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MEDICINAL PLANTS

ANTIOXIDANT, CYTOTOXIC, LARVICIDAL, AND ANTHELMINTIC ACTIVITY AND PHYTOCHEMICAL SCREENING BY HPLC OF *Calicotome villosa* FROM TURKEY

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Phytochemical screening of *Calicotome villosa* ethanolic extracts in respect of phenolic compounds (HPLC method), antioxidant activity (DPPH and β-carotene tests), determination of total phenolic and total flavonoid contents, and evaluation of cytotoxic (against *Artemia salina*), larvicidal (against *Culex pipiens* and *Musca domestica*) and anthelmintic activity (against *Tubifex tubifex*) have been performed. The flower extract exhibited higher biological activity than the stem extracts in all assays (DPPH, 0.6 mg/mL, IC_{50}, β-carotene, 75.12 ± 0.73 %). There was good correlation between the antioxidant activity and total phenolic and total flavonoid contents. The flower extract exhibited significant cytotoxic activity (against *A. salina*) with 0.312 mg/mL, LC_{50} larvicidal activity (against *Cx. pipiens*) with 0.330 mg/mL, LC_{50} and anthelmintic activity (against *T. tubifex*) with 1.32 mg/mL, LC_{50}. HPLC analysis showed that vanillic acid was major component in the flower extract. In conclusion, *C. villosa* has good biological activity for further studies in agriculture, medicine and pesticide industry.

Keywords: *Calicotome villosa*; HPLC analysis; antioxidant activity; larvicidal activity; cytotoxic activity; anthelmintic activity.

1. INTRODUCTION

Reactive oxygen species (ROS) as free radicals are produced in reactions involved in the metabolism of aerobic organisms and play a role in a wide variety of diseases including cancer, diabetes, AIDS[1]. The struggle against ROS is vital in preventing these diseases and one of critical defense mechanisms is based on antioxidants. These substances inhibit the oxidative stress caused by ROS, and especially dietary antioxidants are essential to protect the human body [2 – 4]. The best known dietary antioxidant compounds are vitamins E and C, polyphenols [5]. Synthetic antioxidants are widely used in foods. However, some side effects of synthetic antioxidants have been reported [6]. For this reason, searching for natural antioxidants is necessary to get rid of the side effects of synthetic antioxidants. Plants are the primary source of antioxidants. In addition to their antioxidant properties, they have many different biological activities including larvicidal effects.

Synthetic chemical larvicides are applied to control insects in many parts of the world. However, many of these chemicals are toxic to human, plant and animal life. If the insect gains resistance to this chemistry, then the struggle becomes a problem. The natural substances obtained from plants used as insecticides for larval control continue to be extensively investigated [7]. In recent years, it has been reported that the use of synthetic pesticides is significantly reduced due to increased use of natural compounds (alkaloids, glycosides, volatile oils) in agricultural areas. As a result, natural insecticides are considered safer than synthetic pesticides because they are very quickly degradable and have low toxicity for organisms [8].

The genus *Calicotome* belonging to the family of Fabaceae has five species around the world [9]. There is only one species growing in Turkey, *Calicotome villosa* (Poir.) Link [10, 11]. In addition to being used as an antitumor remedy, *C. villosa* is also used by Sicilian people for treating furuncle, cutaneous abscess and chilblains diseases [12]. Earlier works by Loy, et al.[13] and Dessí et al.[14] reported antioxi-

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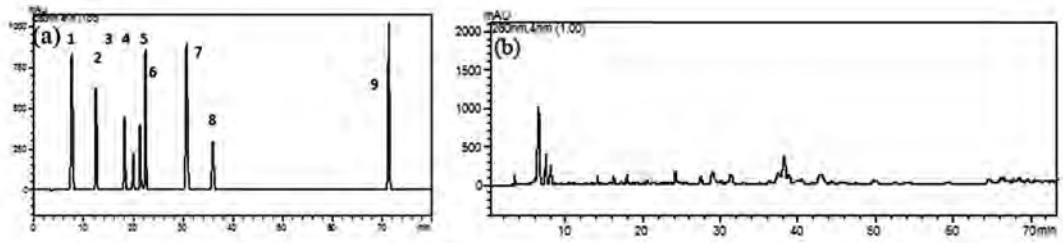

Fig. 1. Chromatograms of (a) standard solution and (b) sample of *C. villosa* extract: (**1**) gallic acid; (**2**) 3,4-dihydroxy benzoic acid; (**3**) 4-hydroxybenzoic acid; (**4**) chlorogenic acid; (**5**) vanillic acid; (**6**) caffeic acid; (**7**) *p*-coumaric acid; (**8**) ferulic acid; (**9** cinnamic acid.

dant, cytotoxic and antimicrobial effects of alcoholic extracts of *C. villosa*. More recent studied reported that *C. villosa* has constituents such as flavones, isoflavones, alkaloids and triterpenes [15, 16].

The present study aimed to define the antioxidant, cytotoxic, larvicidal, and anthelmintic activity of ethanolic extracts from flowers and stems of *C. villosa*. This is the first study to report on the phytochemical screening, cytotoxic activity against *A. salina* L., larvicidal activity against *M. domestica* L. and *Cx. pipiens* L., and anthelmintic activity against *T. tubifex* Müller for the extracts of *C. villosa*, and this study will shed light on future medical studies for this plant.

2. MATERIALS AND METHODS

2.1. Plant Materials and Extraction

Different parts (flowers and stems) of *C. villosa* were collected during the flowering season at Marmaris, Muğla province, Turkey in April 2014. Dr. Olcay Düşen identified the plant sample, and voucher specimens were deposited in Pamukkale University Herbarium (PAMUH) under herbarium number of 2847. Flowers and stems were dried in the shadow at low humidity and room temperature. Dried materials were chopped with blender and then the samples and ethanol (1:10) were put into 250 mL Erlenmeyer flasks. Erlenmeyers were placed in a shaker water bath (Memmert WNB 22) for 6 h at 49 – 50°C. After 6 h, the extraction mixture was filtered with filter paper. This procedure was repeated twice in the same way. The solvent was removed in a rotary evaporator (Ika RV 10) at 50 – 51°C. Water inside extracts was frozen at –80°C and were removed in freeze-dryer (Labconco Freezone 6) at -54°C. Extracts were stored in a refrigerator at –20°C [17].

2.2. Assay of DPPH Free Radical Scavenging Activity

The radical scavenging antioxidant activity of *C. villosa* extracts was assessed with DPPH (2,2-diphenyl-1-picrylhydrazyl) test as described by Wu, et al. [18]. According to this method, 0.004 g DPPH was mixed with 100 mL methanol for DPPH solution. Extracts (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) and BHA (for positive control) were mixed with ethanol in each test tubes. Then, 4 mL DPPH solution was added to each test tube and 1 mL methanol was mixed with 4 mL DPPH solution for negative control. After 30 min exposure, a decrease in the absorbance at 517 nm was measured using a spectrophotometer and the percentage scavenging activity (SA) was calculated using the following formula:

$$SA\% = [(A_c - A_e)/A_c] \times 100, \quad (1)$$

where A_c is the absorbance of the negative control and A_e is the absorbance of sample.

2.3 Assay of β*-Carotene-Linoleic Acid Antioxidant Activity*

The test for β-carotene-linoleic acid antioxidant activity was performed according to Amin, et al. [19]. The stock solution of β-carotene was prepared in concentration of 0.2 mg/mL in chloroform. Then, 1 mL stock solution was appropriately mixed with linoleic acid (40 μL) and 400 μL of Tween 20 in a beaker, chloroform was evaporated, and 100 mL of distilled water was added. The emulsion (4.8 mL) was mixed with 0.2 mg of the sample, and then the absorbance was measured at 470 nm in a spectrophotometer to determine $A0_a$ (sample) and $A0_b$ (control). The beaker was incubated for 2 h at 50°C and then the absorbance was measured as $A2_a$ (sample) and $A2_b$ (control) with BHA used for positive control. The percentage antioxidant activity (AA) of β-carotene-linoleic acid was calculated using the following formula:

$$AA\% = [1 - (A0_a - A0_b/A2_a - A2_b)] \times 100. \quad (2)$$

2.4. Determination of Total Phenolic Content

Total phenolic content (TPC) was determined using the the Folin-Ciocalteu reagent (FCR) method according to Slinkard, et al. [20] and gallic acid was used as a standard for the calibration curve. 46 mL of distilled water and 1 mg/mL of extract solution were mixed with 1 mL FCR. Then, 3 mL of sodium carbonate (2%) was added to the mixture in 3 min and, after 2 h incubation at room temperature, the absorbance at 760 nm was measured and the TPC was expressed in units of gallic acid equivalent (mg GAE/g extract).

TABLE 1. Antioxidant Activity and Total Phenolic and Flavonoid Contents of *C. villosa*

Extract and standard	DPPH[a]	β-Carotene[b]	TPC[c]	TFC[d]
Flower part	86.34 ± 0.16 & 0.6	75.12 ± 0.73	159.47 ± 0.33	66.21 ± 0.09
Stem part	70.09 ± 0.10 & 0.76	60.05 ± 0.23	109.67 ± 0.26	18.41 ± 0.02
BHA	89.46 ± 0.11 & 0.34	89.25 ± 0.33	-	-

[a] DPPH Inhibition (%) at 1 mg/mL, IC_{50} (mg/mL); [b] β-carotene inhibition (%) at 40 mg/mL;
[c] total phenolic content (TPC) expressed in gallic acid equivalents (mg GAE/g extract);
[d] total flavonoid content (TFC) expressed in quercetin equivalents (mg QEs/g extract).

2.5. Determination of Total Flavonoid Content

The total flavonoid content (TFC) in *C. villosa* extract was determined as described by Arvouet-Grand, et al. [21] and expressed in quercetin equivalents (mg QEs/g extract). $AlCl_3$ solution prepared in 1.0 mL of 2.0% methanol was added to test tubes containing 1.0 mL extract solution and incubated at room temperature for 10 min. The blank sample contains 1.0 mL of methanol. Absorbance measurements were performed at 415 nm.

2.6. Phytochemical Screening of Phenolic Compounds with HPLC

Phenolic compounds were analyzed according to the modified method of Caponio, et al. [22] using a reversed phase HPLC system equipped with a UV–VIS Photodiode-Array Detector (SPD-M20A). The mobile phases were solvent A (3.0% formic acid in distilled water) and solvent B (methanol). Samples (0.2 g) of *C. villosa* flower extract were dissolved in the mobile phase. Details of the mobile phase gradient conditions were given in our previous report [17]. The number of phenolic compounds in a sample was determined according to the calibration curve constructed for the same analysis conditions.

2.7. Assay of Cytotoxic Activity against Brine Shrimp (Artemia salina L.*)*

The brine shrimp *(A. salina*) cytotoxicity of the extracts was determined using the method of Krishnaraju, et al. [23]. According to this, *A. salina* eggs (10 mg) were incubated in 500 mL artificial seawater at 28°C for 48 h. After incubation, 10 nauplii were added to each vial containing 4.5 mL of brine solution and 0.5 mL of four concentrations (0.1, 0.25, 0.5, and 1 mg/mL) of extract were added to brine solution, respectively. The mixtures were incubated for 24 h at 28°C. Then, dead nauplii were recorded under the light.

2.8. Assay of Larvicidal Activity against. House Flies (Musca domestica L.*) Larvae*

The larvicidal effect of *C. villosa* extracts on houseflies (*M. domestica*) was studied according to the Çetin, et al. [24] with modified feeding method. In this study*, M. domestica* was used and cultured with milk and sugar in the mixture was prepared as 1:3 and 50 g. Two concentrations (1 and 5 mg/mL) of extracts were prepared with 20 mL milk in beakers and transferred to food containers. Twenty-five housefly larvae were taken from their eggs and transferred to their food containers. After 24 – 36 h, the eggs started to open and the larvae emerged. The larvae were expected to become adult during three weeks and the adult ones were recorded. The larvicidal effect was carried out in 12:12 (L:D) photoperiod at 25 – 26°C and 50 – 60% humidity in a laboratory environment.

2.9. Assay of Larvicidal Activity against Mosquito (Culex pipiens L.*) Larvae*

Larvicidal activity of *C. villosa* extracts against mosquito (*Cx. pipiens*) larvae was investigated according to the method of Çetin, et al. [7]. Four concentrations (0.1, 0.25, 0.5, and 1 mg/mL) of extracts were prepared in 100 mL distilled water in beakers. Ten larvae were transferred to beakers and fish food was given to the larvae. The experiment was carried out in 12:12 (L:D) photoperiod at 25 – 26°C, and

TABLE 2. Content (μg/g) and Retention Times of Standard Phenolic Compounds in the Extract of *C. villosa* Flowers

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
TPL content (μg/g)	110.18	41.04	399.00	392.16	1403.36	80.28	99.70	96.33	313.59
Retention time (min)	7.8	12.2	18.1	19.9	22.1	23	30.3	35.7	71.1

(**1**) Gallic acid; (**2**) 3,4-dihydroxy benzoic acid; (**3**) 4-hydroxybenzoic acid; (**4**) chlorogenic acid; (**5**) vanillic acid; (**6**) caffeic acid; (**7**) *p*-coumaric acid; (**8**) ferulic acid; (**9**) cinnamic acid.

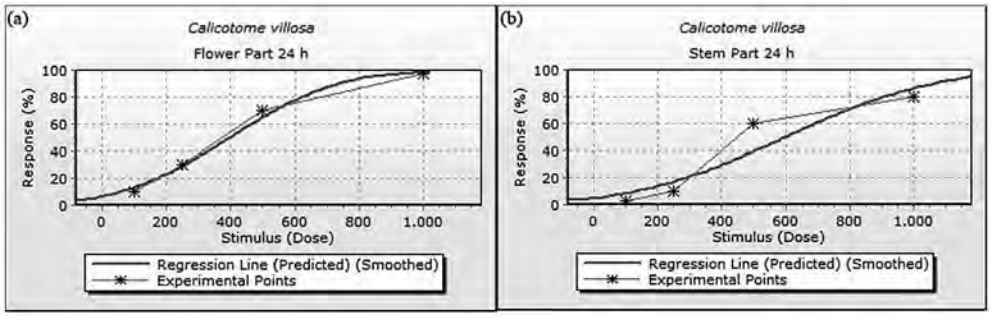

Fig. 2. Graphs of 24-h percentage mortality for (a) flower part and (b) stem part of *C. villosa* in brine shrimp assay.

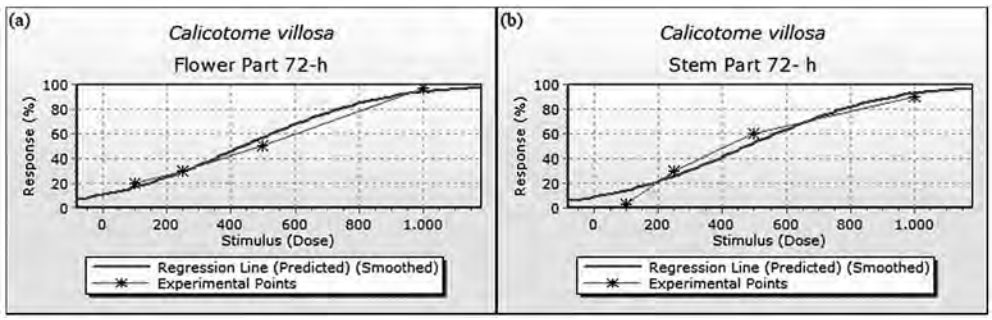

Fig. 3. Plots of 72-h percentage mortality of (a) flower part and (b) stem part (b) of *C. villosa* against *Cx. pipiens* larvae.

50 – 60% humidity. After 24-, 48-, and 72-h exposure, dead larvae were recorded.

2.10. Assay of Anthelmintic (against Tubifex tubifex Müller*) Activity*

The anthelmintic activity of *C. villosa* extracts was determined using a modified method of Ajaiyeoba, et al. [25]. Ten *T. tubifex* of nearly 2 – 3 cm size in each group were taken for the experiment. Six concentrations of extracts in ethanol (1, 2.5, 5, 10, 20, and 40 mg/mL) were mixed with 20 mL distilled water in petri dishes and distilled water was used as negative control. Each petri dish was contained 10 helminths; dead helminths were recorded upon 2-, 4-, and 6-min exposure in room temperature.

TABLE 3. Average Mortality Rates (%) of *C. villosa* Extract Concentrations in Preset Time of Exposure to *A. salina* in Brine Shrimp Assay and Statistical Data

Concentration	Flower part 24 h later	Stem part 24 h later
0.1 mg/mL	10 ± 2.778	0 ± 0.0
0.25 mg/mL	30 ± 5.556	10 ± 2.778
0.5 mg/mL	70 ± 4.811	60 ± 4.811
1 mg/mL	100 ± 0.0	80 ± 2.778
Control (dH_2O)	0 ± 0.0	0 ± 0.0
LC_{50} (min) (mg/mL)	0.203	0.353
LC_{50} (mg/mL)	0.312	0.509
LC_{50} (max) (mg/mL)	0.455	0.773
LC_{90} (mg/mL)	0.770	1. 157
x^2	0.60	1.056

2.11. Statistical Analysis

In all experiments, three replicates of each concentration were run at the same time. The standard errors of mean in experiments were analyzed using Microsoft Excel. The values of $LC_{50\ (min)}$, $LC_{50\ (max)}$, LC_{50}, LC_{90}, and chi-square (χ^2) were calculated with Probit analysis in STATPLUS Pro 5.9.8 package for brine shrimp cytotoxicity and the larvicidal and anthelmintic activity.

3. RESULTS AND DISCUSSION

The antioxidant properties of plants and plant products cannot be determined by a single method due to the complex nature of phytochemicals. It is well known that at least two different methods should be used in order to obtain more reliable results in testing antioxidant activity [26]. For this reason, DPPH, β-Carotene antioxidant assays were performed with ethanol extract of *C. villosa* to show antioxidant properties. The results of DPPH scavenging and β-carotene antioxidant activities showed that the highest activity was inherent

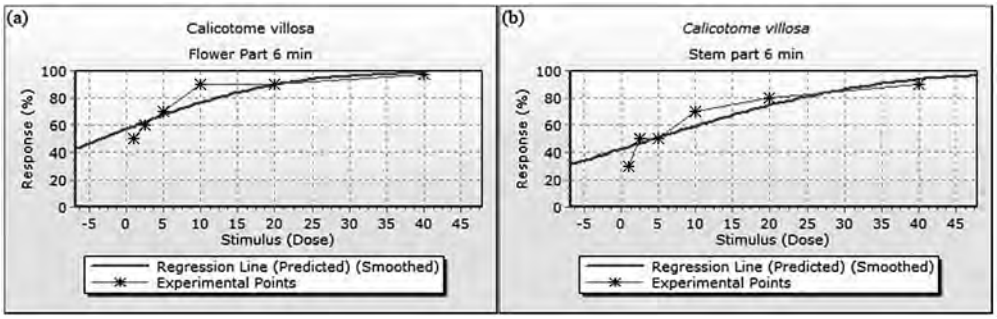

Fig. 4. Graphs of 6-min percentage mortality of (a) flower part and (b) stem part of *C. villosa* in anthelmintic assay.

in flower part (DPPH scavenging assay (86.34 ± 0.16 % & 0.6 mg/mL, IC_{50}) and β-carotene antioxidant assay (75.12 ± 0.73 % mg/mL). These results were very close to those for standard antioxidant, BHA (Table 1). A higher DPPH radical-scavenging activity was associated with a lower IC_{50} value. Similar to our results, ethyl acetate and methanol extracts of *C. villosa* showed high DPPH activity (0.2 mg/mL, IC_{50} for ethyl acetate and 0.34 mg/mL for methanol extract) [27]. In addition to antioxidant activity, total phenolic and flavonoid contents of ethanol extracts obtained from the flowers and stems were also determined in this study (Table 1). Total phenolic and flavonoid contents were higher in flower extract than in stem extract. The results of total phenolic and flavonoid content showed a similar tendency in the antioxidant activity of extracts. Total phenolic and flavonoid contents of ethyl acetate extract of *Calicotome spinosa* leaves were found to be 107.75 ± 0.41 mg GAE/g and 20.87 ± 0.13 mg QE/g extract, respectively [28]. Comparison of these data to our results showed higher total phenolic (159.47 ± 0.33 mg/g GAEs) and flavonoid (66.21 ± 0.09 mg/gQEs) contents in *C. villosa* flower extract, which were probably related to the solvent and part of plant selected for extraction.

In addition to these experiments, phenolic acids were identified by comparing HPLC peak retention times to those of standard compounds (Table 2 and Fig. 1). According to HPLC data, vanillic acid was the major compound in the flower extract of *C. villosa* plant.

The results of cytotoxic activity assay of *C. villosa* extract against *A. salina* are presented in Table 3. The LC_{50} values of the plant extracts were obtained from a plot of the percentage of brine shrimp nauplii killed against the extract concentrations. The best-fit line obtained from experimental data through regression analysis is presented in Fig. 2. The flower extract showed most prominent activity with 0.203 mg/mL, $LC_{50.}$ In study by Krishnaraju, et al. [23], 12 species belonging to the Fabaceae family were tested and different LC_{50} results were obtained (between 60 µg/mL, LC_{50} and >5000 µg/mL, LC_{50}). In comparison to that study, our present results show a lower LC_{50} values (0,203 mg/mL, LC_{50}),

TABLE 4. Average Mortality Rates (%) of *C. villosa* at Various Concentration in Preset Time of Exposure to *Cx. pipiens* and Statistical Data

Concentration	Flower part 72 h later	Stem part 72 h later
0.1 mg/mL	20 ± 0.0	0 ± 0.0
0.25 mg/mL	30 ± 2.778	30 ± 4.811
0.5 mg/mL	50 ± 4.811	60 ± 2.778
1 mg/mL	100 ± 0.0	90 ± 2.778
Control (dH_2O)	0 ± 0.0	0 ± 0.0
LC_{50} (min) (mg/mL)	0.199	0.272
LC_{50} (mg/mL)	0.330	0.404
LC_{50} (max) (mg/mL)	0.549	0.598
LC_{90} (mg/mL)	1.167	0.969
x^2	1.41	0.17

TABLE 5. Mortality Rates (%) of *C. villosa* at Various Concentrations in Preset Time of Exposure to *T. tubifex* (Statistical Data)

Concentration	Flower part 6 min later	Stem part 6 min later
1 mg/mL	50 ± 2.778	30 ± 0.0
2.5 mg/mL	60 ± 4.811	50 ± 2.778
5 mg/mL	70 ± 2.778	50 ± 4.811
10 mg/mL	90 ± 0.0	70 ± 4.811
20 mg/mL	90 ± 4.811	80 ± 2.778
40 mg/mL	100 ± 0.0	90 ± 0.0
Control (dH_2O)	0 ± 0.0	0 ± 0.0
LC_{50} (min) (mg/mL)	0.13	0.74
LC_{50} (mg/mL)	1.32	3.25
LC_{50} (max) (mg/mL)	2.79	6.81
LC_{90} (mg/mL)	13.93	50.67
x^2	0.18	0.17

which indicate that our extracts are much more effective in terms of cytotoxic activity. This activity may be due to the presence of saponins, alkaloids, etc., in the obtained extracts [29].

The larvicidal activity of C. *villosa* ethanol extract (1 and 5 mg/mL) was also studied against *M. domestica* larvae. No statistically significant result was obtained for the control group. For this reason, *C. villosa* did not ehxibit larvicidal activity against *Musca domestica*. Moreover, *Cx. pipiens* was also used for another larvicidal activity assay. The percentage mortality due to ethanolic extracts of flower and stem parts of the *C. villosa* affected the 2nd and 3rd larval stages of *Cx. pipiens* (Table 4 and Fig. 3). The lowest mortality was observed for 1 mg/mL flower extract after 72 h of exposure. The flower extract was found to be more toxic than the stem extract to *Cx. pipiens* larvae. The LC_{50} values at 72 h for flower and stem extracts were 330.07 and 404.32 mg/mL, LC_{50}, respectively. In the study of Govindarajan and Sivakumar [30] on larvicidal activity against *Culex quinquefasciatus*, extract of *Erythrina indica* (Lam.) belonging to Fabaceae family was tested (91.41 ppm, LC_{50}). In the present study, we obtained a lower toxicity with 330.07 ppm, which can be due to differences in the types of plants and flies studied, solvents used, and active antioxidant components. The insecticidal effect of plant-derived products (extracts) against different mosquitoes has been evaluated by many authors [31].

The percentage mortality data for the ethanolic extracts of flower and stem parts of the *C. villosa* against *T. tubifex* are shown in Table 5 and Fig. 4. After 6 minutes, it was found that the flower part (1.32 mg/mL, LC_{50}) was more toxic than the stem part (3.25 mg/mL, LC_{50}). Hossain, et al. [32] studied the anthelmintic activity of *Hopea odorata* against *T. tubifex* and the result was 7.5 ± 0.38 min for 20 mg/mL of methanol extract. We found 6 min for 40 mg/mL with flower extract and believe it to be a good result for anthelmintic activity. Alkaloids and saponins present in plants are known to be powerful antioxidants in the *Calicotome* genus. Therefore, β-sitesterol and stigmasterol-type components in the *Calicotome* genus, may be responsible for their cytotoxic, larvicidal, and anthelmintic activity [29, 33]. These results show that *C. villosa* contains natural phenolic compounds and has a good potential for use in agriculture, medicine, and pesticide industry. There is a need to isolate toxic compounds affecting insects and helminths and it is necessary to investigate their toxicity on more diverse insect species.

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