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The phytochemical constituents and the antioxidant effects of different extracts of *Thea sinensis* Linn. (Tea) leaves

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Antioxidants may play a major role in the prevention of diseases, including cardiovascular and cerebrovascular diseases, some forms of cancer and effective to be long life and anti-aging. Thus, the aim of this study was to evaluate phytochemical constituents and the antioxidant activity of different extracts of *Thea sinensis* Linn. (လက်ဖက်) leaves. It was found that leaves of *Thea sinensis* Linn. contained alkaloids, α-amino acids, basic compounds, flavonoids, phenolic compounds, reducing sugars, saponins, steroids and terpenoids. The chloroform, ethanol, petroleum ether extracts of tea leaves were tested for their antioxidant activity by using thiocyanate method (the inhibition of linoleic acid autoxidation to detect lipid oxidation) in comparison with the synthetic antioxidant butylated hydroxy anisole (BHA). The chloroform, ethanol, petroleum ether extracts and BHA significantly lowered the autoxidation of linoleic acid when compared with that of control ($p<0.01$-$p<0.0005$). The % inhibition of autoxidative activity of the chloroform, ethanol, petroleum ether extracts and BHA were 75.97%, 87.06%, 59.10% and 85.34%, respectively, after 14thday incubation.

INTRODUCTION

Antioxidants are substances that retard oxidation by atmospheric oxygen at moderate temperatures (autoxidation). An important characteristic of antioxidants is that they significantly inhibit or delay oxidative processes at very low concentrations [1]. Antioxidants may reduce the energy of the free radicals, stop the free radicals from forming in the first place or interrupt an oxidizing chain reaction to minimize the damage caused by free radicals [2].

There are two types of antioxidants namely synthetic and natural. Dietary antioxidants (natural) such as α-tocopherol, ascorbic acid, carotenoids, flavonoids, and other phenolics may be effective in protection from oxidative damage. The synthetic antioxidants, such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), tertabutyl hydroquinone (TBHQ) have been developed but their use has begun to be restricted due to their carcinogenicity [3]. Vitamin E (α-tocopherol) is an effective natural antioxidant but has limited usage. As a result, there is considerable interest in preventive medicine in the development of natural antioxidants from plants [4].

Recent developments in biomedicals point to the involvement of free radicals in many diseases [5]. Free radicals attack the unsaturated fatty acids in the biomembranes resulting in membrane lipid peroxidation, a decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane proteins leading to cell inactivation [6]. Free radicals also attack DNA and cause mutation leading to cancer [7]. For these reasons, antioxidants are of interest for treatment of any kind of cellular degeneration [8]. Recently, antioxidants have been found to play an important role in prevention of oxidation-related diseases such

as cardiovascular and cerebrovascular diseases, and some forms of cancer. Flavonoids are antioxidant molecules found in plant sources such as fruits, flowers, roots, stems, tea, wine, grains and vegetables. They may be regarded as a semi-essential food related to human health. They act as antioxidants by directly scavenging free radicals or by inhibiting the lipid peroxidation cascade [9].

Biological organs contain many polyunsaturated fatty acids (PUFA), such as linoleic, linolenic and arachidonic acids, mainly in the form of esters with phospholipids, triglycerides, or with cholesterol. These PUFA can undergo lipid peroxidation which can be interrupted by antioxidants by the donation of electrons. Lipid peroxidation is initiated by active oxygen species attacking unsaturated fatty acids, and is propagated by a chain reaction cycle involving lipids, peroxy radicals and lipid hydroperoxides. Superoxide anion, hydrogen peroxide and hydroxyl radical actively participate in the initiation of lipid peroxidation. These peroxides cause damage and dysfunction in cell and organelle membranes [10].

Phenolic compounds including flavonoids are known to be major antioxidative compounds in various herbs and spices [11]. Among them, tea leaves *(Thea sinensis, Camellia sinensis* (L.) O. Kuntze) are rich in phenolic compounds. Inspite of the uses of plants as antioxidants, no study on antioxidant activity of tea leaves has so far been reported in Myanmar. Therefore, the aim of this study was to evaluate the phytoconstituents and antioxidative activities of its extracts.

MATERIALS AND METHODS

Plant material

The tea leaves were purchased from Pin Pyo Ywet Nu warehouse, Yangon and botanically identified and authenticated by a taxonomist from Botany Department, Yangon University. It was confirmed as *Thea sinensis* Linn. The leaves were made to fine powder and stored in air-tight glass bottle.

Determination of phytochemical constituents of Thea sinensis Linn. (tea) leaves

Both ethanolic extracts of *Thea sinensis* were tested qualitatively for the presence of alkaloid, flavonoid, glycoside, tannin, steroid, phenol, saponin, resin and amino acid by using the method of Physicochemical standards of Unani formulations [12].

Successive extraction of plant material by various solvents

The powder of tea leaves (100g) was percolated with 1 liter of petroleum ether (60-80˚) for 3 days at room temperature. Petroleum ether soluble portion was filtered and then evaporated to dryness by rotary evaporator to get petroleum ether (PE) extract (1.0 g, 1.0%). The residue was further extracted with chloroform for 3 days at room temperature. Filteration and then evaporation of chloroform soluble portion were performed to obtain chloroform extract (1.5 g, 1.5%). Finally, the residue was further extracted with 95% ethanol for 3 days at room temperature. Ethanol soluble portion was filtered and evaporated to obtain ethanol extract (10.0 g, 10.0%).

Screening of anti-oxidative activities of various crude extracts

The antioxidative activities of petroleum ether, chloroform and ethanol extracts from *T. sinensis* were determined by thiocyanate method [13, 15]. Two milligrams of each extract sample were added to a 2.53% linoleic acid solution consisting of 99.5% ethanol (8.1 ml), 0.05 M phosphate buffer (pH 7.0, 8.0 ml), and distilled water (3.9 ml) in a screw-top vial (36 mm i.d.×75 mm). A solution without the sample was used as a negative control and synthetic antioxidant BHA was used as positive control. Duplicate vials were prepared for each sample. Each vial was incubated at 40ºC for 14 days in the dark. During the 0, 3, 5, 7, 10, 14th day incubation, 100 µl of each vial was mixed with 75% ethanol (9.7 ml) and 30%

ammonium thiocyanate (100 µl), 0.02 M ferrous chloride (100 µl) was added and the mixture was vigorously shaken. Absor-bance of the generated red color was mea-sured at 500 nm after 3 minutes. Absorbance measurements were done in triplicate and average values were taken to calculate % inhibition by following equation:

$$\% \text{ Inhibition} = [(A_c - A_b)/A_c] \times 100$$

A_c and A_b are absorbance of control at 500 nm and absorbance of sample at 500 nm, respectively.

The data were expressed as mean ± SE of six determinations and statistical analysis was performed according to the unpaired student 't' test.

RESULTS AND DISCUSSION

Extraction from the tea leaves

Petroleum ether extract (1 gm, 1.0%), chloroform extract (1.5 gm, 1.5%) and ethanol extract (10 gm, 10.0%) from the leaves of *Thea sinensis* Linn. were obtained by successive extraction method, respectively.

Phytochemical constituents of Thea sinensis Linn. (tea) leaves

The results of preliminary phytochemical examination are shown in Table 1. It was found that leaves of *Thea sinensis* Linn. contained alkaloids, α-amino acids, basic compounds, flavonoids, phenolic compounds, reducing sugars, saponins, steroids and terpenoids.

Antioxidative activities of crude extracts by thiocyanate method

This method bases on the inhibition of autoxidation of linoleic acid and measures the absorbance at 500 nm after a colouring reaction with ferrous chloride and thiocyanate at intervals during incubation. According to this method, low absorbance that results in high % inhibition indicates high antioxidative activity at constant concentration. The results of antioxidative activity in absorbance and % inhibition (mean ± SE) of

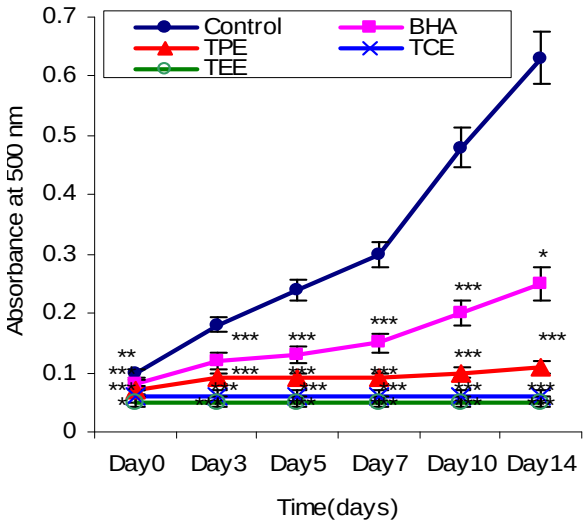
Table 1. Preliminary phytochemical examinations of *Thea sinensis* Linn. samples

No.	Constituent	Extract	Reagent used	Observation	Result
1	Alkaloids	DW	Mayer's reagent	white ppt	+
			Dragendroff's reagent	orange ppt	+
			Sodium picrate	yellow ppt	+
			Wagner's reagent	reddish-brown	+
2	α-amino acids	DW	Ninhydrin reagent	purple color	+
3	Acidic or basic compounds	DW	Bromocresol green	light bluish color	basic com-pound
4	Carbo-hydrates	DW	α-naphthol and conc: H_2SO_4	red ring	-
5	Flavonoids	70% EtOH	Mg turning & conc: HCl	pink color	+
6	Glycosides	DW	10% lead acetate	white ppt	-
7	Phenolic compounds	DW	1% $FeCl_3$ & 1% $K_3Fe(CN)_6$	deep blue	+
8	Reducing sugars	DW	Benedict's solution	red ppt	+
9	Saponins	DW	Distilled water	frothing	+
10	Terpenoids /steroids	P.E	Acetic anhydride & conc: H_2SO_4	greenish color	+
11	Tannins	DW	2% NaCl & 1% Gelatin	white ppt	+

+ =Presence

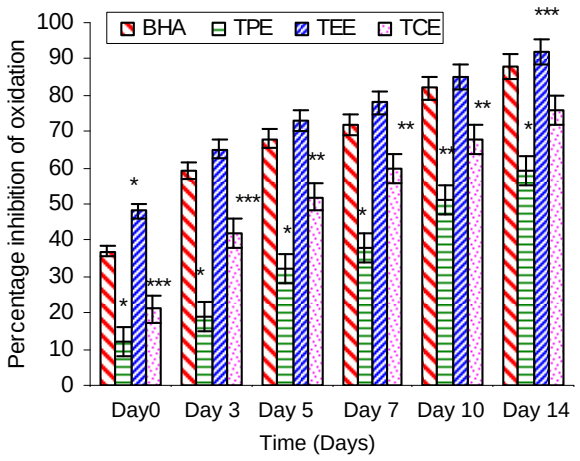
- =Absence

various extracts at various times in days are illustrated in Fig.1 and Fig. 2. It was also found that three extracts had significantly lower absorbance than that of control ($p<0.1$-0.0005) and significant increased % inhibition than that of control ($p<0.1$-0.0005) after 14th day incubation (Table 2). However, while the antioxidative activity of ethanol extract was significantly higher % inhibition than that of BHA, petroleum ether extract and chloroform extract activities were significantly lower % inhibition than that of BHA ($p<0.1$-0.0005) for every reaction time in days at constant concentration (2 mg/ml). Among these three extracts, the antioxidative activity of ethanol extract was higher than that of chloroform extract, which was in turn higher than that of petroleum ether extract, when compared to that of BHA, for every reaction time in days at constant concentration (2 mg/ml). The antioxidative activities of three extracts from the tea



BHA=Butylated hydroxy anisole
TPE=Petroleum ether extract of *T. sinensis* Linn.
TCE=Chloroform extract of *T. sinensis* Linn.
TEE= Ethanol extract of *T. sinensis* Linn.
Concentration (0.02% or 2 mg/ml)
SE=Standard Errors, n=6
Significantly different from control; *p<0.01, **p<0.005, ***p<0.0005

Fig. 1. Antioxidative activities of extracts from tea leaves and BHA on autoxidation of linoleic acid by thiocyanate method



BHA=Butylated hydroxy anisole
PE=Petroleum ether extract of *T. sinensis* Linn.
TCE=Chloroform extract of *T. sinensis* Linn.
TEE=Ethanol extract of *T. sinensis* Linn.
Concentration (0.02% or 2 mg/ml)
SE=Standard Errors, n=6
Significantly different from BHA; *p<0.01, **p<0.005, ***p<0.0005

Fig. 2. Antioxidative activities (in % inhibition) of extracts from tea leaves and BHA on autoxidation of linoleic acid by thiocyanate method

Table 2. Antioxidative activities of extracts from tea leaves as measured by the thiocyanate method after 14th day incubation

Sample[1]	Absorbance at 500 nm	% inhibition[2]
Control	0.619 ± 0.017	0
Butylated hydroxy anisole	0.091 ± 0.003***	85.340 ± 0.445
Petroleum ether extract	0.252 ± 0.007*	59.108 ± 1.410***
Chloroform extract	0.148 ± 0.004**	75.970 ± 1.019***
Ethanol extract	0.080 ± 0.003***	87.055 ±0.529

1=Concentration (0.02% or 2 mg/ml)
2=A high inhibition percent indicates a high antioxidative activity.
Each value represents the mean ± SE (n=6)
Statistically significant; *p<0.01, ** p<0.005, *** p<0.0005

leaves after 14th day incubation are listed in Table 2. These results indicated that ethanol extract showed more antioxidative activity than chloroform extract, which in turn showed more activity than petroleum ether extract. In addition, antioxidative activity of ethanol extract was found to be higher than that of BHA. From the results it was expected that the compounds possessing antioxidative activity may contain in ethanol extract and chloro-form extract, respectively.

Caffeine, from the leaves of tea (*Thea sinensis*) and coffee (*Coffea arabica*) were shown to have antioxidative activity (in a linoleic acid oxidation test) comparable to that of BHA and BHT [16]. Thus, these results were in agreement with results obtained in this present study. The mechanism of their action could be related to their antioxidant function since vascular disorders may be caused by oxidative damage of cell membranes. Polyunsaturated fatty acids, present in cell membranes, are easily oxidized both by enzymatic and autoxidative peroxidation via free radical chain reactions. Lipid peroxidation can be inhibited by flavonoids acting as strong $O_2^{-\circ}$ scavengers and $^1O_2$ quenchers. The autoxidation of linoleic acid leads to the formation of four hydroperoxide isomers: 13-hydroperoxy-9-*cis*, 11-*trans*-octadecadienoic; 9-hydroperoxy-10-*trans*, 12-*cis*-octa-

deca dienoic; 9-hydroperoxy-10-*trans*, 12-*trans*-octadecadienoic acids. The addition of phenolic compounds such as flavonoids partially inhibited the formation of 13-*trans*, *trans* and 9-*trans*, *trans* isomers. The antioxidative activity of flavonoids is related to an inhibition of the formation of *trans*, *trans* hydroperoxide isomers. The inhibition of the formation of *trans*, *trans* isomers by flavonoids showed that these compounds act as H-atom donors to the peroxy radical, thus inhibiting the autoxidation of fatty acids by chain radical termination [17].

The results of this study indicated that tea leaves are indeed a rich source of natural anti-oxidants and have ability to scavenge O_2:-OH and peroxyl radicals. Oxygen-derived free radicals are known to play a significant role in the pathophysiology of many diseases, cardiovascular diseases such as ischemic heart diseases, arrhythmias, stroke, and brain damage; lung injury such as adult respiratory distress syndrome; liver damage; cancer; influenza; malaria and many more [18]. Leaves of tea (*Thea sinensis* Linn.) should be used as "anti-aging herb" or in the formulation of "longivity medicine", owing to their high antioxidative activities.

REFERENCES

1. http://www.bentham.org/cmciema1-1/vaya/vaya-ms.htm
2. http://www.mycustompak.com/healthnotes/supp/Antioxidants
3. Bronen AL.Toxicology and biochemistry of Butylated hydroxy anisole and Butylated hydroxy toluene. *Journal of American Oil Chemistry Society* 1975; 52: 59-63.
4. Tringali C. Bioactive Compounds from Natural Sources: Isolation, Characterisation and Biological Properties, University di Catania, Italy, 2001; 729.
5. Ames BN, Shigenaga MK & Hagen TM. Oxidants, antioxidants and the generative diseases of aging. *Proceedings of Natural Academic Science. USA*, 1993; 90: 7915-7922.
6. Dean RT & Davies MJ. Reactive spicies and their accumulation on radical damaged proteins. *Trends in Biochemical Science* 1993; 18: 437-441.
7. Ceruti P. Oxyradical and cancer. *Lancet* 1994; 344: 862-863.
8. Tutour BL. Antioxidative activities of algal extracts. Synergistic effect with vitamin E. *Phytochemistry* 1990; 29: 3759-3765.
9. Matsuno T. Propolis: Its Pharmacology and Therapeutic Effects, M.P.I. Tokyo, Japan, 1994; 49.
10. Witting LA. Vitamin E and Lipid Antioxidants in Free Radical Initiated Reactions. *Free Radicals in Biology*, New York, Academic Press 1980; 4: 295-319.
11. Carrubba A & Calabrese I. Antioxidant compounds in some herbaceous aromatic plants. *Acta Horticulture* 1998; 457: 85-93.
12. The central council for research in Unani medicine. Physiochemical standards of Unani formulations. Government of India, Nice printing press. New Delhi, part 2 ; 289-292.
13. Hatano *et al*. Effect of the Interaction of Tannins with co-existing Substances. VI. Effect of Tannins and Related Polyphenols on Superoxide Anion Radical. *Chemical Pharmacology Bulletin* 1989; 37: 2016-2021.
14. Sekiwa Y, Kubota K & Kobayashi A. Isolation of Novel Glucosides Related to Gingerdiol from Ginger and their Antioxidative Activities. *Journal of Agriculture and Food Chemistry* 2000; 48: 373-377.
15. Shrififar F, Yassa N & Shafiee A. Antioxidant Activity of *Otostegia persica* (Labiatae) and its constituents. *Iranian Journal of Pharmaceutical Research* 2003; 235-239.
16. Larson RA. Review Article Number 30. The antioxidants of higher plants. *Phytochemistry* 1988; 27(4): 969-978.
17. Torel J, Cillard J & Cillard P. Antioxidant activity of flavonoids and reactivity with peroxy radical 1986; 25(2): 383-385.
18. Mukhopadhyay SN & Das DK. Oxygen responses, reactivities, and measurement in biosystems 1994, CRC press, Florida.