The Haryana Veterinarian (June, 2023) **62(1),** 46-49 ISSN 0033-4359 (P) **Research Article**

DETERMINATION OF *IN-VITRO* GROWTH INHIBITION EFFICACY OF DIFFERENT *ARTEMISIA SCOPARIA* EXTRACTS IN MASP CULTURE SYSTEM AGAINST *THEILERIA EQUI*

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ABSTRACT

The present study was conducted to determine the anti-*Theileria equi* efficacy of *Artemisia scoparia* plants extracts. *Artemisia scoparia* plant material was collected from natural habitat. Extract and its different fractions were prepared. *In-vitro* growth inhibition assessment was carried out as per the standards procedure after establishing the *T. equi* MASP *in-vitro* culture system. *In-vitro* drug trial results indicated that all the fractions of *A. scoparia* (except aqueous fraction) have significant (P<0.05) anti- *T. equi* activity. Methanolic fraction of *A. scoparia* showed the most promising *in-vitro* anti- *T. equi* activity. At lowest concentration (100 µg/mL) the percent parasitemia of N-hexane, Chloroform, Ethyl acetate, Methanol and Aqueous fraction was found as $6.79^{b} \pm 0.39$, $6.45^{a} \pm 0.41$, $6.02^{a} \pm 0.87$, $4.28^{a} \pm 0.87$ and $7.48^{b} \pm 0.38$, respectively. These results indicated that methanolic extract comparatively has better *T. equi* growth inhibition efficacy and needs analysis.

Keywords: *Artemisia scoparia*, Herbal medicine, *Theileria equi*, MASP *in-vitro* culture, Plant extracts

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Equine piroplasmosis (EP) is an acute hemolytic disease of equids characterized by intermittent fever, anemia, jaundice, haemoglobinuria, reduced working capacity and petechiation on nictitating membrane. Disease is distributed worldwide due to widespread distribution of the potential vectors in the different regions of the world (Wise *et al.*, 2013). Reported seroprevalence of *T. equi* in equids from arid and semiarid region of India lies between 50 % to 70 % (Dahiya *et al.*, 2018). Currently imidocarbdipropionate is common drug of choice available for EP treatment but multiple dosing is required which may lead to systemic toxicity. This also entails for the development of drug resistant parasite strain (Schwint *et al.*, 2009; Hines *et al.*, 2015). To combat these limitations, many recent studies explored the use of herbal medicine for treating the disease with minimum or no side effect. Beshbishy *et al.* (2019) evaluated the activity of methanolic and acetonic extract of plant *Oleaeuropaea* and *Acacia laeta*, respectively against piroplasm under specified MASP culture condition and concluded that it can be used an alternate remedy for the treatment of piroplasmosis. They further evaluated antiparasitic potential of combination of extract of *Oleaeuropaea* and diminazene aceturate against *B. microti* and concluded that the combination therapy has increased efficacy. Furthermore, Salama *et al.* (2014) claimed the antibabesial as well as anti-theilerial effect of allicin, an active ingredient of garlic, under *in-vitro* MASP culture system as well as *in-vivo* mouse model. They concluded that growth inhibitory effect of allicin is dose dependent with IC50 value of 818, 675, 470 and 742 µM for *Babesia bovis*, *Babesia bigemina*, *Babesia caballi* or *Theileria equi*, respectively. In this regard, we have selected a plant *Artemisia scoparia* for our study which has previously been identified as an alternate source of artemisinin (a potential antimalarial drug). This plant posse possesses anti-tumor, anti-bacterial, antioxidant, anti-inflammatory, analgesic, anti-viral, and hepatoprotective activities. It has been found very good source of flavonoids, flavonoid glycosides, steroids, volatile oil, coumarin, chromones, phenolic acids, terpenoids and other components with flavonoids and coumarins having widest bioactivity (Ding *et al.*, 2021). Dry leaves of *A. scoparia* are used as an effective anthelmintic agent, while floral buds are used for the treatment of seasonal fever (Sher *et al.*, 2016). In present study we have conducted phytochemical screening and preliminary assessment of anti *T. equi* activity of this plant to evaluate its medicinal potential and pharmacological importance.

MATERIALS AND METHODS

Collection of plant material

The Areal parts of Artemisia scoparia was collected from its natural habitat of Hisar district of Haryana and submitted for identification at department of botany, MDU, Rohtak, Haryana.

Plant extract preparation

Plant material was collected, washed properly in distilled water and air dried for 5-7 days in shade dry

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condition. The plant material was cut into small pieces and milled in electrical grinder to powder. The 100 g powder was sieved through a common strainer to remove the larger sized particles. The 20 g of powder was suspended in 100 ml of methanol and kept overnight with continuous shaking. The supernatant was collected by filtering through Whatman filter paper, and the residue was again suspended in same volume of methanol. The process was repeated for three times and finally collected. The collected extract was concentrated in rotary evaporator and then freezes dried at -55º C and stored at -20º C until use.

Fractionation of methanolic extract of Artemisia scoparia

Freeze dried methanolic extract (1.0 g) was initially dissolved in 20 mL of methanol and final volume was made up to 100 mL by adding more methanol. This re-constituted extract was poured into separating funnel. To this methanolic extract, 100 mL of n-Hexane wasadded and funnel was stirred thoroughly at regular intervals of 3-5 minutes for at least 6-7 times and finally kept for another 10-15 minutes to separate the methanolic and hexane layer. Upper hexane layer (due to lower relative density) was collected, and same process was repeated with other solvents (chloroform, ethyl acetate and aqueous (through distillation) and desired layers were collected based on their freeze relative density. Finally, different fractions collected underwent rotatory evaporation to obtain dried form of the fractionated material which were stored at -20ºC until further use.

Preparation of stock and working concentration

Stock solutions of different fractions of plant extract were prepared by dissolving approximately 50 mg of plant extract into incomplete M-199 medium and making the solution of final concentration 50 mg/mL. These stock solutions were stored at -20° C till further use. For evaluation of *in vitro* growth inhibition activity against *T. equi* working solution of different concentrations ranging from 100 µg/mL to 1000 µg/mL was prepared through serial dilution in complete M-199 culture medium.

Establishment of MASP culture

The continuous propagation of *T. equi* parasites requires low oxygen (3%) and carbon dioxide (5%) environment in *in-vitro* MASP culture conditions. Briefly, the infected whole equine blood from seropositive animal was collected in tube containing anticoagulant (EDTA) and transported aseptically to equine piroplasmosis laboratory, ICAR-National Research Centre on Equine, Hisar, Haryana. The blood was centrifuged at 3000 rpm for 10 min and the plasma was separated. The pelleted erythrocytes were washed by adding Vega Y Martinez (VYM) solution and centrifuged at 3000 rpm for 10 min. The supernatant was aspirated, and the previous procedure was repeated for at least three times. The final washing of erythrocytes was done with media M 199 for conditioning of erythrocytes to media. Culture was started in 24 well culture plate (in duplicate) by pouring 1 mL of complete M-199 media and adding 100 µL of infected erythrocyte in each well was added. The plate was incubated at 37°C under specified MASP culture system.

Theileria equi growth inhibition assay in MASP culture

T. equi growth inhibition assay was performed as per protocol described by Bork *et al.* (2003) to study effect of drug molecules on *T. equi* multiplication. The working solution of 1000 µg/mL, 500 µg/mL, 250 µg/mL and 100 µg/mL of plant extract was prepared on the day of assay. The solution of 100 µM of imidocarbdipropionate (IMD) was prepared in complete M-199 medium to be used as a positive control during assay. The parasitemia of already established MASP culture was recorded, and the infected RBCs were washed at least three times with VYM solution to wash off any remaining media. The assay was initiated at 1% parasitemia. The infected erythrocytes were mixed with fresh healthy equine erythrocytes according to previously determine dparasitemia level to achieve the 1% parasitemia. The growth inhibition assay was performed in a 96 well cell culture plate (Greiner India). The working solution of 100 µL volume of respective working solution of plant extract, imidocarb and negative control (complete media devoid of plant extract) were dispensed in to triplicate wells and the wells were seeded with 5 µL of infected equine erythrocytes to initiate the assay. The overlaid media was replaced every day with similar working solution. The final parasitemia was recorded after 96 h of incubation period by making Giemsa-stained smears from each well. The parasitemia per cent was calculated by the following formulae-

$$\text{Percentage parasitemia (for each drug concentration)} = \frac{\text{Average parasitized RBC}}{\text{Average total number of RBC}} \times 100$$

Statistical analysis

All the data under study were represented as mean ± SEM. Significant differences between various treatments groups were studied by using Two-way analysis of variance (ANOVA) as per standard protocol by using graph pad prism version 7. Descriptive statistical analysis was performed to calculate the mean value and standard error (SE) of data. Results were considered as significant at $P<0.05$ (Snedecor and Cochran, 1994).

Table 1. *Theileria equi* percentage parasitemia after *in-vitro* treatment (96h) with different fractions of *A. scoparia* plant extract in MASP culture system

Fraction	Percent PPE (Mean±SE) observed at different Concentration (µg/ml) of the fraction				No drug Control
	1000	500	250	100	
N-hexane	2.56[a]±0.59	4.97[a]±0.66	5.06[a]±0.89	6.79[b]±0.39	8.76[b]±0.85
Chloroform	3.30[a]±1.05	4.77[a]±0.91	6.40[a]±1.46	6.45[a]±0.41	
Ethyl acetate	3.57[a]±0.54	4.65[a]±0.58	5.63[a]±0.50	6.02[a]±0.87	
Methanol	1.26[a]±0.61	1.60[a]±0.65	2.60[a]±0.50	4.28[a]±0.87	
Aquas	6.88[b]±1.42	6.93[b]±0.99	7.38[b]±0.44	7.48[b]±0.38	

*Different superscript indicates statistically significant (P<0.05) differences between control and drug treated well.

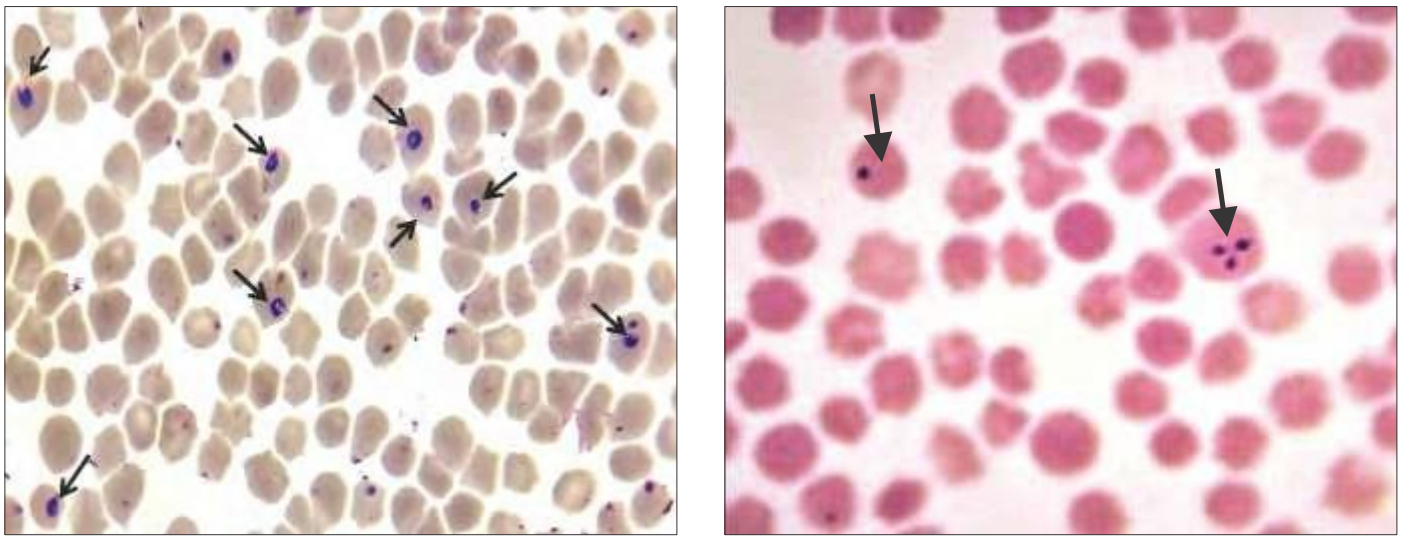

Fig. 1. Smear prepared from (A) negative control well and (B) *T. equi in-vitro* cultured parasite treated with methanol fraction of *A. scoparia* (100 µg/mL conc.) A: showing live multiplying *T. equi* parasites (arrowhead); B: Pyknotic and dead *T.equi* parasite after treatment at 96 h.

RESULTS AND DISCUSSION

In-vitro growth inhibition assay

Percentage of parasitized erythrocytes (PPE) was calculated after 96 hours for each concentration of plant extract used and compared with PPE of negative control well. Details of percentage parasitemia are presented in Table 1. Data analysis revealed that aqueous fraction was ineffective amongst all the fractions underwent for *in-vitro* trial. At 100 µg/mL of concentration n-hexane was not showing any significant activity as indicated by non-significant difference (P>0.05) in parasitemia between controls and treated well while higher concentration showed significant (P<0.05) activity in inhibiting *T. equi* multiplication (Fig 1).

Previously, drug trial for equine piroplasmosis was attempted in *in-vivo* trials involving splenectomized horses or donkeys but risk of accidental transmission and limitation of screening large number of drug molecules creates the *in-vitro* drug activity assessment to develop novel drug molecules created. Also, *in-vitro* MASP culture system eliminates the need of keeping experimental infection for research purpose. The MASP culture system enabled the *in-vitro* screening of various other drug molecules like pyrimethamine, artesunate and pamaquine (Nagai *et al.*, 2003), triclosan (Bork *et al.*, 2004), harmaline hydrochloride dihydrate (HHD), hexadecyltrimethyl-ammonium bromide (HDTAB), hesperidin methyl chalcone (HMC), andrographolide (Gopalakrishnan *et al.*, 2016) etc. The encouraging results in *in-vitro* assay have better chance of success when testified in *in-vivo* system. *A. scoparia* extracts contain various phytoconstituents (Ding *et al.*, 2021). Caffeoylquinic acids are one of the important phenolic acid presents in *A. scoparia* showing potent anti-inflammatory activity. Quercetin, rutin, naringenin, luteolin, hypericin, cirsiliol etc. are important flavonoids isolated from these plants (Ding *et al.*, 2021). Besides, more than hundred volatile oil constituents were also identified from this plant by advance analytical techniques (Singh and Sarin, 2010). The anti-*T. equi* activities may be due to anti-piroplasmic nature of these phytoconstituents. This requires further analysis.

It can be concluded that different *Artemisia scoparia* plant extract has variable anti-*T. equi* activity in *in-vitro* MASP culture system. The preliminary data indicated that all the fractions except aquas fraction are active against *T. equi* and further needed for evaluation in detail to analyze the pattern of changing parasitemia in concentration and time dependent manner.

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Declarations

Prior approval was taken for equine sampling in the present study from the Institutional Animal Ethics Committee of ICAR-NRCE, Hisar.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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