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Evaluation of toxicity and antioxidant activities of the crude leaf extracts of *Cnidoscolus chayamansa*

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Abstract. The crude ethanol extract (CcE) of the leaves of *C. chayamansa* was partitioned to obtain crude hexane (CcH), chloroform (CcC), and aqueous (CcA) extracts. The decoction (CcD) of *C. chayamansa* leaf sample was also prepared. The bioactivities of these extracts were tested using brine shrimp lethality test (BSLT) and various *in vitro* antioxidant assays (phosphomolybdenum method, DPPH radical scavenging assay, inhibition of linoleic acid peroxidation using Ferric Thiocyanate assay, and test for the total phenolic contents using Folin-Ciocalteu method). The CcD was the most toxic extract against *Artemia salina* with LC_{50} of 316.2 ppm. The CcD and CcA exhibited good antiradical activities (44.5% for CcD and 51.2% for CcA) which can be validated by their high total phenolic contents of 66.8 gallic acid equivalence (GAE) and 50.4 GAE, respectively. The extract CcC exhibited the highest total antioxidant activity (79.7 ppm ascorbic acid equivalents and 79.0 ppm butylated hydroxytoluene equivalents). The results can provide additional scientific confirmation for the traditional use of *C. chayamansa* as medicine or for being a nutritious vegetable and can guide the isolation and characterization of the bioactive components.
Key Words: *C. chayamansa*, *in vitro*, DPPH radical, total antioxidant, total phenolics.

Introduction. The leafy perennial shrub *Cnidoscolus chayamansa* is one of the significant species of Euphorbiaceae family that grows naturally in thickets and open forests. This plant is originated by the Maya in Southeast Mexico's dry topics in the Yucatan peninsula and only recently distributed to other areas in post-conquest times (Kolterman et al 1984; Martin & Ruberte 1978). This leafy vegetable was consumed by Mayan Indians and is traditionally incorporated in salads as regional dishes (Martin et al 1977). *C. chayamansa* or commonly known as Chaya in Central America is an outstanding vegetable especially in Mexico City that offers greater amounts of nutrients than other leafy green vegetables (Ranhotra et al 1998; Kuti & Kuti 1999). The plant is also called spinach tree and is rich in essential amino acid, vitamins and minerals (Booth et al 1992; Booth et al 1993; Yang 1979). The leaves of this plant contain undetermined amounts of cyanogenic glycosides which can be reduced or removed through heat treatment thus minimizing the risk of poisoning (Martin & Ruberte 1978). This nutritious plant has also been used as traditional medicine for diabetes, rheumatism, gastrointestinal disorders, and inflammation-related diseases (Kuti & Torres 1996; Berkelaar 2006; Ensen 2012). It is also believed that Chaya cleans the circulatory system, stimulates lactation, improves eyesight, strengthens nails, improves digestion, and is a diuretic and laxative agent (Diaz Bolio 1974). Several studies have been conducted to establish scientific basis of its folkloric uses. One of these studies focused on the anti- hyperglycemic property of *C. chayamansa* wherein the ethanolic extract of Chaya at high dose (250 mg kg^{-1}) and low dose (100 mg kg^{-1}) exhibited significant anti-hyperglycemic activity in normal and alloxan- diabetic rats (Pillai et al 2012). In addition, Yakubu et al (2008) reported that Chaya is capable to induce hormonal imbalance or disorders such as infertility and contraception in hormone- dependent organs like the ovary and mammary glands. Moreover, the phytochemical analysis of Chaya revealed the

presence of various phytochemicals (alkaloids, flavonoids, and saponins) that shown to produce sedation and prolong sleeping time in mice (Sánchez-Jiménez & Estrada-Lugo 1989; Adebiyi et al 2012).

The toxicity of extracts and substances can be primarily assessed using brine shrimp lethality test (BSLT) in which the biological responses to monitor is lethality to *Artemia salina* Leach, the test organism. This test also can be used as a preliminary screening for the presence of pharmacologically active extracts (Montanher et al 2002). Another prominent method in assessing the bioactivity of plants is to examine the antioxidants contained in plants. Antioxidants are compounds that inhibit the oxidation of an oxidizable substrate in a chain reaction and balance the production of reactive oxygen species (ROS) (Jamuna et al 2011). The *in vitro* antioxidant activities of the crude extracts of *C. chayamansa* were evaluated using the phosphomolybdenum method, DPPH radical scavenging activity, ferric thiocyanate method, and the total phenolic contents-Folin Ciocalteu method. Using different methods is logical since this approach offers a more comprehensive picture of the mechanisms of the extracts involved in the oxidation processes.

This study was pursued to bring additional knowledge on the ability of *C. chayamansa* as one of the significant medicinal plants and to compare the bioactivities of the crude leaf extracts of *C. chayamansa* by evaluating their toxicities and antioxidant activities.

Material and Method

Sample preparation. After field collection and botanical identification of *C. chayamansa*, the leaf samples were cleansed and dried in an oven at temperature of 35-40^{o}C for three days. The *C. chayamansa* leaf samples were percolated with 95% ethanol for another three days. The solution was filtered, concentrated *in vacuo*, and obtained crude ethanol extract labeled as CcE (*Cnidoscolus chayamansa* ethanol extract). Scheme 1 shows the serial partitioning to obtain crude hexane (CcH), chloroform (CcC), and aqueous (CcA) extracts. Fort he decoction, clean leaves were freshly cut into pieces, boiled in adequate amount of distilled water (1:2 ratio) for five min, filtered, cooled, and stored in glass containers. The mixture was labeled as CcD, concentrated and weighed.

Toxicity assay using brine shrimp lethality test. The procedure was carried out, with slight modifications, based on the principle and protocol previously described by various researchers (Meyer et al 1982; McLaughlin et al 1998). In this test, 1000, 500, 100, and 10 ppm concentrations of the extracts were prepared in three replicates. The prepared test solutions were then tested for toxicity test against the brine shrimp *A. salina*. The number of dead and alive nauplii were monitored, counted, and recorded after 6 and 24 hours. The results were then processed using of the Reed-Muench method and the acute and chronic lethal concentrations (LC_{50}) were determined (Miya et al 1973).

Total antioxidant assay using phosphomolybdenum method. The protocol of Prieto et al (1999) was employed for this assay. The five crude leaf extracts of *C. chayamansa* were prepared at 200 ppm concentrations. 300 µL of the prepared solutions was mixed with 3000 µL of phosphomolybdenum reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the mixture were screw-capped and incubated at 95°C for 90 minutes. Then, the absorbance of the cooled solution (at room temperature) was measured at 695 nm. The known antioxidants ascorbic acid and butylated hydroxytoluene (BHT) were used as the standard references. The results were then expressed by ascorbic acid equivalents (AAE) and butylated hydroxytoluence equivalents (BHTE) obtained from their respective calibration curves.

Antioxidant assay using DPPH radical scavenging method. For this assay, the protocol of Lee & Shibamoto (2001) was followed. A 500 ppm concentration solution was prepared for each of the four extracts of *C. chayamansa*. After putting 300 µL of the prepared solutions into screw-capped test tubes, 3000 µL of methanolic solution of 0.1

mM DPPH (1,1-Diphenyl-2-picrylhydrazyl) was added. The mixtures were shaken thoroughly and permitted to stand at room temperature for one hour. Then, the absorbance for each mixture was measured at 517 nm against methanol as a blank in the spectrophotometer. The percent of DPPH decoloration of the samples was calculated according to the formula:

$$\text{Antiradical activity} = \frac{A_{\text{control}} - A_{sample}}{A_{control}} x100$$

where A_{sample} is the absorbance of the extracts of *C. chayamansa* and $A_{control}$ is the absorbance of the methanol as the control. The examination of DPPH radical scavenging activity of the samples was compared with the known antioxidants ascorbic acid and BHT.

Antioxidant assay for the total phenolics content. The protocol of Makkar et al (1993) was employed for this assay. 100 µL of 500 ppm of the five crude leaf extracts of *C. chayamansa* was mixed with 2800 µL of 10% Na_2CO_3 and 100 µL of 2 N Folin-Ciocalteu reagent. The mixture was permitted to stand for 40 minutes and then the absorbance was measured at 725 nm. The results were expressed as gallic acid equivalence (GAE) in milligrams per gram of sample obtained from a standard calibration curve constructed for different concentrations of gallic acid (25-500 mg g^{-1}).

Results and Discussion. The mortality rate of the brine shrimp after 24 h of exposure to various doses of the leaf extracts of *C. chayamansa* and the concentration of the leaf extracts that kills 50% of brine shrimp (LC_{50}) are shown in Table 1.

Table 1

Results of the lethality test of the various leaf extracts of *Cnidoscolus chayamansa*

Extract	*Mortality percent (%)*				*LC_{50} (ppm)*
	1000 ppm	*500 ppm*	*100 ppm*	*10 ppm*	
CcE	61.5	20.0	0.0	0.0	854.1
CcH	50.0	23.5	0.0	0.0	1000.0
CcC	51.4	14.3	1.4	0.0	1000.0
CcA	12.9	1.8	0.0	0.0	>1000.0
CcD	98.1	74.2	0.0	0.0	316.2

CcE - *Cnidoscolus chayamansa* ethanol extract, CcH - crude hexane extract, CcC - chloroform extract, CcA - aqueous extract, CcD – decoction.

Among the five leaf extracts of *C. chayamansa,* its decoction (CcD) showed the largest percent mortality for doses 1000 and 500 ppm after 24 hours of exposure. Whilst the CcA showed the smallest percent mortality at 1000 and 500 ppm so it had the least probability to contain any toxic substances to kill the brine shrimps. For leaf extracts CcE, CcH, and CcC, they had comparable percent mortality at 1000 ppm while the percent mortality at 500 ppm only ranges from 14.3% to 23.5% which is not so significant. At 100 and 10 ppm, almost all of the *C. chayamansa* extracts showed no toxicity against brine shrimps. Thus, the leaf extracts of *C. chayamansa* is not toxic or harmful to brine shrimps when prepared at lower concentration (100 and 10 ppm). It also has been shown in the results that the greater the value of the percent mortality, the lesser is the value of the LC_{50}. Based on the results, it took higher concentrations of the organic leaf extracts of *C. chayamansa* to kill 50% of the brine shrimps. Among the five leaf extracts, CcD has the smallest LC_{50} value of 316.2 ppm and is considered then to be bioactive.

The total antioxidant activity is based on the reduction of Mo (IV) to Mo (V) by the extract. Table 2 shows the total antioxidant activities of the various leaf extracts of *C. chayamansa*.

Table 2

Total antioxidant activities of the leaf extracts of *Cnidoscolus chayamansa* expressed as ascorbic acid equivalents (AAE) and butylated hydroxytoluene equivalents (BHTE)

Extract	*Ascorbic acid equivalence (ppm)*	*BHT equivalence (ppm)*
CcE	54.4	54.8
CcH	61.6	61.7
CcC	79.7	79.0
CcA	57.0	57.3
CcD	54.2	54.6

CcE - *Cnidoscolus chayamansa* ethanol extract, CcH - crude hexane extract, CcC - chloroform extract, CcA - aqueous extract, CcD – decoction.

All of the extracts give more than 50 ppm standard equivalence and CcC gives the highest total antioxidant activity with 79.7 ascorbic acid (AA) and 79.0 BHT equivalents. This suggests that the CcC has the potential comparable antioxidant constituents because potent antioxidants ascorbic acid and BHT have been used as reference standards.

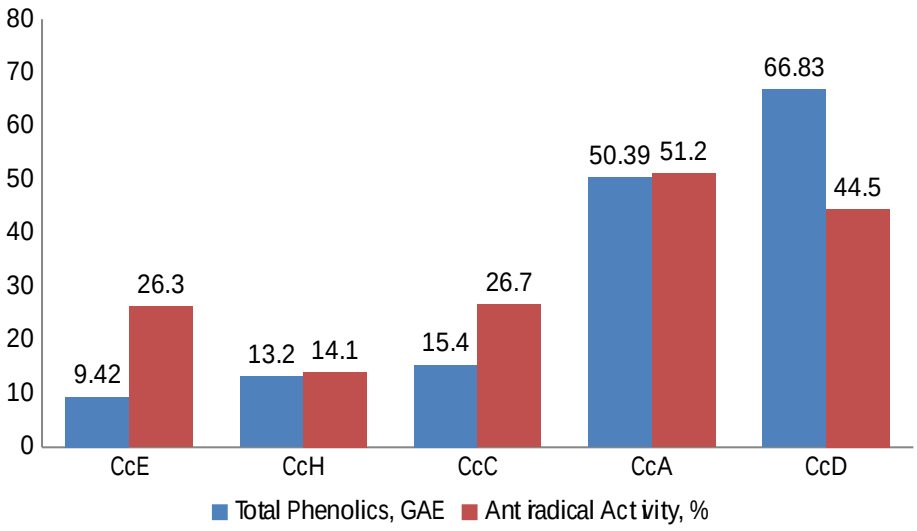

Figure 1. The antiradical activity and estimation of total phenolics content of the leaf extracts of *Cnidoscolus chayamansa*.

The radical scavenging assay is based on the reduction of DPPH, a stable free radical. When a free radical scavenging antioxidant donates an electron or hydrogen to DPPH, the absorption strength is decreased and the resulting decolorization is stoichiometric with respect to the number of electrons captured (Blios 1958). As shown in Figure 1, the radical scavenging activity of the crude leaf extracts of *C. chayamansa* is not significant compared to those of the standards. Compared to the standard references, CcD gives the highest antiradical activity in almost all of the concentrations among the leaf extracts tested and the lowest EC_{50} of 408.1 ppm. This suggests that CcD might contain compounds like polyphenols that can donate electron or hydrogen easily. Polyphenolic compounds have high redox potentials which allow them acts as reducing agents, hydrogen donors and singlet oxygen quenchers (Kahkonen et al 1999). Hence, high phenolics content of these extracts indicates high antioxidant potentials because the phenolics constituents can react with ROS. Moreover, the results as shown in Figure 1

indicate that the polar extracts relatively contain more phenolics than the medium- polar and non- polar extracts. This explains the poor radical scavenging activity of the CcE, CcH, and CcC extracts. Whilst CcA and CcD exhibited good antiradical activity since they give high total phenolic contents with 51.2 GAE and 41.5 GAE, respectively.

Conclusions. The crude leaf extracts of *C. chayamansa* exhibited toxicity and antioxidant activity. Among the five leaf extracts, the polar extracts showed promising bioactivities. The CcD was the most toxic extract against *A. salina* with LC_{50} of 316.2 ppm. The significant antioxidant activity of CcD and CcA is correlated to the estimation of total phenolics. This study supports the traditional use of *C. chayamansa* as effective medicine and signifies the excellent nutritional value of *C. chayamansa* leaves as outstanding vegetable. Further work on the isolation and characterization of the bioactive components especially in the polar extracts needs to be done.

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