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In vitro phytochemical screening, antioxidant activity and cytotoxicity of *Coffea liberica* ethanolic leaf extract

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Abstract
Coffea liberica are small trees, native from the Ethiopian mountains and varieties grow in tropical and sub-tropical areas. It is one of the 6500 species one of the largest plant family, the family Rubiaceae. Coffea plant species are known to carry a bioactive chemical caffeine and and related species are known to be positive in polyphenols and tannin. This study investigates the phytochemical contituents, antioxidant activity and cytotoxicity in one of the Coffea plant species, the *Coffea liberica* leaf in ethanolic extract. Phytochemical screening showed high concentration of alkaloids, flavonoids and saponins. DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity possess poor free radical scavenging activity with LC50 value of 33.48 µg/mL and is somehow dependent to concentration. Cytotoxicity test, using brine shrimp lethality assay, also showed potent and active effects with an LC_{50} value of 74.16ppm. The result shows the present bioactive compounds found in the leaf extract of *Coffea liberica* and its effective anti-oxidative and cytotoxic property. These results are promising and can be a useful reference for future studies especially in the field of medicine and pharmaceuticals.

Keywords: Rubiaceae, free radical, alkaloids, anti-oxidative, brine shrimp

Introduction

Plants are the indispensable storehouse of many chemical metabolites. The medicinal value of plants have assumed a more important dimension in the past two decades owing largely to the discovery that extracts from plants contain many minerals, primary metabolites and secondary metabolites with antioxidant potential (Akinmoladun *et al*., 2007) [4]. Reactive oxygen species, reactive nitrogen species, and free radicals are linked to a variety of pathological problems, including aging of the cells, inflammation, metabolic abnormalities, and cancer (Robak *et al*., 1998 [37]; Ames *et al*., 1993) [6].

The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and protective/disease preventive properties (phytochemicals). These phytochemicals, often secondary metabolites present in smaller quantities in higher plants, include the alkaloids, steroids, flavonoids, terpenoids, tannins, and many others (Peteros and Uy, 2010) [33]. Coffee has long been used to cure a variety of conditions, including vertigo, fever, headache, jaundice, asthma, atropine poisoning, migraine, narcosis, ulcers, and malaria. Coffee is said to have several good health qualities, including diuretic, antioxidant, and antibacterial effects. It has been observed that components with antioxidant and antibacterial properties include caffiene, chlorgenic acid, caffeic acids, condensed pro anthocyanidins, quinic acid, and ferulic acid (Nayeem *et al*., 2011) [28].

Small trees called *Coffea liberica* are native to the Ethiopian highlands. Varieties of the plant occur in tropical and subtropical regions near the Equator. They belong to the Rubiaceae family, which is the largest plant family in the world with 450 genus and 6500 species species (Patay *et. al*., 2016) [31]. Coffea plant species are considered small shrubs which can be up to 8 meters tall and have simple leaves that are opposite or sometimes whorled (https://sca.coffee/research/botany/). The Coffea plant species are widely popular worldwide due to the production of coffee beans that is being enjoyed by millions of people all throughout the world. Coffea plant species carries bioactive chemical caffeine that is a member of the alkaloid group (Rao and Nadunmane, 2015) [35]. Also, an experiment conducted by Patay and colleagues (2017) [30] for her dissertation on Coffea plant species further shows other positive polyphenols and tannin in their results from phytochemical and antioxidant screening. A study conducted by Rao and Nadunmane (2015) [35] provided result on the possible anti-cancer property of *C. robusta* and *C. arabica*. Results from the study showed positive cytotoxic effect of the extracts of two Coffea plants species to Hela and PA-1 cell lines.

The medicinal properties of plants have been investigated, in the light of recent scientific developments, throughout the world due to their potent pharmacological activities and economic viability (Abass *et al*., 2015) [2]. Thus, the aim of the present work was to evaluate the phytochemical composition, antioxidant properties and cytotoxicity of the leaf extract of *Coffea liberica*.

Materials and methods

Plant selection

Criteria for the selection of plants were based on published articles, personal interviews and documentation of various medicinal plants used by traditional healers. Collected plant was be subjected for identification and was be confirmed by a Botanist in the Department of Biological Sciences, MSU-Iligan Institute of Technology, Iligan City, Lanao del Norte, Philippines.

Collection of plant samples

Coffea liberica (leaves) plant parts were collected from Barangay Inagongan, Municipality of Tagoloan, Lanao del Norte, Philippines (Fig.1). Collection of plants samples followed local government protocol, while sampling

certificate was applied from the government's agency Department of Natural Resources (DENR). Initial identification was prepared for botanical authentication by a Botanist at the Department of Biological Sciences, College of Science and Mathematics, MSU -IIT, Iligan City, Philippines

Fig 1: Map of the sampling site, Barangay Inangonan, Municipality of Tagoloan, Lanao del Norte

Preparation of plant parts extracts

Ethanolic crude extract

In the preparation of the ethanolic crude extract, the following methods was followed: fresh selected plant parts was cleaned and washed off of any foreign matter. Plants parts was placed in shade until totally dried for 2-3 weeks. When the plants were dried it was macerated into fine pieces by the used of blender. Finely macerated parts was soaked with 100% absolute ethanol with a ratio of 1g: 3ml and was stored in a dark place for 3-7 days. Extract was filtered with filter paper Whatman no. 1. Ethanolic extract was then subjected for rotary evaporation to acquire crude extracts.

Phytochemical screening

The ethanolic leaf extract were screened for phyto-constituents using standard procedures as described by Sheel *et al*. 2014 [38].

DPPH radical scavenging activity

By measuring the UV absorbance at 517 nm, the radical scavenging ability of the *Coffea liberica* leaf extract against 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was ascertained. The method was carried out as described by Nayeem *et al*. 2011 and Shekhar and Anju, 2014 [39], with slight modifications. The working solutions (500 µg/mL, 300 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 30 µg/mL, 20 µg/mL, 10 µg/mL) of the leaf extracts were made from the stock solution by making appropriate dilutions. The DPPH was prepared as 0.002% solution in ethanol and mixed with 1ml of both the standard and the samples. Using a UV-VIS Shimadzu spectrophotometer, the produced solutions were exposed to a ½ hour of darkness before the absorbance at 517nm was measured. By utilizing the Log dosage inhibition curve, the IC_{50} value of the sample—the concentration of the sample needed to inhibit 50% of the DPPH free radical—was determined. There was more free radical activity in the reaction mixture when its absorbance was lower. The percent DPPH scavenging effect was calculated by using following equation:

$$\% \text{ DPPH scavenging activity} = (A_{control} - A_{sample} / A_{control}) \times 100$$

Where, $A_{control}$ and A_{sample} are the absorbance values of the test and of the blank sample, respectively.

Brine shrimp lethality assay

The procedure was based according to the principle and protocol previously described by Meyer *et al*. (1982) [26] with slight modifications. Brine shrimp eggs were hatched in rectangular dish filled with artificial sea water prepared with commercial salt mixture. About 500 milligrams of the crude extracts was dissolved with 5 ml of ethanol to prepare 10 000 ppm stock solutions added with few drops of dimethyl sulfoxide (DMSO) to aid in dissolution. A volume of 5 µl, 50 µl, 250 µl and 500 µl was pipetted out from the stock solution to previously cleaned and dried test tubes labeled 10 ppm, 100 ppm, 500 ppm and 1,000 ppm respectively. It was then diluted with sterilized sea water to produce the required concentrations. Three replicates was prepared for each concentration.

A small plastic container was used for hatching the brine shrimp eggs with partition for smaller (illuminated) and larger (darkened) areas. The eggs was sprinkled into larger area which was darkened, while the smaller area was illuminated. After 48 hours, nauplii larvae from the lighted side was collected by pipette and transferred to the vials for treatments of different concentrations for the extracts. The number of surviving shrimps was counted and recorded after 24 hours. The percentage mortality (% mortality) was calculated using the formula below:

$$\% \text{ mortality} = (\text{no. of dead nauplii} / \text{initial no. of live nauplli}) \times 100$$

Plant extracts with an LC_{50} value of less than 100 ppm was considered as potent or active (Peteros and Uy, 2010). LC_{50} value of less than 1000 µg/ml is toxic while LC_{50} value of greater than 1000 µg/ml is non-toxic (Meyer *et al*., 1982).

Results and discussions

Phytochemical screening of leaf extract of *Coffea liberica*

Phytochemical screening of the leaf extract of *Coffea liberica* revealed the presence of alkaloids, cyanogenic glycosides, flavonoids, saponins, steroids and tannins (Table 1). Alkaloids, flavonoids and saponins were present at high concentration in the leaf extract of *Coffea liberica*. The medicinal values of the plant leaves may be related to their constituent phytochemicals (Agbafor and Nwachukwu, 2011) [3]. According to Varadarajan *et al.* (2008) [41], the secondary metabolites (phytochemicals) and other chemical constituents of medicinal plants account for their medicinal value.

Alkaloids that are derived from medicinal plants have been reported that these compounds show biological activities like, anti-inflammatory (Augusto *et al*., 2011) [8] antimalarial (Dua *et al*., 2013) [14], antimicrobial (Benbott *et al*., 2012) [10], cytotoxicity, antispasmodic and pharmacological effects (Ameyaw and Duker-Eshun, 2009 [7]; Thite *et al*., 2013) [40]. Flavonoids are naturally occurring

plant components with possible biological functions, such as antioxidant capabilities (Jiangseubchatveera *et al*., 2017) [20] and since they have been linked to benefits in a wide range of illness states, they are regarded as a prospective source for therapeutic development (Havsteen, 2002) [16], such as anticancer, antioxidant, and anti-inflammatory (Pandey *et al*., 2009 [29] and Rathee *et al*., 2009) [36]. Naturally occurring surface-active glycosides, saponins are mostly made by plants (Marrelli *et al*., 2016) [25] are also well known for their association with cytotoxic, antitumor and anti-inflammatory activity (Perrone *et al*., 2005) [32]. If administered intravenously, saponins may be harmful (Milgate *et al*., 1995) [27] and depending on the kind of aglycone and sugar chains, these substances are recognized for their hemolytic action on human erythrocytes. This property is due to the interaction with sterols present in the erythrocyte membrane, which lead to an increase of membrane permeability and the consequent loss of haemoglobin (Baumann *et al*., 2000) [9]. Many pharmacological properties, such as antifungal, insecticidal, anthelmintic, cytotoxic, anti-inflammatory, immunostimulant, hypocholesterolemic and hypoglycemic, have been ascribed to these compounds (Francis *et al*., 2002). Interestingly, this class of phytochemicals has been also investigated for its potential anti-diabetic properties, with the aim to find new effective drugs in the treatment of diabetes mellitus. Saponins' hypoglycemic effects appear to be caused by a variety of processes, including the activation of insulin production from the pancreas, the restoration of the insulin response, and an increase in plasma insulin levels (Elekofehinti *et al*., 2015) [15].

Table 1: Phytochemical screening of the crude extract of leaves of *C. liberica*

Phytochemical	*Coffea Liberica* (Leaves)
Alkaloids	+++
Anthraquinones	-
Cyanogenic Glycosides	+
Flavonoids	+++
Saponins	+++
Steroids	+
Tannins	+

* (+++) Present in high concentration; (++) Present in moderate concentration; (+) Present in low concentration;
(-) Absent

Three phytochemicals compounds were also present in the leaf extract of *Coffea liberica*: cyanogenic glycosides, steroids and tannins. Foods that contain chemical components called cyanogenic glycosides produce hydrogen cyanide after chewing or digestion. The capacity of cyanogenic glycosides to hydrolyze either spontaneously or with the aid of an enzyme to create cyanide as a byproduct of the hydrolysis process is linked to their toxicity (Bolarinwa *et al*., 2016) [11]. It is well known that plant-based steroids have cardiotonic effects in addition to antibacterial and insecticidal qualities (Alexei *et al*., 2009) [5]. Because of their well-known biological properties, they are frequently employed in pharmaceuticals (Iqbal *et al*., 2015) [19]. For tannins, according to research, they are known to have antibacterial (Hisanori *et al*., 2001), antitumor and antiviral activities (Kumari and Jain, 2012) [23]. They function by precipitating microbial protein, which prevents them from accessing nutritious protein. Tannins are also well known for their astringent properties (Zucker *et al*., 1983) [43] and they also promote formation of tissues on wounds and also used in case of varicose veins (Pravin *et al*., 2012) [34]. The present of these compound in the plant extract, as evident from phytochemical screening, may be responsible for the antioxidant action (Hossain *et al*., 2013) [18].

DPPH radical scavenging activity of leaf extract of *Coffea liberica*

The DPPH test showed the ability of the test compound to act as a free radical scavenger. DPPH assay method is based on the ability of 1, 1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants (Kumarasamy *et al*., 2007) [24]. This characteristic is frequently used to assess how well natural antioxidants scavenge free radicals (Jao *et al*., 2002) [21]. Basically, a higher DPPH radical-scavenging activity is associated with a lower LC50 value (Jothy *et al*., 2011) [22]. Thus, in our results the leaf extracts of *C. liberica* possess poor free radical scavenging activity with LC50 value of 33.48 µg/mL (Table 2). According to de Vargas *et al*. (2016) [13], samples only exhibiting LC50 < 10 µg/mL are considered very active antioxidants as they have the potential comparable to the antioxidant standards of quercertin, β-carotene, ascorbic acid, gallic acid and Trolox® and the plant extracts exhibiting the greatest antioxidant potential were those with the highest levels of total polyphenols. Flavonoids exhibit their antioxidative qualities through multiple methods, including the scavenging of free radicals, the chelation of metal ions like copper and iron, and the suppression of enzymes that generate free radicals. Additionally, reports exist about the function of flavonoids, which are potent antioxidants (Brown and Rice-Evans, 1998 [12] and Vinson *et al*., 1995) [42].

Table 2: DPPH radical scavenging assay of the leaves extracts of *C. liberica*

% Inhibition at Different Concentration	Control	0.00±0.01
	10µg/ml	18.10±0.01
	20µg/ml	30.19±0.01
	30µg/ml	45.61±0.01
	50µg/ml	70.73±0.01
	100µg/ml	95.38±0.00
	200µg/ml	95.57±0.00
	300µg/ml	95.48±0.00
	500µg/ml	96.31±0.00
IC_{50}µg/mL		33.48

*Mean ± SD

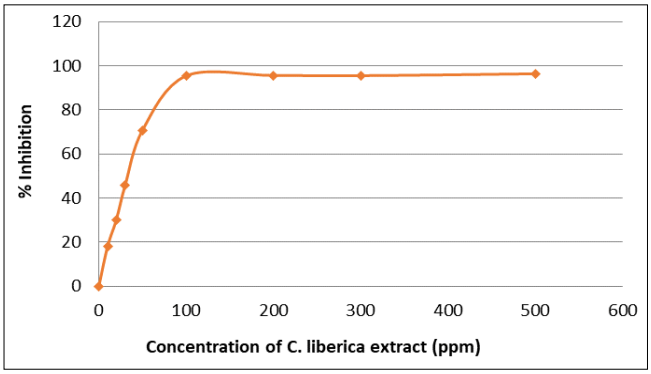

Fig 2: Plot of % inhibition vs. concentration in ppm of *C. liberica* extract

Brine shrimp lethality assay of *Coffea liberica* leaf extract

To test the toxicity of the *C. liberica* leaf extract, 3 level of increasing concentrations (10, 100 and 1000ppm) and a control were prepared and was exposed to the brine shrimp nauplii. Accordingly, as shown in the graph (Figure 2), average mortality increases as the concentration of the extract increases. Probit analysis revealed a percentage of 6.13 in relation to the response of log dose of *C. liberica* leaf extract (Table 3). The LC_{50} value is 74.16ppm, which means 50% of the nauplii population will die at 74.16ppm. According to Peteros and Uy (2010), plant extracts with an LC_{50} value of less than 100 ppm are considered as potent or active.

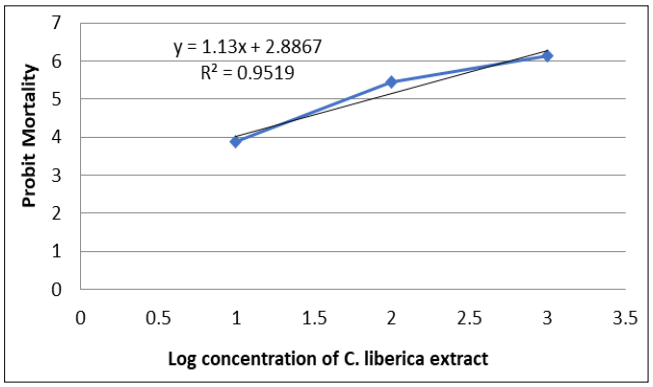

Fig: 3 Plot of probit mortality and log concentration of *C. liberica* extract

Table 3. Values of % mortality and probit mortality on different concentrations of *C. liberica* extract and the LC_{50} value

Concentration	% Mortality	Probit Mortality
Control	0.00	
10ppm	13.00	3.87
100ppm	67.00	5.44
1000 ppm	87.00	6.13
LC_{50} Value	74.16ppm	

Conclusion and recommendation

The result shows the present bioactive compounds found in the leaf extract of *Coffea liberica* and its effective anti-oxidative and cytotoxic property. This provenly show the possible medicinal quality of this plant species. With possible more elaborate analysis such as GC-MS or LC-MS, to determine and quantify all possible bioactive compounds present within the plant species, will thus, be solidifying basis for future pharmacological study for this plant species such as antiproliferative, anticancer and antidiabetic, etc.

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