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Antioxidant, Antiprotoscolices Activity of Ethanolic Extracts of Some Medicinal Plants Against *Echinococcus granulosus* as Eco-friendly System

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Abstract: The principal objective of the study is to investigate the use of *Mentha spicata, Thymus vulgaris* and *Ocimum basilicum* as a good alternative option to Antiprotoscolices *in-vitro*. Hydatid disease, a zoonotic parasitic infection caused by *Echinococcus granulosus,* an important economic and human public health problem with a wide geographical distribution**.** Fertile hydatid cysts were collected from livestock and the viability of the protoscolices was confirmed. Protoscolices were subjected to four different concentrations of organic extracts (12.5, 25, 50 and 75 mg/ml) for 10, 20 and 30 min. Each extract was investigated and viability of the protoscolices was tested using 0.1% eosin staining. The highest efficacy was by *T. vulgaris*, *M. spicata* (100%), and *O. basilicum* (98.8%) respectively, after exposure of 20 minutes at 75 mg/ml, that lead to the significant reduction in the viability of protoscolices. The extract had time-dependent effect. Phytochemical were identified qualitatively and weighted quantitatively, that help in the identification of bioactive compounds involved in selective action on the protoscolices tegument layer. In conclusion, all the selected medicinal plants could be a promising source of potent antiprotoscolices effect. The mechanism by which plant extracts killed protoscolices and also their safety for living cells are unclear and need to be investigated further.

Key words: Protoscolices**,** *Cystic echinococcosis***,** phytochemical**,** zoonotic.

Introduction

Cystic echinococcosis (CE) is the most important zoonotic parasitic diseases, causing high mortality and morbidity in human and livestock by the larval stage of *Echinococcus granulosus* (Neumayr et al., 2013; Pensel et al., 2014; Rajabloo et al., 2012). The adult worms occur in the dogs small intestine, and other carnivores, while the larval stage in intermediate hosts widely (cattle, sheep, pigs, horses and humans) (Gholami et al*.*, 2013). Hosts Infection occur after ingestion of infective eggs in contaminated feed, food or water (Moazeni and Roozitalab, 2012). As a result of CE, Hydatid cysts developed in internal organs mainly in the liver and lungs of intermediate hosts and also in humans as unilocular fluid filled bladder (Budke et al., 2009; Thompson and McManus, 2002). This infection remains asymptomatic for years or permanently (Liu et al., 2014). Eventually, malfunction and even death may occur due to continuous cysts growth, without efficient treatment (Adas et al., 2009; Brunetti et al., 2010).

Surgical removal of the hydatid cyst is still the preferred therapy method (Gholami et al., 2013) but increases the scolices spillage chance, the major cause of recurrence is secondary echinococcosis (Kilicoglu et al., 2008; Moro and Schantz, 2009). The efficient

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utilizing protoscolicidal agent is necessary as spillage prophylactic of the cyst contents (Blanton et al., 1998; Junghanss et al., 2008; Spicher et al., 2008). The most protoscolicidal agents are formalin, hypertonic saline, and povidone iodine (Karaoglanoglu et al., 2011), but unsafe due to sclerosing cholangitis, liver necrosis, fatal hyperthermia and methemoglobinemia (Karaoglanoglu et al., 2011; Gholami et al., 2013), that led to scolicidal agent less patient harmful, and more effective for use in hydatid cyst (Adas et al., 2009). Recently, discovery efforts for new anti-scolicidal compounds from plants (Moazeni et al., 2012), that attempt us to underline the *in-vitro* effect of *M. spicata, T. vulgaris* and *O. basilicum* against the viability of *E. granulosus* protoscolices, analyse selection medicinal plants extracts qualitatively and quantitatively and identifies major biologically active phytoconstituents.

Materials and Methods

Plant Material

Organic solvent extraction of the *Mentha spicata, Thymus vulgaris* and *Ocimum basilicum* leaves was carried out by using 95% ethyl alcohol (BDH England) by using Soxhlet apparatus. For this 50 g plant leaves powder was put inside the thumble and 500 ml (95%) ethanol in the flask extract for 24 hrs., by heating at (50-60)°C until a clear colourless solvent appeared, dried via electric oven (40-45)°C, placed in an incubator (38-40)°C and kept frozen (–20)°C until use (Effraim et al.).

Phytochemical Analysis

All plants extracts were prepared through ethanol extraction, standard procedures to identify qualitative and quantitative screening (Prabhavathi et al., 2016; Ullah et al., 2018). All chemical reagents and solutions supplied by BDH, England.

Qualitative Phytochemical Analysis

Flavonoids

Few drops (20%) NaOH were added to 2 ml of each extract, a yellow colour appeared. Then the addition of few drops of 70% HCL, the yellow colour disappeared.

Alkaloids

Marquis Reagent (1ml) was added to 1 ml of each extraction, few drops (40%) CH_2O and 2 mL (conc. H_2SO_4) were added and mixed; the dark orange or purple colour appeared.

Phenolic Components

Plant leaves powder or ethanolic extraction (10 g) added to (50 ml) distilled water, heated till boiling; the solution left to cool, filtered, and 1% $FeCl_3$ sol added.

Tannins

Added (2 mL) extraction to (10%) $FeCl_3$, the brownish black or blue colour represents tannins (Prabhavathi et al., 2016).

Saponins

A big foam for a long time because of the aqueous solution stirring in the test tube.

Quantitative Phytochemical Analysis

Total Flavonoids

Each plant extract 4 gm with 40 ml (80% aqueous methanol), at 37ºC, filtered, transferred to the crucible, kept in the water bath, evaporated to dryness and weighed (Prabhavathi et al., 2016).

Total Alkaloids

Extract sample (1gm) mixed with 40 ml (10% acetic acid in ethanol), for 4 hrs., filtered, kept in water bath, then, drops of NH_4OH added until the precipitation was complete. Precipitate washed via NH_4OH, filtered, dried and weighed (Prabhavathi et al., 2016).

Total Tannins Content

About 3.75 ml distilled water was added to each extraction, 0.25 mL of Folin-Ciocalteu's phenol reagent and 0.5 ml sodium bicarbonate, then spectrophotometer measuring (725 nm) and standard solutions as tannic acid (Prabhavathi et al., 2016).

Hydatid Cysts Collection and Protoscolices Viability Determination

Hydatid cysts are collected from the infected liver naturally. Many times, cyst washed with sterile phosphate buffer solution (pH: 7.2), surface-sterilised (70%) ethanol and their fertility was examined, then aspirated with proper care and hydatid cyst fluid (HCF) evacuated containing protoscolices (15 Ml) falcon tubes completely, left (60 min) to precipitate at room temperature to obtain hydatid sand.

The sterile preservative solution made from a mixture of Kreb-Ringer solution and hydatid cyst sand (4:1) was utilised for the viability of protoscolices by amoeboid (Smyt and Barrett, 1980). Results were confirmed by eosin solution staining (0.1% aqueous). The protoscolices are classified as dead (stained) and viable (not stain) (Macpherso and Smyth, 1985). Viable protoscolices in the sediment considered appropriation for experiment and counting 100 protoscolices minimum, the number of viable protoscolices to total protoscolices. When the samples have 95% or more will be appropriate for further experimentation, transferred to the container having normal saline and stored at 4˚C.

Plant Extraction Concentrations

Stock solutions (1g) dried extraction with 10ml (50%) C_2H_6OS-DMSO (Fluka, Germany), that sterilised via Millipore membrane filter (0.20 µm). The concentrations (12.5, 25, 50,75 mg/ml) were prepared by mixing known volume from the stock solution with 50% DMSO.

Determination of *In-vitro* Assay

In the tube dilution assay, standard hydatid cyst fluid (1.0 ml) was added to tubes (1 ml) of different concentrations of each extraction in comparison with the control for (10, 20, 30) min with triple replication, the mortality % measuring of the dead protoscolices (Vijay et al., 2013) via the following formula:

$$\% \text{ Mortality} = \text{ODP} \div \text{TP} \times 100$$

Antioxidant Activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay (Ullah et al., 2018) was used for evaluating the antioxidant potential of each alcoholic extract at different concentrations (50, 100, 150, 200, 250 µg/ml). Plant extracts incubated with 3 ml freshly prepared purple coloured DPPH (0.004%) solution turned yellowish after 30 min at room temperature, and colour changes were measured via spectrophotometer (514 nm). Methanol (1 ml) was utilised as blank. The antioxidant activity was calculated by using the formula :

Antioxidant Activity (%) =

$$\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Statistical Analysis

The data were analysed by one-way analysis of variance (ANOVA). All analysis was accomplished by using Minitab software, version 16.

Results and Discussion

Phytochemical Analysis

Qualitative Phytochemical Analysis

Oualitative screening of the extracts showed the presence of alkaloids, saponin, which were found to be present in high levels, compared to tannin (Table 1).

Quantitative Analysis

The quantity of alkaloid was high in *Ocimum basilicum* (4.81±0.01%), *Thymus vulgaris* (3.14±0.01%) and *Mentha spicata* (2.92±0.01%) respectively. Flavonoids were higher in *Thymus vulgaris* (5.72±0.04%), *Mentha spicata* (3.85±0.12%) and *Ocimum basilicum* (3.43±0.02%), respectively. The highest tannin quantities were found in *Thymus vulgaris*, *Ocimum basilicum* (3.01±0.08%), (2.64±0.02%), and *Mentha spicata* (0.87±0.01%) (Table 2).

Table 1: Qualitative phytochemical analysis of the ethanolic plant extract

Plant extract	*Alkaloids*	*Phenolic*	*Flavonoids*	*Tannin*	*Saponin*
Mentha spicata	++	+++	++	+	++
Thymus vulgaris	++	++	+++	++	++
Ocimum basilicum	+++	+++	++	++	+++

+ = positive, ++ = good present, +++= strongly present, – = not detected.

Table 2: Quantitative detection of important secondary metabolites in the plant extract

Plant extract	*Alkaloids %*	*Flavonoids %*	*Tannin %*
Menthaspicata	2.92±0.01	3.85±0.12	0.87±0.01
Thymus vulgaris	3.14±0.01	5.72±0.04	3.08±0.02
Ocimum basilicum	4.81±0.01	3.43±0.02	2.64±0.02

Antioxidant Activities

The antioxidant activities for the extracts of *Mentha spicata, Ocimum basilicum* and *Thymus vulgaris* increased with sample concentration increasing. The highest antioxidant concentrations at 250 µg/mL were 68.41, 59.15 and 42.02 for *Thymus vulgaris, Mentha spicata, Ocimum basilicum*, respectively. After incubating in sterile preservative solution (negative control), the protoscolices remain viable for 24 hrs., without treatment, with minimum differences in distinct movement, order of hooks and membrane integrity of protoscolices. The microscopic images of dead protoscolices showed distortion and degeneration in morphology and structure, which meant loss of motility, hooks and calcareous capsules (Table 3).

Phytochemicals are non-nutritive plant constituents that have protective properties from diseases beneficial to human health. Qualitative phytochemical analysis shows the presence of active compounds including alkaloids, flavonoids, tannins, phenol and saponins, thus showing the highest efficacy against the viability of protoscolices. Flavonoids inhibit the initiation, promotion and tumours progression, reduce coronary heart disease (Hertog et al., 1993; Kim et al., 1994) through chelation and effecting the membrane permeability and membrane bound enzymes (Li et al., 2015). The phenolic compounds have scavenging ability due to the hydroxyl group (Dillar and German, 2000). Similarly, saponins are less toxic to mammals and have antimicrobial activity, highly used as mild detergents (Sheikh et al., 2013). Alkaloids have a broad range of medicinal activities including anticancer, antiasthma antimalarial (Kittakoop et al., 2014), vasodilatory (Russo et al., 2013), analgesic (Raymond et al., 2010) and antibacterial properties (Cushnie et al., 2014).

The Viability of Protoscolices

Before the experiments, the viability of free protoscolices placed in the wet mount of hydratic cyst fluid was assessed using eosin stain (aqueous, 0.1%). The protoscolices appeared red/purple coloured indicating it to be non-viable as shown in Figure 1. However, viable protoscolices remain colourless and show amoeboid like movement and flame cell activity under light microscope. Untreated protoscolices remain viable for 60 min, with no significant difference between 0 and 60 min.

Table 3: Antioxidant activities via alcoholic plant extracts

Plant extract	*50 ug/ml*	*100 g/ml*	*150 ug/ml*	*200 ug/ml*	*250 ug/ml*
	Antioxidant activity (%)				
M. spicata	42.18	48.9	50.61	52.12	59.41
O. basilicum	32.5	38.4	40.20	40.81	42.02
T. vulgaris	38.9	41.5	49.7	57.5	68.41

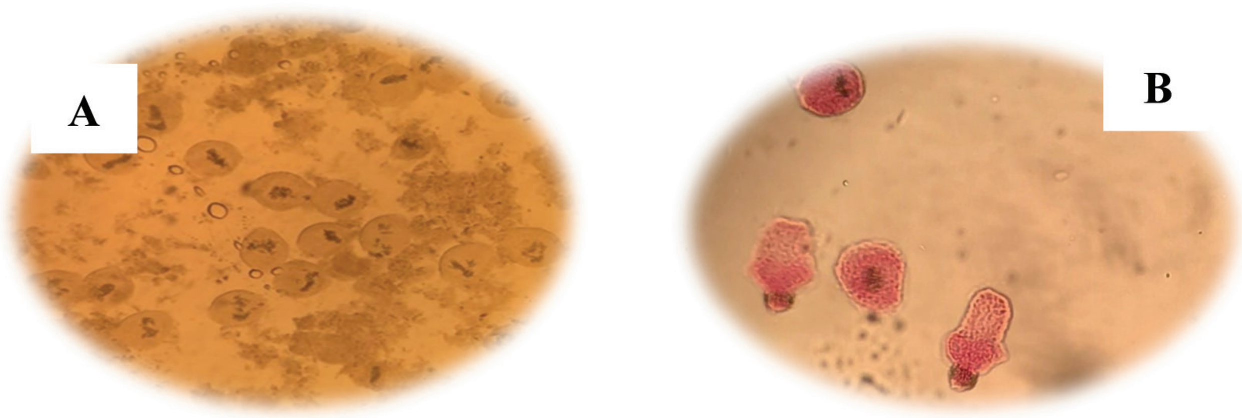

Figure 1: (a) Dead protoscolices staining by eosin stain (0.1%) (×20) and (b) viable protoscolices (×20).

In-vitro Antiprotoscolices Activity

Different concentrations of ethanolic extract of *Mentha spicata, Ocimum basilicum* and *Thymus vulgaris* utilised in the tube diffusion assay, caused different mortality % degrees for protoscolices. The protoscolices mortality differed due to extraction concentration and time exposure, the mortality rate was proportionally increased with increasing concentration of ethanolic extract (Tables 4-6). The highest mortalities (100.0%) of protoscolices with alcoholic extract of *Thymus vulgaris* at (50mg/ml, 30 min), and (100.0%) at (75mg/ml, 10 min) (Table 4). The significant effect when protoscolices of *E. granulosus* treated with *Mentha spicata* alcoholic extraction (83.8%, 100.0% and 100.0%) at (75 mg/ml) for (10, 20, 30) min, respectively (Table 5). *Ocimum basilicum* showed the mortalities (72.7%, 89.9%, 100.0%) at (75 mg/ml) at (10, 20, 30 min) respectively (Table 6).

Cystic echinococcosis caused a major health problem in many countries and was declared as emerging and re-emerging infection despite some progress in control of CE (Moro and Schantz, 2000). Surgical removal is the preferred method for the treatment of hydatid cysts (Gholami et al., 2013), and inactivation of the parasite with protoscolicidal agents is an important component of surgical treatment to avoid recurrence and multiple secondary echinococcosis (Haghani et al., 2014).

Many protoscolicidal agents are utilised, including hypertonic saline, alcohol, and povidone–iodine solution (Karaoglanoglu et al., 2011), but it is argued that there is

Table 4: Antiprotoscolices effect of *Thymus vulgaris*

Conc. mg/ml	*10 min.*			*20 min.*			*30 min.*		
	Total protoscolices	*Dead Protoscolices*	*Mortality rate (%)*	*Total protoscolices*	*Dead Protoscolices*	*Mortality rate (%)*	*Total protoscolices*	*Dead Protoscolices*	*Mortality rate (%)*
12.5	97	30	30.9	99	45	45.5	98	52	53.0
25	99	38	38.3	99	56	56.5	99	62	62.6
50	98	49	50.0	98	88	89.7	99	99	100
75	99	99	100	99	99	100	99	99	100

Table 5: Antiprotoscolices effect of *Mentha spicata* extracts

Conc. mg/ml	*10 min.*			*20 min.*			*30 min.*		
	Total protoscolices	*Dead protoscolices*	*Mortality rate (%)*	*Total protoscolices*	*Dead protoscolices*	*Mortality rate (%)*	*Total protoscolices*	*Dead protoscolices*	*Mortality rate (%)*
12.5	98	29	29.5	98	40	40.8	97	52	53.6
25	98	35	35.7	98	51	52.0	98	75	76.5
50	97	42	43.2	98	72	73.4	99	86	86.8
75	99	83	83.8	98	98	100	99	99	100

Table 6: Antiprotoscolices effect of *Ocimum basilicum* extracts

Conc. mg/ml	*10 min.*			*20 min.*			*30 min.*		
	Total protoscolices	*Dead protoscolices*	*Mortality rate (%)*	*Total protoscolices*	*Dead protoscolices*	*Mortality rate (%)*	*Total protoscolices*	*Dead protoscolices*	*Mortality rate (%)*
12.5	97	28	28.8	98	35	35.7	99	52	52.5
25	98	32	32.6	99	48	48.4	100	64	64.0
50	98	45	45.9	98	69	70.4	99	82	82.8
75	99	72	72.7	99	89	89.8	98	98	100

no ideal agent that is both effective and safe (McManus et al., 2003). It is important to identify an effective alternative protoscolicidal agent to overcome the severe side-effects of synthetic pharmaceuticals (Abdel-Baki et al., 2016; Moazeni and Nazer, 2011). Herbal extracts are recognised as effective and safe alternative agents (Elissondo et al., 2008), so we sought to investigate the protoscolicidal effect of ethanolic extracts of *Menthaspicata*, *Thymus vulgaris* and *Ocimum basilicum* utilising against protoscolices in hydatid cysts.

The 0.1% eosin stain was used for getting access to the protoscolices viability. The non-viable protoscolices appear red with eosin dye while viable appear colourless. The antimicrobial and antiparasitic potential of many plants has been well explored against a wide range of harmful microbes and parasites, via diverse procedures. The *Thymus vulgaris* showed the highest activity against the viability of protoscolices, *Mentha spicata*, *Ocimum basilicum* at 12.5, 25, 50, and 75 mg/ml after 10, 20, and 30 min, respectively. Similarly, considerable attention to alternative therapies particularly using natural sources derived compounds for the treatment of hydatid disease. Many reports included the in-vitro scolicidal effect of aqueous extract of *Z. multiflora* (Moazen and Roozitalab, 2011), *Oleaeuropaea* (Zibaei et al., 2012), *S. ebulus* (Gholami et al., 2013), *Z. officinale* (Lakshm and Sudhakar, 2010) and musk (Kamil et al., 2016).

Thymus vulgaris showed a slightly higher effect against *E. granulosus* due to differences in qualitative and quantitative compositions. The phytochemical composition can differ according to harvesting seasons, geographical sources, age, and vegetative cycle stage (Angioni et al., 2006), and for constant composition, should be extracted under the same conditions from the same organ of the plant (Bakkali et al., 2008).

Otherwise, the inherent activity of the phytochemicals can relate to the proportions of the components that are present and the interactions between them (Burt, 2004), i.e., the observed anthelmintic activity of *T.vulgaris*, *Mentha spicata*, and *Ocimum basilicum* the possibility antagonic interactions between the components of the active ingredients. Antagonic effects between the components depend on essential oils and phytochemicals (Liolios et al., 2009; Sumbal et al., 2019).

The phytochemical mechanism action and their components have not been studied as their essential oils are complex mixtures, their antimicrobial activity is not attributable to one specific mechanism but to several targets in the cell (Carson et al., 2002; Skandamis and Nychas, 2001).

In eukaryotic cells, phytochemicals can provoke the depolarisation of the mitochondrial membranes by decreasing the membrane potential (Novgorodov and Gudz, 1996; Richter and Schlegel, 1993; Vercesi et al., 1997). Phytochemical and essential oils change the fluidity of the membrane to become abnormally permeable, thereby resulting in leakage of radicals, cytochrome C, calcium ions, and proteins, as in the case of oxidative stress and bioenergetics failure. Permeabilisation of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Armstrong, 2006). Apoptosis induced by thymol in cancer cells is associated with the mitochondrial pathway (Chang et al., 2011; Deb et al., 2011).

Similar considerable attention to alternative therapies particularly use natural sources derived compounds for the treatment of hydatid disease. Many literatures have reported the in-vitro scolicidal aqueous extract of *Oleaeuropaea*, S. (Zibaei et al., 2012), *Z. multiflora* (Moazen and Roozitalab, 2011), *S. ebulus* (Gholami et al., 2013), *Z. offocinale* (Lakshm and Sudhakar, 2010) and musk (Kamil et al., 2016).

Conclusion

All the plants, *Thymus vulgaris*, *Mentha spicata*, and *Ocimum basilicum,* show antiprotoscolices against protoscolices of *E. granulosus*. We recommend utilising these plants as a complementary treatment after investigation on the animal models and clinical trials in the human population.

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