An Elsevier Indexed Journal ISSN-2230-7346

*Journal of Global Trends in Pharmaceutical Sciences*

# EFFECT OF ETHANOL EXTRACT OF *SYZYGIUM AROMATICUM* ON FUMARATE REDUCTASE AND SUCCINATE DEHYDROGENASE OF *HAEMONCHUS CONTORTUS*

**S. Sathish Kumar and L. Veerakumari***

PG and Research Department of Zoology, Pachaiyappa's College, Chennai – 600 030, Tamilnadu, India.

***Corresponding author E-mail**: veerakumari_2002@yahoo.co.in

**ARTICLE INFO**

**Key Words**

Fumarate reductase, Succinate dehydrogenase, *Syzygium aromaticum*, *Haemonchus contortus*.

## ABSTRACT

Parasitic infestation exerts adverse effects on the health and productivity of livestock. *Haemonchus contortus* is one of the most pathogenic, economically important and ubiquitous nematode parasites of small ruminants which causes haemonchosis. Haemonchosis is mainly controlled by synthetic anthelmintic compounds; however, indiscriminate use of anthelmintics has led to the emergence of resistant to *H. contortus* worldwide, which has awaken interest to use medicinal plants as an alternative source of anthelmintic drugs. Carbohydrate is the major source of energy for parasites. The pathway of carbohydrate metabolism is essentially anaerobic and involves the glycolytic and part of the reversed tricarboxylic acid (TCA) cycle. The enzymes Fumarate reductase (FR) catalyses the reduction of fumarate to succinate and Succinae dehydrogenase (SDH) catalyses oxidation of succinate to fumarate. Reduction of fumarate to succinate results in ATP production. In the present study, anthelmintic efficacy of *Syzygium aromaticum* ethanol extract (*Sa*EE) against the *H. contortus* was analyzed based on its effect on FR and SDH. *H. contortus* were exposed to five different sub-lethal concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) of *Sa*EE for 2, 4 and 8h. FR and SDH was assayed in control and drug-treated worms using standard procedure. Maximum inhibition of FR and SDH activity was observed at 0.5 mg/ml of *Sa*EE after 8h of exposure. Inhibition of FR and SDH interferes with the terminal electron acceptor and prevents succinate formation which leads to deprived ATP synthesis. Energy deprivation results in the death of the worms.

## INTRODUCTION:

India has the largest livestock population, in the world, which contributes nearly 7% towards its national income. Livestock sector provides the exclusive source of animal protein to 300 million rural people[1]. Haemonchosis, an important gastrointestinal nematodiasis caused by *Haemonchus contortus* is prevalent wherever sheep and goats are raised, and produces the greatest economic loss in temperate climate[2]. *H. contortus* is a blood sucking nematode found in abomasum of the sheep and causes significant blood loss, resulting in anaemia, loss of body weight and wool growth[3]. Thus it leads to production loss

and death of the infected animals. Anthelmintics drugs are used to treat parasitic diseases. The most common drugs are niclosamide, oxyclosamide, triclabendazole and albendazole[4]. The current efficacy of these drugs has been reduced, because of resistant strain development in parasites[5,6]. It is imperative to decrease the reliance on these chemotherapeutic drugs for parasite control, not only because of resistance, but also because of growing concerns about the adverse consequences of these antiparasitic drugs on the ecosystem and biodiversity. Hence there is a search for alternative methods to control parasitic disease using plants as herbal remedy. Phytotherapy has become a solution to control gastrointestinal helminths[7,8].

*Syzygium aromaticum* Linn. is commonly known as clove belong to the family Myrtaceae. Clove bud oil has biological activities, like anthelmintic, antibacterial, antifungal, analgesic, antispasmodic, anticancerous, anticarminative and antioxidant properties[9,10,11,12,13,14]. The predominant constituents in clove bud oil are eugenol and B-caryophyllene[15]. *S. aromaticum* possess saponins, tannins, phenols, cardiac glycoside, flavonoids, alkaloids and anthracene [16]. Manoj Dhanraj and Veerakumari, [17] reported the anthelmintic activity of *S. aromaticum* against *Cotylophoron cotylophorum*. Helminth parasites derive energy for their survival mainly through the degradation of carbohydrate. The pathway of carbohydrate catabolism is essentially anaerobic and involves the glycolytic and part of the reversed tri carboxylic acid (TCA) cycle. The reduction of malate to succinate occurs in two reactions that reverse part of the Krebs cycle, and the reduction of fumarate is essential NADH consuming reaction to maintain redox balance. 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). Reduction of fumarate to succinate complex results in mitochondrial ATP synthesis. Therefore FR-SDH system acts to be vulnerable point for the phytotherapeutic interference. Inhibition of these enzymes prevents ATP formation. Decreased production of ATP leads to the death of the parasites. Hence, the present investigation was carried out to assess the anthelmintic potential of ethanol extract of *Syzygium aromaticum* (*Sa*EE) on FR and SDH of *H. contortus*.

## MATERIALS AND METHODS

### Collection of Parasites

Adult live *Haemonchus contortus* were collected from the abomasum of the sheep slaughtered at a local abattoir in Chennai. The worms were washed thoroughly in physiological saline and maintained in Hedon-Fleig solution. Hedon-Fleig solution (pH 7.0) is the best medium for *in vitro* maintenance of *C. cotylophorum*[18]. It is prepared by dissolving 7 g of sodium chloride, 0.1 g of calcium chloride, 1.5 g of sodium bicarbonate, 0.5 g of disodium hydrogen phosphate, 0.3g of potassium chloride, 0.3 g of magnesium sulphate and 1 g of glucose in 1000 ml of distilled water.

### Extract preparation of plant material

*Syzygium aromaticum* was purchased from local store, then cleaned and coarsely powdered. Successive soaking of plant extract was done for 48 h with hexane, chloroform, ethyl acetate and ethanol. Aqueous extract was also prepared. Intermittent agitation was necessary while soaking in various solvents. Wattman filter paper no.1 was used for filtrations. Distillations have been done with rotary evaporator

(EQUITRON). Extracts were kept in Lyodel lyophilizer (DELVAC) to remove the solvents and dried up.

**Assay of Fumarate reductase**

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[19].

$$\text{Fumarate} + \text{NADH} + H^+ \longrightarrow \text{Succinate} + \text{NAD}$$

The reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.6)[18], 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 minutes at an interval of 15 seconds. The enzyme activity was calculated by using the millimolar coefficient of 6.22 and expressed in n moles of NADH oxidised/min/mg protein.

**Assay of Succinate dehydrogenase**

The activity of succinate dehydrogenase (SDH) (EC 1.3.99.1) was assayed according to the method of Singer[20]. Succinate is oxidised to fumarate by the flavoprotein SDH, which contains covalently bound flavin adenine dinucleotide. This reducible co-enzyme functions as hydrogen acceptor in the following reaction.

$$\text{Succinate} + \text{E. FAD} \longrightarrow \text{Fumarate} + \text{E. FADH}_2$$

The reduced enzyme can donate electrons to various artificial electron acceptors e.g reducible dyes. SDH assay is based on the reduction of phenazinemethosulphate (PMS) by SDH. Reduced PMS is immediately reoxidised by dicholoro phenol indophenol (DCPIP). Bleaching of later dye is estimated spectrophotometrically. The reaction mixture included 0.5 ml of 300 mM phosphate buffer (pH 7.5)[18], 0.3 ml of 0.1 M succinate, 0.1 ml of enzyme, 0.3 m 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 minutes 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 600nm. The enzyme activity was calculated using millimolar extinction coefficient of 19.1 and expressed in n moles of dye reduced/min/mg protein.

**Statistical Analyses**

Statistical analyses were performed with the statistical program for the social sciences SPSS version 16.0. The significance of drug induced inhibition in the FR and SDH was assessed using analysis of variance (ANOVA) for different concentrations of ethanol extract *Syzygium aromaticum* (*Sa*EE). The term significant had been used to indicate difference for which $P \leq 0.05$.

**RESULTS**

The current investigation reveals that *S. aromaticum* ethanol extract significantly inhibited both FR and SDH activies in the nematode *H. contortus*. The percentage of inhibition in FR activity of *Sa*EE-treated worms was 53.86, 71.21 and 90.88% (Fig. 1) and whereas, in SDH, the percentage of inhibition was 51.81, 79.39 and 95.76% (Fig. 2) after 2, 4 and 8h of incubation respectively at 0.5 mg/ml concentration. Inhibition of FR and SDH in *Sa*EE worms is directly proportional to concentration of the drug and period of exposure. The inhibition of both FR and SDH was statistically significant ($P<0.05$).

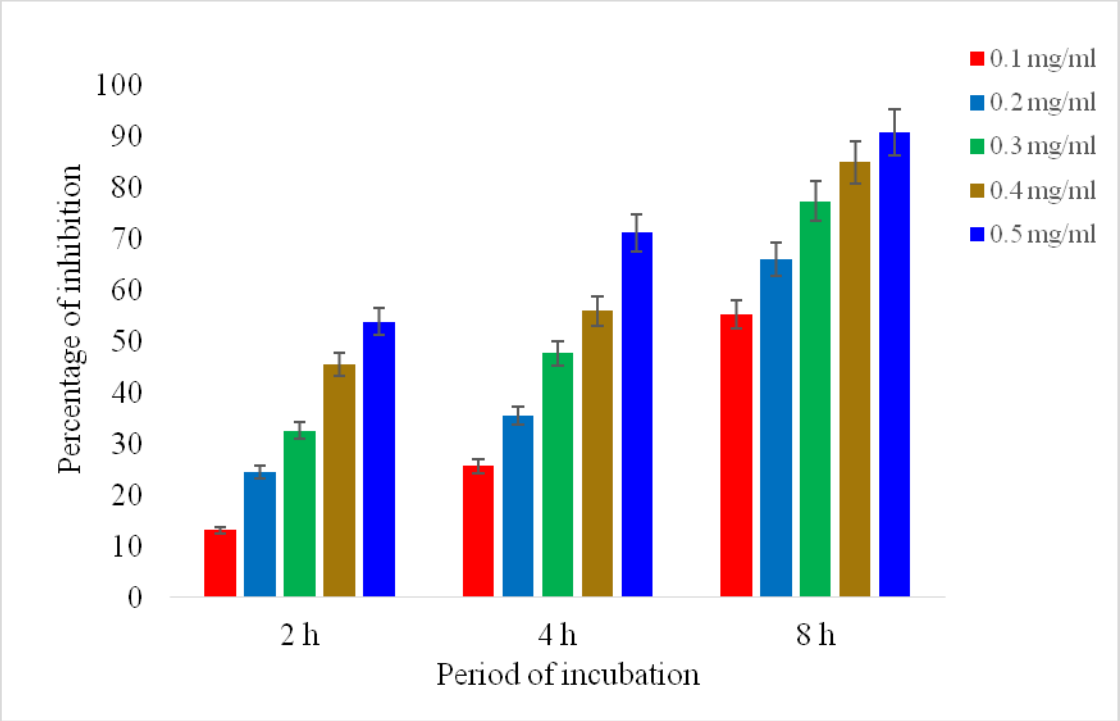


Figure 1: Effect of *Sa*EE on FR activity of *H. contortus*

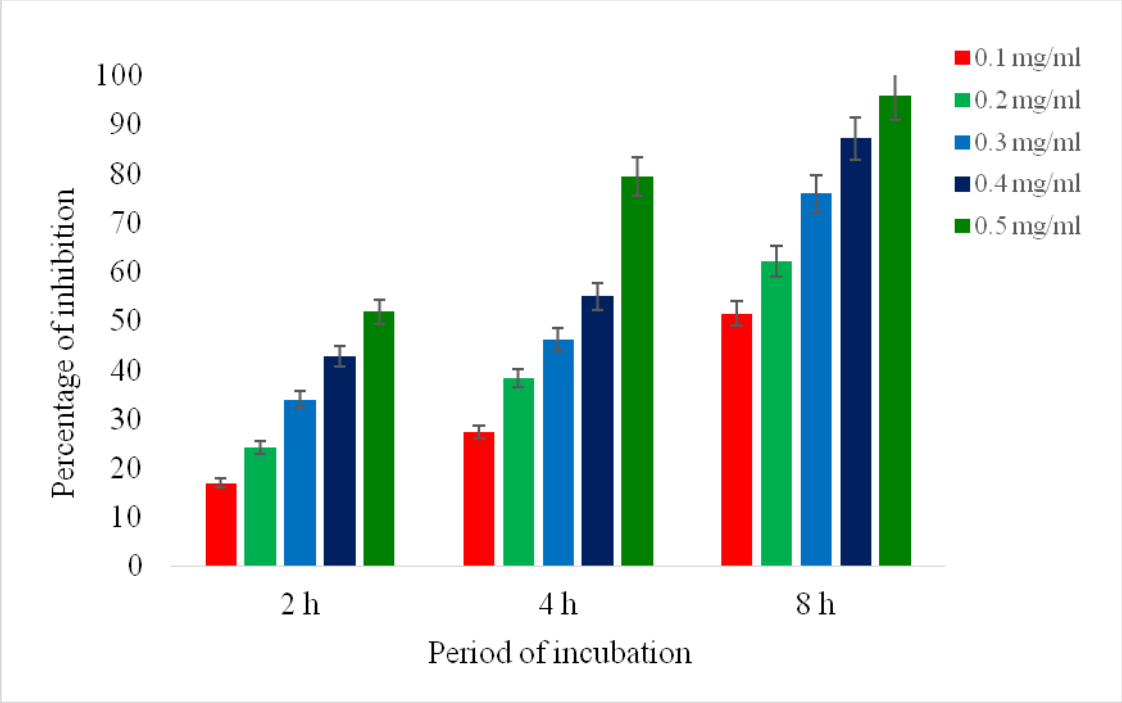


Figure 2: Effect of *Sa*EE on SDH activity of *H. contortus*

## DISCUSSION

The universal feature of endoparasitic organism is their dependence upon anaerobic carbohydrate metabolism to obtain energy and glycogen is considered as the chief energy reserve[21]. During energy metabolism the various level of metabolic reactions are happening and this significant pathways paves a loads of effective targets for a novel therapeutics. Consequently, glycolysis is the major energy yielding pathway in helminth parasites[22]. Scientists have studied the influence of anthelmintics on the carbohydrate metabolism of helminth parasites[23-25]. In the present study, ethanolic extract of *Syzygium aromaticum* (*Sa*EE) significantly inhibited the fumarate reductase (FR) and succinate dehydrogenase (SDH) activity of *H. contortus*. Anthelmintic drugs affect the energy metabolism in helminth parasites [26-28]. Hence the depletion in the glycogen reserve arrest the ATP synthesis in helminths[18]. Helminths use unsaturated organic acids as terminal electron acceptors, instead of oxygen and enzymes during energy metabolism, whose properties often differ significantly from those of their host. From the current study, it is evident that the *Sa*EE significantly inhibited the FR activity in *H. conortus*. FR is an enzyme that converts fumarate to succinate. It is the terminal electron acceptor in the energy metabolism of helminthes. Maule and Marks[29] opined that the fumarate is reduced to succinate using NADH as reducing equivalent and succinate formation is the final step of the glycolytic pathway. Satoshi et al.[30] reported the inhibitory effect of nafuredin, a novel compound isolated from *Aspergillus nigers*. Likewise, nafuredin inhibited NADH-fumarate reductase of nematode *Ascaris suum*[31]. Other chemotherapeutic drugs such as tetramisole, rafoxanide, thiabendazole, cambendazole, mebendazole, morantel tartrate and disophenol are also inhibit the FR activity of the nematode *H. contortus*[32-35].

Investigation of the current study, reveals that *Sa*EE consequently inhibit the SDH activity in *H. contortus*. SDH is an enzyme complex, bound to the inner mitochondrial membrane. SDH has the ability to transfer electrons to the respiratory chain by catalyzing the formation of fumarate and succinate[36]. SDH inhibition by anthelminthics could prevent the utilization of the chemical energy derived from electron transport for the net phosphorylation of ADP to ATP and deprive the parasite for its normal source of energy[37-39]. Disturbance in the terminal electron acceptor prevents succinate formation thereby curtail the ATP synthesis. Decreased production of ATP leads to the death of the parasite. Priya and Veerakumari[40] reported similar inhibition of FR and SDH in *Acacia concinna*–treated *C. cotylophorum*. In addition, Manoj Dhanraj and Veerakumai[41] described the inhibition of the enzyme FR and SDH in *Areca catechu* treated with *Cotylophoron cotylophorum*. The current study reveals that FR and SDH provide biochemical target for *Sa*EE which ultimately disrupt the carbohydrate metabolism, glycolysis in particular of *H. contortus*, results in lower production of ATP. Due to this parasite unable to sustain in abomasum and get expelled from the host. Hence the study strongly suggest the use of *S. aromaticum* as an herbal anthelmintics for the control of the nematode, *H. contortus*.

## ACKNOWLEDGEMENTS:

Inspire fellowship funded by Department of Science and Technology (DST) is gratefully acknowledged.

## REFERENCES:

1. Abraham, S. K., 2001. Anti-genotoxicity of trans-anethole and

eugenol in mice. Food Chem. Toxicol. **39**, 493–498.

2. Dutta, B., Konch, P., Rahman, T., Upadhyaya, T. N., Pathak, D. C., Tamuli, S. M., Phangchoo, C. V. and Begum, S. A. 2017. Occurrence and pathology of *Haemonchus contortus* infection in Goats. *J. of Ent.* and *Zoo. Studies*. **5(3)**: 1284-1287.
3. Mortensen LL, Williamson LH, Terrill TH, Kircher R, Larsen M, Kaplan RM (2003). Evaluation of prevalence and clinical implications of anthelmintic resistance in gastrointestinal nematodes in goats. *Jou. Am. Vet. Med. Assoc*. **223(4)**: 495–500.
4. Galdhar, C. N. and Roy, S., 2004. Clinico-haematological and biochemical changes in cattle during paramphistomiasis. *Ind. Vet. Med. J*., **28**: 245-248.
5. Jabbar, A., Iqbal, Z., Kerboeuf, D., Muhammad, G., Khan, M.N. and Afaq, M. 2006. Anthelmintic resistance: The state of play revisited. *Life Sci*., **79**: 2413-2431.
6. Artho, R., Schnyder, M., Kohler, L., Torgerson, P. R. and Hertzberg, H. 2007. Avermectin resistance in gastrointestinal nematodes of Boer goats and Dorper sheep in Switzerland. *Vet. Parasitol*. **144**: 64-73.
7. Bizimenyera, E. S., Githiori, J. B., Eloff, J. N. and Swan, G. E., 2006. *In vitro* activity of *Peltophorum africanum* Sond (Fabaceae) extracts on the egg hatching and larval development of the parasitic nematode *Trichostrongylus colubriformis*. *Vet. Parasitol*. **142**: 336-343.
8. Manolaraki, F., Sotiraki, S., Stefanakis, A., Skampardonis, V., Volanis, M., Hoste, H., 2010. Anthelmintic activity of some Mediterranean browse plants against parasitic nematodes. *Parasitol*. **137**: 685-696.
9. Cuman, R. K. N., Bersani-Amado, C. A., Caparroz-Assef, S. M., Schmidt, G., Sartoretto, S. M., Daniel A. N. 2009. Anti-inflammatory and antinociceptive activities of eugenol essential oil in experimental animal models. *Braz. J. of Pharma*. **19(1B)**: 212-217.
10. Githiori, J. B., Athanasiadou, S., Thamsborg, S. M. 2006. Use of plants in novel approaches for control of gastrointestinal helminthes in livestock with emphasis on small ruminants. *Vet. Parasitol*. **139**: 308-320.
11. Hussain, A., Sasidharan, T., Ahmed, M., Sharma, C. 2009. Clove (*Syzygium aromaticum*) Extract Potentiates Gemcitabine Cytotoxic Effect on Human Cervical Cancer Cell Line. *Int J Cancer Res*. ISSN 1811- 9727.
12. Park, M. J., Gwak, K. S., Yang, I., Choi, W. S., Jo, H. J., Chang, W. J., Jeung, E. B., Choi, I. G. 2007. Antifungal activities of the essential oil in *Syzygium aromaticum* (L.) Merr. Et Perry and *Leptospermum betersonni* Bailey and their constituents against various dermatophytes. *J Microbiol*. **45(5)**: 460-465.
13. Ponce, A. G., Fritz, R., del Valle, C., Roura, S. I. 2003. Antimicrobial activity of essential oils on the native microflora of organic Swiss chard. *J Lebensmittel-Wissenschaft and-Technologie*. **36**: 679-684.
14. Pinto, E., Vale-Silva, L., Cavaleiro, C., Salgueiro, L. 2009. Antifungal activity of the clove essential oil from *Syzygium aromaticum* on Candida, *Aspergillus* and dermatophyte species. *J Med Microbiol*. **58**: 1454-1462.
15. Srivastava, A. K., Srivastava, S. K. and Syamasundar, K. V. 2003. Bud

and leaf essential oil composition of *Syzygium aromaticum* from India and Madagascar. *Flav.* and *Frag. Jou*. **20**(1):51-53.

16. Emmanuel, 2015. Phytochemical Screening and Antimicrobial Sensitivity of Clove Flower (*Syzygium aromaticum*, L. Merrill and Perry) Bud on Dental Pathogens. *Int. J. of Pharma. and Pharma. res*. Vol.:3, Issue: 2.
17. Manoj Dhanraj, K. and Veerakumari, L. 2014. *In vitro* effect of *Syzygium aromaticum* on the motility and Acetylcholinesterase of Cotylophoron cotylophorum. *Ind. J. Vet. & Anim. Sci. Res.*, **43(3)**: 187-194.
18. Veerakumari, L.., 1996. *In vitro* studies on the effect of some anthelmintics on *Cotylophoron cotylophorum* (Fischoeder, 1901) (Digenea: Paramphistomidae). A structural and biochemical analysis. Ph.D. thesis, University of Madras. Chennai.
19. Sanadi, D. R. and Fluharty, A. L., 1963. On the mechanism of oxidative phosphorylation. VII-The energy requiring reduction of pyridine nucleotide by succinate and energy yielding oxidation of reduced pyridine nucleotide by fumarate. *Biochemistry*, **2**: 523-528.
20. Singer, T. P., 1974. Determination of activity of succinate, NADH, choline and β-glycerophosphate dehydrogenase. In: *Methods in Biochemical Analysis*. (Glick, D., ed.), Vol. 22, Interscience publications. John Wiley and Son, New York, pp. 133-161.
21. Richard Komuniecki and Aloysius Tielens, G.M. 2002. Carbohydrate and energy metabolism in helminths. Research gate.
22. Roy, 2012. Lead Optimization Studies towards the Discovery of Novel Carbamates as Potent AChE Inhibitors for the Potential Treatment of Alzheimer's disease. *Bioorganic & Med. Chem*., Volume 20, Issue 21; pp. 6313-6320.
23. Bueding, E. and Fisher, J., 1970. Biochemical effects of niridazole on *Schistosoma mansoni*. *Mol. Pharmacol*., **6(5)**: 532–539.
24. Schulman, M. D., Ostlind, D. A. and Valentino, D., 1982. Mechanism of action of MK–401 against *Fasciola hepatica*: inhibition of phosphoglycerate kinase. *Mol. Biochem. Parasitol*, 5 (3): 133–45.
25. Donahue, M. J., Masaracchia, R. A. and Harris, B. G., 1983. The role of cyclic AMP–mediated regulation of glycogen metabolism in levamisole–perfused *Ascaris suum* muscle. *Mol. Pharmacol*., **23**: 378–383.
26. Bose C., Agarwal S.K., Chatterjee R.K. and Srivastava V.M. 1994. Carboline antifilarials: effects on carbohydrate metabolizing enzymes in *Litomosoides carinii* female. *Ind. J. of Exp. Bio*. **32**: 431-433.
27. Manoj Dhanraj, 2015. *In vitro* Studies on the Effect of Ethanol Extract of *Syzygium aromaticum* on the Carbohydrate Metabolism of *Cotylophoron Cotylophorum*. *Int. J. Adv. Vet. Sci. and Technol*., Volume 4, Issue 1, pp. 199-210. Article ID Sci-384, ISSN 2320-3595.
28. Jeya, S. and Veerakumari, L., 2015. Effect of *Piper betle* on pyruvate kinase and Phosphoenolpyruvate carboxykinase of *Cotylophoron cotylophorum*. *World journal of pharmacy and pharmaceutical sciences*. Vol. 5, Iss.1, 1537-1548. Research Article ISSN, 2278 – 4357.

29. Maule, A. G. and Marks, N. J., 2006. Parasitic Flatworms: Mole. Biol. Bioch. Immun. and Physiol., CABI, pp. 448.
30. Satoshi, O., Mura-Hiroko Miyadera., Hideaki Ui., Kazuro Shiomi., Yuuichi Yamaguchi., Rokuro Masuma., Tohru Nagamitsu., Daisuke Takano., Toshiaki Sunazuka., Achim Harder., Heinz Ko lbl., Michio Namikoshii., Hideto Miyoshi., Kimitoshi Sakamoto., and Kiyoshi Kita. 2001. An anthelmintic compound, nafuredin, shows selective inhibition of complex-I in helminth mitochondria. Proceedings of the National Academy of Sciences. **98(1)**:60-62.
31. Sakai, C., Tomitsuka, E., Esumi, H., Harada, S. and Kita, K. 2012. Mitochondrial fumarate reductase as a target of chemotherapy: from parasites to cancer cells. *Biochem Biophys Acta*. **1820(5)**:643-51.
32. Bueding, E., 1963. Electron transport and fermentation in *Ascaris lumbricoides*. In: Control mechanisms in respiration and fermentation (Wright, B.ed.). Ronald Press, New York, pp.167-177.
33. Malkin, M. F. and Camacho, R. M., 1972. The effect of thiabendazole on fumarate reductase from thiabendazole-sensitive and resistant *Haemonchus contortus*. *J. Parasitol*., **58**: 845-846.
34. Prichard, R. K., 1973. The fumarate reductase reaction of *Haemonchus contortus* and the mode of action of some anthelmintics. *Int. J. Parasitol*., **3**: 409-417.
35. Kaur, R. and Sood, M. L., 1983. The effects of DL-tetramisole and rafoxanide on tricarboxylic acid cycle enzymes of *Haemonchus contortus, in vitro*. *Vet. Parasitol*., 13: 333-340.
36. Barrett, J., 1981. Biochemistry of Parasitic Helminths. Macmillan Press, London. pp. 308.
37. Kumari, Y. S., 2006. Effect of tolzan on carbohydrate metabolism and protein metabolism of an *Acantho cephalan* parasite *Pallisentis nagpurensis* parasitising the fresh water fish *Channa striatus*. 2006
38. Lemke, T. L., Williams, D. A., Roche, V. F. and Zito, S. W., 2013. FOYE‟S Principles of Medicinal Chemistry. Seventh Edition Copyright © 2013 Lippincott Williams & Wilkins, 351 West Camden Street, Baltimore, MD 21201.
39. Manoj Dhanraj, K. and Veerakumari, L., 2015. *In vitro* Studies on the Effect of Ethanol Extract of *Syzygium aromaticum* on the Carbohydrate Metabolism of *Cotylophoron Cotylophorum*. *Int. J. Adv. Vet. Sci. and Technol*., Volume 4, Issue 1, pp. 199-210. Article ID Sci-384, ISSN 2320-3595.
40. Priya, P. and Veerakumari, L., 2011. *In vitro* effect of *Acacia concinna* on the key enzymes of carbohydrate metabolism of *Cotylophoron cotylophorum* (Fischoeder, 1901). *Biomed*, **31 (3)**: 329-333.
41. Manoj Dhanraj, K. and Veerakumari, L., 2016. Effect of ethanol extract of Areca catechu on Fumarate reductase and Succinate dehydrogenase of *Cotylophoron cotylophorum*. *Int. J. Res. Dev. Pharm. L. Sci*., **5 (3)**: 2117-2123.