The Pharma Innovation Journal 2019; 8(1): 508-512

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Ratings: 5.03
TPI 2019; 8(1): 508-512
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www.thepharmajournal.com
Received: 15-11-2018
Accepted: 20-12-2018

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Abstract
Equine piroplasmosis incurs economic loss to equine industry through prolonged illness and embargo on international movements. The current therapeutics are becoming resistant because of its continuous use for decades. The search for a new drug molecule which is safer and effective in combating disease condition is the need of hour. The Acacia nilotica (kikar plant) is well recognised for its traditional therapeutic usage in India. The plant is bestowed with different active secondary metabolites and phytochemicals. Therefore, in this study, the methanolic extract of bark of A. nilotica was tested against T. equi parasite in in vitro system at a concentration of 500 µg/ml. The imidocarb dipropionate at concentration of 10 µg/ml was used as standard control drug. The results of the current study established the anti-piroplasm activity of A. nilotica bark extract against T. equi parasites.

Keywords: Thelileria equi, Acacia nilotica, Kikar, MASP

Introduction
Equine piroplasmosis is a tick borne haemoproteozoon disease of horses, donkey, mules and zebras. The disease is clinically manifested as intermittent fever, lethargy, loss of working capacity, weight loss, peripheral oedema, pallor mucous membrane, petechiation on nictitating membrane, etc. Subsequently, the signs of haemolytic anaemia like jaundice, haemoglobinuria, tachycardia etc. were followed in later stage of infection. The Ixodide ticks of fourteen species of Dermacentor, Rhipicephalus and Hyalomma genus were identified as capable vectors for transmission of the disease. The presence and distribution of the potential vectors in the different regions of the world is the reason for wide spread prevalence of equine piroplasmosis in equine population [1]. In India, the states coming under arid to semi-arid climatic zones were having 48-70 percent of seroprevalence of equine piroplasmosis protozoan parasites among equids [2]. The Babesia caballi and Thelileria equi are the two important apicomplexan parasites are reckoned as etiological agents for equine piroplasmosis [3]. In India, the equine piroplasmosis is mostly caused by T. equi parasite. There are several drugs used for treatment of equine piroplasmosis caused by T. equi parasites with partial success. The drug molecules of imidocarb dipropionate, buparvaquone, dimazene aceturate, amicabalis were tried for this purpose. The imidocarb dipropionate is till now the preferred drug for clinical recovery of animal. However, no drug till now able to completely clear the parasite from host. The current group of chemotherapeutic drugs possess severe side effects like hepatotoxicity, tissue toxicity, untoward anticholinergic effects upon therapeutics use on equines [1, 4].

The plant kingdom is largely remained unexplored for its phytoconstituent and potential ameliorative agents for different infectious and non-infectious diseases. In the alternative therapy like Ayurveda, Unani medicine, Siddha medicine, etc. the plant-based therapy constitutes the major principle of treatment. The phytochemicals like alkaloids, flavonoids, polyphenols and their derivative, triterpens, etc. were a vast group of natural molecules those remained unexplored and lack scientific validation as well.

The Acacia nilotica plant is a medium size perineal tree of 15-18-meter tall, spread in arid and semiarid climatic zones of Indian subcontinent. The plant bears 10-30 pairs of small size (4.5-7cm) compound leaflets, yellow colour flowers at the terminal points of branches and greyish coloured pods [5]. Acacia nilotica plant is commonly called as “Kikar”, “Babul” in hindi, “Babli” in kannada, “Karivelam” in malayalam, “Karuelai” in tamil and “Babaria” in gujarati. The different parts of the plant are used by local healers to treat various ailments in man and
The continuous propagation of *T. equi* parasites in erythrocytes required media M 199, hypoxanthine and equine serum (Sigma Aldrich, India). The streptopenicillin stock solution was added to the media (Gibco chemicals, USA). The standard drug molecule, imidocarb dipropionate (Sigma Aldrich, India), was included as control drug. The methanol (SRL chemicals, India) was used for preparation of extract of *A. nilotica*. The dimethyl sulfoxide (DMSO), dextrose, potassium chloride, magnesium sulphate hepta hydrate, sodium chloride, calcium chloride dihydrate, potassium dihydrogen phosphate, adenine, guanosine was used for preparation of complete culture media or dissolving methanolic plant extract. All the solvents used in the research work are of analytical grade.

Collection of plant material
The bark of *A. nilotica* was collected from its native tract of Hisar district of Haryana and submitted for identification at department of Plant physiology, Department of Botany and Plant Pathology, CCSHAU, Hisar, Haryana.

Plant extract preparation
The barks from mature plant were collected, washed properly in distilled water and air dried for 7-10 days in shade dry condition. The barks were cut into small pieces and milled in electrical grinder into powder. The 100 g powder of the bark was sieved through a common strainer to remove the larger sized bark remnants. The 20 g of bark powder was immersed in 100 ml of methanol and kept for 24 h with occasional shaking. The supernatant containing active principles were collected by filtering through whatman filter paper (no 41) and the residue was remerged 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 freeze dried at -55 °C and stored at -20 °C until use.

Propagation of *T. equi* in MASP culture
The continuous propagation of *T. equi* parasites required low oxygen (3%) and carbon dioxide (5%) environment in *in vitro* culture conditions. The MASP culture technique was first introduced by Holman et al. (1994) [10] followed by Zweygarth et al. (1995) [7] and Avarzed et al. (1997) [8] using different media and culturing conditions. Briefly, the infected blood from seropositive animal was collected in anticoagulant added (EDTA) tube and transported aseptically to National research centre on equine, Hisar laboratory facility. The blood was centrifuged at 2000 g for 10 min and the plasma was separated. The pelletted erythrocytes were washed by adding double volume of Vega Y Martinez solution and centrifuged at 2000 g for 10 min. The supernatant was aspirated, and the previous procedure repeated for three times. The final washing of erythrocytes was done with media M 199 to condition the erythrocytes to media. In a 24 well cell culture plate (Greiner India) add 1 ml of prepared complete M199 media into wells. The infected erythrocyte of 100 µl volume was added to the wells and every day the supernatant media was replaced by fresh complete M199 media. The complete M 199 media was prepared by adding 40% of horse serum, 0.1 mM hypoxanthine and 100 µg/ml of strepto-penicillin to media M 199. The sub-culturing of culture was performed as per requirement after estimating parasitaemia in the wells.

**Theileria equi** growth inhibition assay in MASP culture
In this study we have followed the growth inhibition assay protocol of Bork et al. (2004) [9] to study effect of drug molecules on *T. equi*. The stock solution of methanolic extract of *A. nilotica* was prepared by dissolving 50 mg of extract in 100 µl of DMSO. The stock solution of 100 µg/ml of imidocarb dipropionate was prepared in distilled water. The working solution of 500 µg/ml and 10 µg/ml of plant extract and standard control drug was prepared by diluting the appropriate volume of stock solution in 6 ml of complete M 199 media to get the working concentration on the day of assay. All tests were performed in triplicate wells. The parasitaemia of MASP culture (maintenance culture) well was recorded and the infected RBCs were washed thrice with VYM solution to clear any remaining media. The assay was initiated with 1 per cent parasitaemia. The infected erythrocytes were mixed with fresh noninfected erythrocytes according to previously determine parasitaemia level to lower the parasitaemia to 1 per cent. The growth inhibition assay was performed in a 48 well cell culture plate (Greiner India). The working solution of 500 µl volume of respective working solution of plant extract, imidocarb and negative control (complete media without added plant extract/drug) were dispensed in to triplicate wells and the wells were seeded with 50 µl of infected erythrocytes to initiate the assay, the supernatant media was replaced with similar working solution on every 24 h. The final parasitaemia was recorded after 96 h of commencement of assay by making Giemsa stained smears from each well. The parasitaemia per cent was calculated by the following formulae;

\[
\text{Parasitaemia per cent} = \frac{\text{Number of infected erythrocytes}}{\text{Number of total erythrocytes observed}} \times 100
\]

Minimum 1000 no. of erythrocytes were counted to calculate the parasitaemia per cent in the growth inhibition assay.

**Theileria equi** parasite’s morphology studies
The morphological changes observed in Giemsa-stained thin smear of treated culture parasites and damaged parasites were compared with the morphology of intact *T. equi* parasites (control cultures) using light microscopy.

**Statistical analysis**
The results were analysed by performing one-way analysis of variance (ANOVA) and differences among the means were determined for significance at *p*≤ 0.05 using GraphPad Prism 7.0 software.

**Results and Discussion**
The effectiveness of drugs for equine piroplasmosis was studied previously in *in vivo* trials involving splenectomised horses or donkeys. The procedure was not suitable for screening of large number of drug molecules simultaneously. Moreover, there was always a risk of accidental transmission of infection to other animals of the farm or localities. The *in vitro* MASP culture system eliminate such risk of maintain infected animals for research purpose. Moreover, the infected
The currently available drugs are not free from side effects. The imidocarb dipropionate has anti-cholinesterase activity, leading to sweating, diarrhoea, colic problems [13]. The toxicity of drug lead to periportal and renal tubular necrosis. The injection site necrosis is common after imidocarb injection. The diminazene aceturate also produce injection site muscle damage and found effective particularly in B. caballi infection [1]. The other drugs were in development phase. The drugs in use in current time not able to provide chemo-sterilisation from T. equi infection from host.

The results of this study clearly indicate the potential of A. nilotica methanolic extract in restricting the parasitic growth. The extract reduced the parasitaemia per cent to 0.53 ± 0.044 percent at treatment concentration of 500 µg/ml (Fig. 1). The 0.5 per cent parasitaemia is the lowest limit of sensitivity in microscopic detection. However, standard drug imidocarb dipropionate could reduce the parasitaemia level to 1.46 ± 0.042 per cent after 96 hr of treatment (Fig. 1). At the end of the study, the parasitaemia percent of negative control reached the height of 7.53 ± 0.277 per cent (Fig. 1). There was significance difference (p<0.05) of parasitaemia per cent of plant treated extract, imidocarb dipropionate and negative control wells.

Fig 2: Giemsa stained smears of treated wells erythrocytes showing morphological changes in T. equi parasites after treatment A. Acacia nilotica methanolic extract, B. Imidocarb dipropionate and C. Negative control without added drug/plant extract.

The morphological comparison of parasites after completion of assay period revealed the parasites in negative control culture exhibited various intraerythrocytic stages including merozoites, trophozoites, dividing parasites with two nuclei located each to oppositely within cytoplasm of parasite and very clear demarcation between nucleus and cytoplasm of parasite. The merozoites of T. equi occurred in erythrocytes as a typical pyriform body, frequently in pairs at acute angles to each other. Trophozoites were single and spherical or pleomorphic in shape. The characteristic maltase cross (tetrad) of dividing parasites can be appreciated in negative control wells (Fig. 2). But the treated culture parasites or
damaged parasites appeared in dot like as pyknotic nucleus which was usually irreversible condensation of chromatin in the nucleus and there was no demarcation between nucleus and cytoplasm of parasite. Also, the dead parasites have changed colour into blackish from pink (normal parasite). *Acacia nilotica* plant has been traditionally used for treatment of fever, cold, bronchitis, diarrhoea, leukoderma, diabetes etc [14]. The twig of the plant is being used as tooth brush and believed to be helpful in bleeding gums, mouth sore, mouth ulcers, loose teeth etc. The leave of the tree is being used as fodder for livestock [15]. The pharmaceutical properties like anti-cancer, antimitogenic [16], anti-pyretic [17], anti-plasmodial [18], anti-asthamatic, molluscicidal, anti-fungal [19], inhibitory activity against Hepatitis C virus (HCV) [20] and human immunodeficiency virus (HIV)-I [21] of different parts of *A. nilotica* claimed by different researcher in current time.

*Acacia nilotica* plant is rich in polyphenolic compounds, tannins, saponins etc.? The pharmaceutical properties of the plant mostly attributed to its rich polyphenolic components. The poly phenols mostly consist of tannins, flavonoids, gallic acids, ellagic acids, catechin, epigallocatechin-7-gallate etc [22]. The plant extracts of *A. nilotica* also effective against other protozoan parasites like leishmanial [23], anti-giardial [24], antiplasmodial [18]. The effectiveness of *A. nilotica* bark methanolic extract against *T. equi* parasites in vitro culture might be attributed to presence of high polyphenolic component of the plant.

Conclusions

The preliminary study of *Acacia nilotica* (kikar) bark methanolic extract has shown encouraging results against *T. equi* parasite in *in vitro* assay. However, further detailed study is under process to ascertain the IC₅₀ determination, cytotoxicity potentials and *in vivo* studies to validate its efficacy in treatment of equine piroplasmosis.

Acknowledgements

Authors are grateful to the Director, ICAR-National Research Centre on Equines, Hisar, Haryana, India for providing necessary facilities to conduct the research and Head, Division of Veterinary Medicine, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India for administrative support and proper guidance to the first author.

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