Cloud Publications
International Journal of Advanced Veterinary Science and Technology
2015, Volume 4, Issue 1, pp. 199-210, Article ID Sci-384
ISSN 2320-3595

Research Article | Open Access

In Vitro Studies on the Effect of Ethanol Extract of *Syzygium Aromaticum* on the Carbohydrate Metabolism of *Cotylophoron Cotylophorum*

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Publication Date: 24 December 2015

Article Link: http://scientific.cloud-journals.com/index.php/IJAVST/article/view/Sci-384

Abstract Paramphistomosis is one of the major pathogenic diseases in domestic animals and responsible for heavy economic loss in terms of reduced milk, meat and wool production. *Cotylophoron cotylophorum* is more prevalent in Tamilnadu. In the present investigation, the effect of *Syzygium aromaticum* ethanol extract on the enzymes of carbohydrate metabolism viz. pyruvate kinase, phosphoenolpyruvate carboxykinase, lactate dehydrogenase, malate dehydrogenase, fumarate reductase and succinate dehydrogenase of *Cotylophoron cotylophorum* was studied *in vitro*. The parasites were incubated in five different sub-lethal concentrations of ethanol extract of *Syzygium aromaticum* viz. 0.005, 0.01, 0.05, 0.1 and 0.5mg/ml for 2, 4 and 8 h. The activity of the enzymes was assayed using standard procedures. The enzyme activity was expressed in terms of protein. The data obtained were analyzed statistically. Ethanol extract of *Syzygium aromaticum* significantly inhibited the enzymes of carbohydrate metabolism and the percentage of inhibition was dose and time dependent. Inhibition of these enzymes leads to decreased ATP production which may be fatal to the parasites. The present study validates the anthelmintic property of ethanol extract of *Syzygium aromaticum* against *C. cotylophorum.*

Keywords *Syzygium Aromaticum; Cotylophoron Cotylophorum; Pyruvate Kinase; Phosphoenolpyruvate Carboxykinase; Lactate Dehydrogenase; Malate Dehydrogenase; Fumarate Reductase; Succinate Dehydrogenase*

1. Introduction

The most important and reliable source of animal proteins in India is meat from goat and sheep. Sheep production has remained an integral part of cultural life and farming system of the rural population. Helminthiasis affects the production potential through mortality, weight loss, reduced milk yield and wool production [1]. Paramphistomosis is a major disease caused by amphistomes. The paramphistome *Cotylophoron cotylophorum* lives in the rumen and reticulum of sheep, goats, cattle and other domestic ruminants [2, 3].

Vast array of synthetic anthelmintics are used to combat paramphistomosis. However, problems have emerged with the use of synthetic anthelmintics, notably the development of resistance in helminths to various anthelmintic compounds and classes, as well as chemical residues and toxicity problems [4, 5]. In addition, recognition of the antigenic complexity of parasites has slowed vaccine development. These disadvantages have stimulated a search for alteration control methods such as the use of traditional medicinal plants. Plants are known to provide a rich source of potent botanical anthelmintics. The use of medicinal plants for the prevention and treatment of gastro intestinal parasitism has its origin in ethno veterinary medicine [6, 7].

Syzygium aromaticum commonly called clove belongs to the family myrtaceae. Clove bud oil has biological activities, such as antibacterial, antifungal, antiviral, antimicrobial, anticancer, antiseptic, anesthetic, insecticidal, analgesic, antispasmodic, anticarminative and antioxidant properties [8]. Clove oil is also active against plant-parasitic nematodes [9]. The major constituents in bud oil are eugenol and *β*-carophyllene, vanillin, crategolic acid, gallotannic acid, methyl salicylate (pain-killer) eugenin, kaempferol, rhamnetin, eugenitin, oleanolic acid, stigmasterol, campesterol and several sesquiterpenes [10, 11] Kumar and Singh, Manoj Dhanraj and Veerakumari reported the anthelmintic activity of *Syzygium aromaticum* against *Fasciola gigantica* and *Cotylophoron cotylophorum* [12, 13].

Carbohydrate is an essential energy source in all adult parasitic helminths and its metabolism is often predominantly anaerobic, even in the presence of oxygen. They depend on carbohydrate either in the form of glycogen or glucose. The inhibition of energy metabolism is the most important mode of anthelmintic action of various groups of drugs since the parasitic trematodes depend on carbohydrates for their energy metabolism and glucose is the only direct source of energy [14, 15]. Glucose is absorbed from the host via the glucose transporters located in the tegument and intestinal epithelium of trematodes [16, 17]. The main storage of carbohydrate in parasitic helminths is glycogen [18].

Carbohydrate metabolism of the helminth parasite resembles Embden-Meyerhof glycolytic pathway of their host animals, until the formation of phosphoenol pyruvate (PEP). PEP obtained from glycolysis can either be carboxylated to oxaloacetate (OAA) by phosphoenolpyruvate carboxykinase (PEPCK), or dephosphorylated to pyruvate by pyruvate kinase (PK). Pyruvate so formed is further reduced to lactate by lactate dehydrogenase (LDH) and OAA is reduced to malate by malate dehydrogenase (MDH). Malate permeates into the mitochondrion where it undergoes dismutation in which one-half of malate is oxidized to pyruvate by malic enzyme (ME) and the other half is dehydrated to fumarate by fumarase (FM), which is further reduced to succinate by fumarate reductase (FR). Succinate oxidized to fumarate by succinate dehydrogenase (SDH). Decarboxylation of pyruvate and succinate results in the final end products of acetate and propionate respectively [18, 19, 20]. Keeping this in view, an attempt has been made to the assess the anthelmintic efficacy of ethanol extract of *Syzygium aromaticum* against *Cotylophoron cotylophorum* based on its effect on the enzyme involved in carbohydrate metabolism.

2. Materials and Methods

2.1. *In Vitro* Maintenance of *Cotylophoron Cotylophorum*

Cotylophoron cotylophorum were collected from the rumen of infected sheep, slaughtered at Perambur abbatoir, Chennai. Adult live worms were collected, washed thoroughly in physiological saline and maintained in Hedon-Fleig solution, which is the best medium for *in vitro* maintenance [21]. It is prepared by dissolving 7gm of sodium chloride, 0.3gm of potassium chloride, 0.1gm of calcium chloride, 1.5gm of sodium bicarbonate, 0.5gm of disodium hydrogen phosphate, 0.3gm of magnesium sulphate and 1gm of glucose in 1000ml of distilled water.

2.2. Preparation of Plant Extracts

The buds of *Syzygium aromaticum* were collected from a local shop at Chennai, and were authenticated in the Department of Botany, Pachaiyappa's college; Chennai and vouchered specimens are deposited in the herbarium of Pachaiyappa's College, Chennai-30. The extraction of plant materials was done following the method of Harborne [22].

2.3. Sample Preparation

Adult *C. cotylophorum* were incubated in various concentration of *Syzygium aromaticum* ethanol extract (*Sa*EE) (0.005, 0.01, 0.05, 0.1 and 0.5mg/ml) for 2, 4 and 8h. Simultaneously, control was also maintained in Hedon-Fleig solution without the plant extract. After incubation, the parasites were rinsed in distilled water. The parasites were weighed wet and a 10% (W/V) homogenate was prepared by homogenising the flukes in ice-cold 0.25 M sucrose solution containing 0.15 M Tris-HCl (pH-7.5). This homogenate was centrifuged at 1000 rpm for 10 min. The supernatant was used as the enzyme source. The cytosolic and mitochondrial fractions of *C. cotylophorum* were prepared following the method of Fry et al. [23].

2.4. Enzyme Assay

2.4.1. Pyruvate Kinase (PK)

Pyruvate kinase (PK, EC 2.7.1.4) activity in the cytosolic fraction was assayed following the method of McManus and Smyth [24]. The reaction mixture contained 1 ml of 300 mM Tris-HCl buffer (pH 7.8) [25], 0.5 ml of 42 mM magnesium sulphate (MgSO4), 0.5 ml of 450 mM potassium chloride (KCl), 0.3 ml of 50 mM adenosine diphosphate (ADP), 0.3 ml of 50 mM PEP, 0.3 ml of 2 mM nicotinamide adenine dinucleotide reduced (NADH), 0.025 ml of 48 mM fructose biphosphate (FBP), 0.025 ml of 15 units of LDH and 0.05 ml of enzyme sample. The reaction was started by the addition of the enzyme sample and the decrease in absorbance at 340 nm was recorded for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NADH and was expressed in n moles NADH oxidised / min / mg protein.

2.4.2. Phosphoenolpyruvate Carboxykinase (PEPCK)

The activity of phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) was assayed according to the method of McManus and Smyth [24]. PEPCK catalyses the formation of oxaloacetate (OAA) from PEP. The assay mixture contained 1ml of 300 mM imidazole buffer (pH 6.2) [25], 0.4 ml of 300 mM MgSO4, 0.3 ml of 400 mM KCl, 0.3 ml of 70 mM sodium bicarbonate (NaHCO3), 0.3 ml of 20 mM ADP, 0.3 ml of 40 mM PEP, 0.3 ml of 2 mM NADH, 0.05 ml of 15 units of MDH and 0.05 ml of enzyme sample. The reaction was started by the addition of the enzyme sample and the decrease in absorbance was read at 340 nm for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NADH and was expressed in n moles NADH oxidised / min / mg protein.

2.4.3. Lactate Dehydrogenase (LDH)

The activity of lactate dehydrogenase (LDH, EC 1.1.1.27) was assayed according to the method of Yoshida and Freese [26]. LDH catalyses the oxidation of lactate and reduction of pyruvate. For oxidation of lithium lactate, 0.8 ml of 60 mM phosphate buffer (pH 7.5) [27], 0.1 ml of 0.5 M lithium lactate, 0.05 ml enzyme sample and 0.05 ml of 20 mM NAD were placed in 1 ml cuvette. The increase of absorbance at 340 nm was recorded for 3 min at an interval of 15 sec. For the reduction of pyruvate, 0.05 ml of enzyme sample was added to 0.8 ml of 60 mM phosphate buffer (pH 6.5) [27],

0.01 ml of 1 mM NADH, 0.01 ml of 10 mM sodium pyruvate and final volume was adjusted to 1 ml by the addition of distilled water in 1 ml cuvette. The decrease in absorbance at 340 nm was measured for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NAD and NADH and was expressed in n moles NAD reduced or NADH oxidised / min / mg protein.

2.4.4. Malate Dehydrogenase (MDH)

Malate dehydrogenase (MDH, EC 1.1.1.37) catalyses the oxidation of malate and reduction of OAA. The activity of this enzyme catalysing the malate oxidation and OAA reduction was assayed in both cytosolic and mitochondrial fractions following the procedure of Yoshida [28]. For the oxidation of malate, the reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.4 for cMDH and pH 7.2 for mMDH) [21] 0.1 ml of 100 mM sodium malate, 0.1 ml of 10 mM NAD, 1.7 ml of distilled water and 0.1 ml of enzyme sample. For MDH catalysing the reduction of OAA, the reaction mixture contained 2.5 ml of 100 mM Tris-HCl (pH 7.4 for both cMDH and mMDH) [21], 0.05 ml of 100 mM oxaloacetate, 0.05 ml of 10 mM NADH, 0.3 ml of distilled water and 0.1 ml of the enzyme sample. The activity of the enzyme catalysing oxidation and reduction reaction was measured at 340 nm for 3 min at an interval of 15 sec each. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NAD and NADH and was expressed in n moles NAD reduced or NADH oxidised / min / mg protein.

2.4.5. Fumarate Reductase (FR)

Fumarate reductase (FR, EC 1.3.1.6) catalyses the reduction of fumarate to succinate. The enzyme was assayed as detailed by Sanadi and Fluharty [29].The reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.6) [21], 0.3 ml of 10 mM KCN (neutralised with 0.01 N HCl), 0.3 ml of 1 mM ethylene diamine tetra acetic acid (EDTA), 0.3 ml of 50 mM fumarate, 0.7 ml of distilled water, 0.1 ml of enzyme sample and 0.3 ml of 1.6 mM NADH in a 3 ml cuvette. After the addition of NADH, decrease in absorbance at 340 nm was measured for 3 min at an interval of 15 sec. The enzyme activity was calculated by using the millimolar coefficient of 6.22 and expressed in n moles of NADH oxidised / min / mg protein.

2.4.6. Succinate Dehydrogenase (SDH)

The activity of succinate dehydrogenase (SDH, EC 1.3.99.1) was assayed according to the method of Singer [30]. The reaction mixture included 0.5 ml of 300 mM phosphate buffer (pH 7.5) [21], 0.3 ml of 0.1 M succinate, 0.1 ml of enzyme, 0.3 ml of 10 mM KCN (neutralised with 0.01 N HCl), 0.1 ml of 0.75 mM calcium chloride and 1.3 ml of water. The enzyme was incubated for 5 - 7 min to permit full activation. After incubation, 0.1 ml DCPIP (0.05 %) (W/V) and 0.3 ml of PMS (0.33 %) were added to initiate the reaction and decrease in absorbance was recorded at 600 nm. The enzyme activity was calculated using millimolar extinction coefficient of 19.1 and expressed in n moles of dye reduced / min / mg protein.

Protein in the sample was determined by the method of Lowry *et al.* [31].

2.5. Statistical Analysis

All the data obtained in the present study were statistically analysed using the statistical software SPSS version 16.0. One-way Anova using Bonferroni test was applied to find out the significant difference between the different concentrations of plant extracts and periods of incubation.

3. Results and Discussion

Helminth parasites derive energy for their survival mainly through the degradation of carbohydrate. Several scientists [25, 32, 33, 34, 35] have studied the influence of anthelmintics on the carbohydrate metabolism of helminth parasites. A good understanding on different carbohydrate metabolic reactions forms solid basis for choosing appropriate targets for new chemotherapeutic agents. Investigations on the effect of ethanolic extracts of *S. aromaticum* on the cytosolic and mitochondrial fraction of *C. cotylophorum* revealed a significant inhibition of the key regulatory enzymes involved in carbohydrate metabolic pathway.

PK and PEPCK activity was found to be inhibited in *Sa*EE treated flukes (Table 1a). Inhibition of both PEPCK and PK activities arrests the PEP-succinate/PEP-lactate pathways. Consequently, the energy yielding process is impaired and deprives the parasite of its ATP production. Decreased generation of ATP proves fatal to the parasites [36].The inhibition of PK and PEPCK activities treated with anthelmintics has been observed in other helminths [37, 38, 39]. Also, Navaneetha Lakshmi and Veerakumari [40] reported the inhibitory effect on the PK and PEPCK activities in *Haemonchus contortus* treated with *Allium sativum*.

The action of PK on PEP results in the production of pyruvate. Pyruvate so formed comes under the influence of LDH, which catalyses, the reduction of pyruvate to lactate and the oxidation of lactate to pyruvate. It is evident from the present investigation that *Sa*EE inhibited the LDH catalysing both the lactate oxidation and pyruvate reduction (Table 1b). It is interesting to note that LDH exhibits a peculiar type of chemotherapeutic response. Inhibition of LDH activity catalyzing pyruvate reduction was found to be higher compared to LDH inhibition catalysing the oxidation of lactate. Similar findings were also reported by various workers [41, 42, 43]. Inhibitory effect of albendazole on LDH activity of *Fasciola hepatica* reported by Ozcelik [44]. Veerakumari and Munuswamy elucidated the inhibitory effect of PZQ and LEV on LDH activity of *C. cotylophorum* [27]. Similar inhibitory effect of *A. sativum* on the LDH activity catalysing both the oxidation and reduction reactions in *H. contortus* has been reported by Veerakumari and Lakshmi [45]. The inhibition of lactate dehydrogenase might arrest the carbon influx in the glycolytic pathway and the generation of the necessary energy through oxidative phosphorylation [36]. Consequently, production of malate, which serves as main substrate for mitochondrial phosphorylation is reduced, which leads to reduced production of ATP [39].

Malate dehydrogenase (MDH) has been a rate–limiting enzyme in the phosphoenolpyruvate metabolism. *Sa*EE significantly inhibited the cytoplasmic MDH (cMDH) and mitochondrial MDH (mMDH) catalysing both the oxidation and reduction reactions in *C. cotylophorum* (Table 1c & 1d). Inhibition of MDH activity of *Ascaris suum*, *F. hepatica* and *Moniezia expansa* by mebendazole, albendazole and parbendazole was reported by Tejada et al. [46]. Also, Oztop et al. [47] reported the alteration of MDH and LDH activities of *Trichuris saginata* by albendazole and niclosamide. Similar inhibitory effect of *Acacia concinna* on the cMDH and mMDH activity of *C. cotylophorum* was reported by Priya and Veerakumari [25]. Anthelmintics may disturb the transmembrane proton gradient severely, leading to drop in cellular ATP levels [48]. Reduction in the MDH activity of the flukes exposed to *Sa*EE suggest that, plants act transtegumentally to target vital tegumental enzymes and interfere with the energy generating pathways depriving the parasite in acquiring ATP, thereby leading to paralysis and death [49]. The inhibition of both cMDH and mMDH observed in the present study suggests the declined production of oxaloacetate (OAA) and malate. The inhibition of MDH might subsequently result in the inhibition of FR, as OAA is essential for production of fumarate [50, 51].

Fumarate is reduced to succinate using NADH as reducing equivalent and succinate formation is the final step of the glycolytic pathway [52]. In the present study, *Sa*EE inhibited the FR and SDH activity of *C. cotylophorum* (Table 1e). Priya and Veeerakumari [25] reported similar inhibition of FR in *A.*

concinna treated *C. cotylophorum*. The FR activity of *H. contortus* was also inhibited by other drugs such as tetramisole, thiabendazole, cambendazole, mebendazole, morantel tartrate and disophenol [53, 54, 55]. Barrowman *et al.* [56] demonstrated the inhibitory effect of benzimidazole and albendazole sulphoxide on the FR activity of *Ascaris suum*. Antiparasitic drugs, inhibit fumarate binding to FR, slowdown the synthesis of body constituents, curtail the energy production in the parasites [57], uncouple oxidative phosphorylation, hamper ATP production [58] and present an excellent biochemical target in the treatment of helminthic infections [59]. The SDH activity of *Heterakis, Trichuris, Ascardia, Chabertia, Bunostomum* and *Nematodirus* was inhibited by tetramisole has been reported by Van den Bossche and Janssen [60]. SDH has the ability to transfer electrons to the respiratory chain by catalysing the formation of fumarate and succinate [61]. SDH inhibition by anthelmintics could prevent the utilization of the chemical energy derived from electron transport for the net phosphorylation of ADP to ATP and deprive the parasite of its normal source of energy [62]. In addition, anthelmintics, affect tubulins bound in mitochondrial membrane of the parasites by influencing SDH–FR complex negatively inhibit succinate metabolism and diminish ATP–synthesis [63, 64]. Hence, SDH could potentially be an important target for anthelmintics against the gastrointestinal parasites of livestock [65].

The inhibition of enzymes of carbohydrate metabolism of *C. cotylophorum* by *Acacia concinna* was also reported by Priya and Veerakumari [25]. Impairment of carbohydrate metabolism in parasitic helminths may be disastrous since they depend almost entirely on it for their energy supply [66]. Present study manifested that PK, PEPCK, LDH, MDH, FR and SDH provide biochemical target for *Sa*EE which disrupt energy generation process in *C. cotylophorum*, resulting in decreased production of ATP. Consequently, the energy deprived parasite unable to sustain themselves *in situ* may be expelled from the host. The results of the present study holds a potential promise in the future use of active principles of *S. aromaticum* as effective anthelmintics and may help in designing assimilated solutions for the control of paramphistomosis.

4. Conclusion

The present study elucidated the anthelmintic effect of *Sa*EE on *C. cotylophorum. Sa*EE blocked the energy metabolism of the parasites by inhibiting the enzymes PK, PEPCK, LDH, MDH, FR and SDH. *Sa*EE possesses a remarkable anthelmintic activity against *C. cotylophorum*. It may serve as an alternative for anthelmintic chemotherapeutic agents to avoid their toxic side effects and development of resistance in a safe and ecofriendly manner. In depth field trials of plant based anthelmintics along with best farm management practices can play a great role in parasite control strategies and in enhancing productivity of livestock farming.

Table 1: *In vitro effect of SaEE on the enzymes involved in Carbohydrate metabolism of C. cotylophorum*

Table 1a PK and PEPCK						
Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	PK			**PEPCK**		
	2h	**4h**	**8h**	**2h**	**4h**	**8h**
0.005	9.95±0.03	13.72±0.04	30.72±0.03	9.08±0.10	26.57±0.08	47.97±0.06
0.01	11.75±0.01	16.84±0.03	48.33±0.05	14.47±0.08	38.51±0.04	56.11±0.01
0.05	17.26±0.01	23.04±0.05	59.89±0.01	21.98±0.08	45.44±0.09	62.37±0.13
0.1	20.55±0.06	32.33±0.12	65.37±0.17	30.01±0.07	52.38±0.12	66.80±0.18
0.5	24.18±0.07	46.12±0.13	76.92±0.19	44.16±0.01	63.70±0.06	90.67±0.11
Table 1b LDH						
Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	Oxidation			**Reduction**		
	2h	**4h**	**8h**	**2h**	**4h**	**8h**
0.005	9.70±0.05	23.06±0.09	59.21±0.01	9.08±0.10	26.57±0.08	47.97±0.06

0.01	21.12±0.01	48.85±0.06	61.03±0.06	14.47±0.08	38.51±0.04	56.11±0.01
0.05	24.63±0.02	53.26±0.04	72.62±0.07	21.98±0.08	45.44±0.09	62.37±0.13
0.1	28.76±0.04	56.08±0.06	76.51±0.09	30.01±0.07	52.38±0.12	66.80±0.18
0.5	46.84±0.02	66.81±0.03	80.28±0.13	44.16±0.01	63.70±0.06	90.67±0.11

Table 1c Cmdh

Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	Oxidation			Reduction		
	2h	4h	8h	2h	4h	8h
0.005	19.59±0.07	45.40±0.24	61.12±0.08	7.50±0.17	40.49±0.22	61.09±0.18
0.01	31.44±0.11	49.27±0.01	74.44±0.01	12.58±0.28	43.73±0.14	75.94±0.40
0.05	40.00±0.03	55.26±0.10	83.71±0.04	23.68±0.13	56.23±0.24	79.69±0.17
0.1	46.23±0.13	64.21±0.13	87.72±0.14	33.62±0.22	63.85±1.21	88.46±0.23
0.5	53.22±0.14	72.18±0.16	98.77±0.18	59.03±0.35	75.06±0.70	92.91±0.39

Table 1d mMDH

Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	Oxidation			Reduction		
	2h	4h	8h	2h	4h	8h
0.005	5.54 ± 0.03	32.25±0.06	62.96±0.17	15.40±0.14	35.12±0.01	54.00±0.07
0.01	15.25±0.04	40.45±0.06	69.46±0.24	19.22±0.07	44.05±0.06	58.51±0.05
0.05	22.96±0.03	43.05±0.14	78.34±0.19	23.48±0.04	51.87±0.05	65.73±0.06
0.1	35.79±0.01	54.49±0.03	81.77±0.16	27.38±0.03	55.55±0.03	72.68±0.07
0.5	53.40±0.06	64.67±0.12	93.41±0.04	42.66±0.11	60.22±0.13	84.29±0.18

Table 1e FR and SDH

Conc. mg/ml*	% inhibition (mean ± SD of n = 5) at various periods of incubation**					
	FR			SDH		
	2h	4h	8h	2h	4h	8h
0.005	28.60±0.18	41.10±0.18	63.28±0.02	12.79±0.05	37.33±0.06	58.02±0.04
0.01	31.65±0.27	47.25±0.13	71.93±0.01	22.43±0.03	49.82±0.08	69.44±0.01
0.05	37.29±0.01	56.14±0.04	79.10±0.03	35.42±0.02	55.20±0.11	73.52±0.12
0.1	45.78±0.01	68.13±0.16	83.24±0.01	38.00±0.15	63.61±0.09	76.08±0.03
0.5	62.27±0.14	70.28±0.11	87.39±0.21	48.41±0.08	67.34±0.14	81.46±0.05

* Inhibitory effects of the extracts among the different concentrations of the respective plant are significantly different for each duration of incubation (Bonferroni test; $P < 0.05$)

** Inhibitory effects of the extracts among the different hours of incubation is significantly different for each concentration of the respective plants (Bonferroni test; $P < 0.01$)

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