o)(A_1 \text{ x } M_1)$$

wherein, X is the flavonoid content, mg/mg extract in *Tinospora cordifolia*; A_1 is the absorbance of the fraction, while A_o as the absorbance of the standard Quercetin solution. M_1 is the weight of the fraction in mg while M_o as the weight of Quercetin in the solution, in mg.

Free radical scavenging activity by DPPH method

Quantitative assay was performed on the basis of the modified method of Choi Y., Jeong H., & Lee J. (2007). Ascorbic acid and α-tocopherol were used as standards for the test. Twenty (20) µL of each concentration of the fractions and standards were placed into their assigned wells and 180 µL of 0.004% DPPH in absolute ethanol was added. The plates were incubated for 30 minutes at room temperature and the absorbance was read at 517 nm [2,14-16].

The % mean inhibition was calculated using the following formula:

$$\% \, inhibition = \frac{A_{DPPH} - A_{Sample}}{A_{DPPH}} \, x \, 100$$

wherein, A_{DPPH} represents the mean absorbance of the DPPH and A_{Sample} represents the mean absorbance of the fraction at each concentration subtracted by the mean absorbance of the blank, which in this case, is absolute ethanol.

Statistical analysis

The mean and its standard error were used to summarize the % inhibition and mean flavonoid content of different fractions F1, F2, F3, F4, and F5 with DPPH and TFC, respectively. Two-factor analysis of variance was used to compare the mean % inhibition and mean flavonoid content of the different extracts and concentrations.

Furthermore, the 50% inhibitory concentration (IC_{50}) was calculated using four-parameter logistic regression. All the statistical tests were performed, under 5% level of significance, using SPSS version 20.0 and Prism 6.

RESULTS AND DISCUSSION

Thin-Layer Chromatographic Test

The five extracts, namely the hexane fraction (F1), the methanol-water fraction (F2), the dichloromethane fraction (F3), the water-butanol fraction (F4), and the water fraction (F5) showed the best TLC profiles observed under UV light using Chloroform:Methanol (5:1), DCM:Acetic Acid:Water (10:3:1), Methanol:Water (5:1), Chloroform:Acetic Acid:Water (10:9:1), and Chloroform:Methanol (5:1), respectively, as mobile phases as shown in Table 1.

When viewed under ultra-violet (UV) light, the water-methanol fraction (F2), had the most number of constituents present (Table 1). The water fraction (F5), on the other hand, has the most number of eluted light orange spots which denote the presence of alkaloids while the methanol-water (F2) fraction exhibited the most evident bluish yellow spots which represent the presence of flavonoids (Tables 2 and 3).

Table 1: Thin-Layer Chromatography Profile of Tinospora fractions under UV light

THIN LAYER CHROMATOGRAPHY UNDER UV LIGHT						
FRACTION	SOLVENT SYSTEM	SPOT NO.	DISTANCE OF SOLUTE (cm)	DISTANCE OF SOLVENT (cm)	Rf VALUE	COLOR (fluorescence)
F1	Chloroform:Methanol (5:1)	1	1.0	7.0	0.1429	white
		2	1.7	7.0	0.2429	light orange
		3	2.5	7.0	0.3571	orange
		4	3.0	7.0	0.4286	light orange
		5	3.7	7.0	0.5286	orange
		6	4.2	7.0	0.6000	white
		7	4.7	7.0	0.6714	white
		8	5.7	7.0	0.8143	orange
F2	DCM:Acetic Acid:Water (10:3:1)	1	0.5	7.0	0.0714	white
		2	0.8	7.0	0.1143	white
		3	1.0	7.0	0.1429	light green
		4	1.5	7.0	0.2143	light green
		5	1.9	7.0	0.2714	white
		6	2.3	7.0	0.3286	white
		7	3.0	7.0	0.4286	white
		8	3.5	7.0	0.5000	purple
		9	4.5	7.0	0.6429	light orange
		10	5.6	7.0	0.8000	light green
		11	6.3	7.0	0.9000	light orange
		12	6.7	7.0	0.9571	light orange
F3	Methanol:Water (5:1)	1	0.4	7.2	0.0556	white
		2	0.8	7.2	0.1111	white
		3	1.2	7.2	0.1667	white
		4	3.9	7.2	0.5417	white

		5	5.2	7.2	0.7222	white
		6	5.9	7.2	0.8194	white
F4	Chloroform:Acetic Acid:Water (10:9:1)	1	0.8	7.0	0.1143	white
		2	1.5	7.0	0.2143	white
		3	3.3	7.0	0.4714	white
		4	4.2	7.0	0.6000	bluish
		5	4.5	7.0	0.6429	white
		6	5.1	7.0	0.7286	white
		7	5.5	7.0	0.7857	purple
		8	5.9	7.0	0.8429	white
		9	6.7	7.0	0.9571	light orange
F5	Chloroform:Methanol (5:1)	1	1.2	7.0	0.1714	white
		2	3.2	7.0	0.4571	white
		3	4.0	7.0	0.5714	white

Table 2: Detection of Alkaloids by Thin-Layer Chromatography using Dragendorff's spray reagent

FRACTION	SOLVENT SYSTEM	SPOT NO.	DISTANCE OF SOLUTE (cm)	DISTANCE OF SOLVENT (cm)	Rf VALUE	COLOR
F1	Chloroform:Methanol (5:1)	1	2.4	7.0	0.3429	light orange
		2	3.6	7.0	0.5143	light orange
		3	6.1	7.0	0.8714	light orange
F2	DCM:Acetic Acid:Water (10:3:1)	1	0.8	7.0	0.1143	light orange
F3	Methanol:Water (5:1)	1	3.9	7.2	0.5417	light orange
		2	6.0	7.2	0.8333	light orange
F4	Chloroform:Acetic Acid:Water (10:9:1)	1	3.2	7.0	0.4571	light orange
		2	5.1	7.0	0.7286	light orange
F5	Chloroform:Methanol (5:1)	1	0.5	7.0	0.0714	light orange
		2	2.0	7.0	0.2857	light orange
		3	3.0	7.0	0.4286	light orange
		4	4.3	7.0	0.6143	light orange

Table 3: Detection of Flavanoids by Thin-Layer Chromatography using $FeCl_3$ and $K_3[Fe(CN)_6]$ spray reagent

FRACTION	SOLVENT SYSTEM	SPOT NO.	DISTANCE OF SOLUTE (cm)	DISTANCE OF SOLVENT (cm)	Rf VALUE	COLOR
F1	Chloroform:Methanol (5:1)	1	5.5	7.2	0.7639	blue green
		2	6.0	7.2	0.8333	blue green
F2	DCM:Acetic Acid:Water (10:3:1)	1	6.2	7.1	0.8732	bluish yellow
		2	6.5	7.1	0.9155	bluish yellow
F3	Methanol:Water (5:1)	1	5.9	7.0	0.8429	bluish yellow
		2	6.1	7.0	0.8714	bluish yellow

F4	**Chloroform:Acetic Acid:Water (10:9:1)**	**1**	**0.2**	**7.1**	**0.0282**	**blue**
		2	**1.3**	**7.1**	**0.1831**	**blue**
		3	**5.5**	**7.1**	**0.7746**	**blue**
F5	**Chloroform:Methanol (5:1)**	**1**	**0.4**	**7.0**	**0.0571**	**bluish yellow**
		2	**1.0**	**7.0**	**0.1429**	**blue**
		3	**3.1**	**7.0**	**0.4429**	**blue**
		4	**3.8**	**7.0**	**0.5429**	**blue**

Determination of total flavonoid content

The total flavonoid content (mg/mg) ranged from 0.006 to 1.443. The DCM fraction (F3) has the highest amount of TFC ranging from 0.033 to 1.443, while the lowest amount (0.006 to 0.074) was observed in the water fraction (F5) as shown in Figure 1.

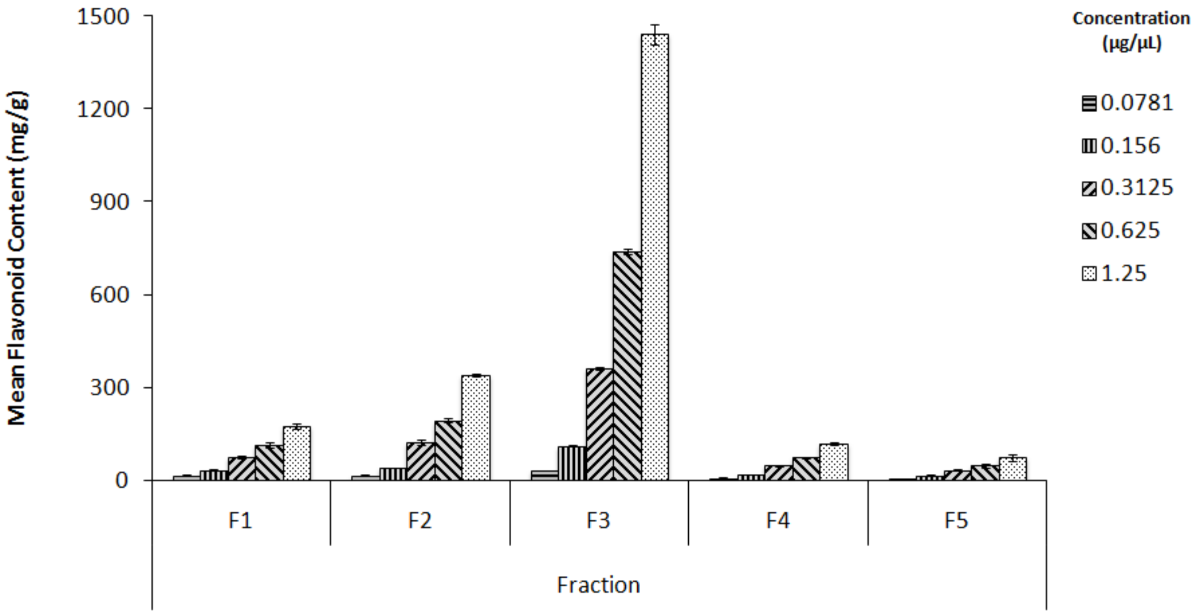

Figure 1: Flavonoid content of *Tinospora* Stem Methanolic fractions

Free radical scavenging activity by DPPH method

Anti-oxidant activity of the different stem fractions of *T. cordifolia* was determined. The water-methanol fraction (F2) showed potential anti-oxidant activity where the IC_{50} was 0.861 µg/µL ($p < 0.001$), as compared to that of ascorbic acid (IC_{50} 0.209 µg/µL; $p < 0.001$) and alpha-tocopherol (IC_{50} 0.267 µg/µL; $p < 0.001$), which are well-known anti-oxidants. However, the antioxidant activity of each fraction is not comparable with Vitamin C and E at the same concentration as shown in Figure 2 and Table 4. The concentration of each fraction can be increased to exhibit comparable antioxidant activity as with the standards mentioned.

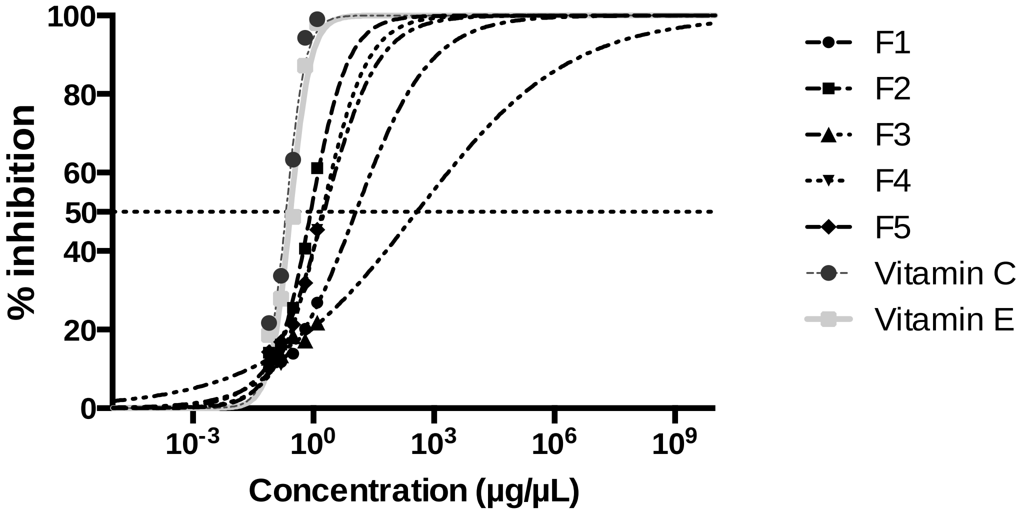

Figure 2. The 4-parameter Logistic Regression Curve of DPPH % inhibition by the Different *Tinospora* Stem Methanolic Fractions

Table 4. % Inhibition of DPPH by Different Tinospora Stem Methanolic Fractions According to Concentrations

	Concentrations (µg/µL)				
	0.0781	0.156	0.3125	0.625	1.25
F1	10.3 ± 1.3	11.7 ± 0.5	13.9 ± 0.7	20.2 ± 0.5	26.9 ± 0.9
F2	14.1 ± 0.7	16.2 ± 2.3	25.6 ± 0.8	40.6 ± 0.6	61.1 ± 1.1
F3	12.8 ± 0.7	13.5 ± 0.7	18.3 ± 3.1	17.2 ± 0.4	21.8 ± 0.6
F4	12.7 ± 0.7	11.0 ± 5.2	20.3 ± 0.8	30.7 ± 0.9	45.4 ± 0.7
F5	14.3 ± 0.7	16.9 ± 0.6	21.3 ± 0.6	31.9 ± 1.2	45.4 ± 1.5
Vit. C	21.7 ± 1.4	33.7 ± 3.6	63.3 ± 7.4	94.3 ± 3.2	99.1 ± 0.8
Vit. E	18.6 ± 0.5	27.9 ± 1.0	48.7 ± 2.1	87.2 ± 1.1	98.1 ± 0.9

Values expressed as mean ± SEM, n = 8.

DISCUSSION

An antioxidant is a chemical that neutralizes free radicals like superoxides and hydroxyl radical. Antioxidants intervene with the oxidative processes through free radical scavenging activity, chelation of catalytic metals and as electron donating agents [1,2].Through these mechanisms, lipid peroxidation may be inhibited and thus may prevent certain degenerative diseases [2,16-17].

Antioxidant supplementations, particularly vitamin E, have been proven to reduce cardiovascular events in patients with Diabetes Mellitus [18]. Increased levels of exogenous antioxidants are proven to inhibit certain types of free radical damage which are related cancer development and metastasis. Inadequate levels of antioxidants in the body may cause oxidative stress caused by peroxidation and free radical activation which tends to lead to accelerated tissue and organ damage [19,20].

Based on the study conducted by Sivakumar, Rajan, and Riyazullahi, (2010), the presence of high amounts of glycosides, alkaloids, tannins, phenolics, and other all the principal secondary metabolites were detected in methanolic extract of *Tinospora cordifolia*. In the phytochemical screening using TLC of the present study, all fractions were proven to contain both alkaloids and flavonoids, the constituents proven to have potential anti-oxidant activities. The alkaloids that were present in the fractions as seen in Table 2 may be attributed to the quaternary alkaloids like berberine, palmatine and magnoflorine from *Tinospora* sp. which are proven to exhibit various biological activities. Berberine and its salts have antibacterial, antifungal, antiprotozoal and antipyretic properties. Berberine has cytotoxic and neoplasm inhibitory effects [22].

In the determination of total flavonoid content, F3 (DCM) was seen to have the most number of flavonoids (1442.68 ± 32.53), followed by F2 (water- methanol) (340.03 ± 5.76).

Sivakumar *et al.* (2010) mentions plant extracts' activity against DPPH wherein a decrease in the absorbance at 517 nm was observed which could be due to the free radical scavenging activity of the extract. The methanol water fraction exhibited the most free radical scavenging activity among the five fractions which may be associated with the antiradical scavenging activity of 85% and 100% exhibited by the methanolic stem extract of *Tinospora cordifolia* as reported by Sivakumar *et al.*, 2010 and Ibahim, 2011 respectively.

Quercetin has the ability to scavenge free radicals, bind transition metal ions, and inhibit lipid peroxidation [24,25]. Lipid peroxidation may generate detrimental effects in the body, such as cellular injury, cardiovascular and neurodegenerative diseases which may hindered by quercetin's action with the radicals formed [24, 26].

The experimental result shows the direct relationship between the percentage (%) scavenging of DPPH radical and dose of the *Tinospora* fraction. IC_{50} value of F2 (0.861 µg/µL; $CI_{95\%}$: 0.80 to 0.93, R^2 = 0.987) was found to be significant when compared to the IC_{50} value of the standards ascorbic acid (0.209 µg/µL; $CI_{95\%}$: 0.19 to 0.23, R^2 = 0.934) and α-tocopherol (0.267 µg/µL; $CI_{95\%}$: 0.25 to 0.29, R^2 = 0.976).

The free scavenging activity of the fractions of *Tinospora* stem extract may be also attributed to the inhibition of oxidative stress by *Tinospora cordifolia* through the suppression of thiobarbituric acid reactive substances (TBARS). Premanath and Lakshmidevi (2010) reported that the *Tinospora ethanolic* leaf extract showed the most antioxidant activity as compared with other solvent extracts. The immunomodulatory effects of *Tinospora* particularly interleukin -8 level reduction in scabies patients after treatment with the prepared *Tinospora* lotion may be attributed to the antioxidant property of *Tinospora* as well as the decreased in lipid peroxidation and inhibition of oxidative stress. These said activities may have prevented further cellular injury in scabies infected patients [28-30].

CONCLUSION

In conclusion, the % inhibition is directly proportional to the concentration of the fractions and F2 (water-methanol) exhibited the highest anti-oxidant activity among the fractions since it contains both alkaloids and flavonoids and it exhibited the highest amount of total flavonoids next to F3 (DCM). It was also tested to have a significant IC_{50} as compared to the standards, vitamins C and E. The researchers recommend to increase the concentration of each *Tinospora cordifolia* fraction and to perform other methods which will confirm anti-oxidant property like hydroxyl radical, nitric oxide and hydrogen peroxide scavenging assays.

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