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Evaluation of selected medicinal plants for their *in vitro* activity against trypanosomiasis

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Abstract:- Trypanosomiasis, Surra, sleeping sickness, African lethargy is a parasitic disease of people and animals such as cattle, sheep, goats, pigs, horses and donkeys, caused by protozoa of the species *Trypanosomabrucei* and transmitted by the tsetse fly. The disease is characterized by severe anaemia, weight loss, reduced productivity, infertility and abortion, with death occurring in some animals during the acute phase of the disease. Off late, the parasite has gained resistance towards the conventional drugs. The vaccines currently used have low efficacy which lead to the development of alternative drugs. Natural drug from the plants are gaining popularity because of several advantages such as fewer side effect, better patient tolerance, relatively less expensive and its acceptance. Ethanol extracts of Punicagranatum and Catharanthusroseus and Ethyl acetate extracts of Ocimumtenuiflorum are tested for their potential in possessing the anti-trypanosomal activity. The extracts of these three plants were obtained through the solid-liquid extraction using a Soxhlet apparatus. Thus obtained extracts were concentrated in a rotary vacuum evaporator and used for the *In vitro* studies. *In vitro* testing of these three extracts in three different concentrations of 20 mg/ml, 10mg/ml and 2 mg/ml in 10% DMSO was carried out in duplicates in 96 well micro titre plates on the blood infected with *Trypanosomaevansi* by Rapid matching method. Punicagranatum extract of 20mg/ml concentration was found to be most effective showing maximum decline in the motility of trypanosomes. Thin Layer Chromatography, carried out to screen and determine the compounds present in each extract revealed the presence of flavonoids, alkaloids and the bitter compounds.

Keywords: *Trypanosomaevansi*, Punicagranatum, Catharanthusroseusm, Ocimumtenuifloru

I. INTRODUCTION

Trypanosomiasis is one of the major haemoparasitic diseases of domestic animals. The tsetse (Glossina)-transmitted form of the disease is endemic throughout the tropical regions of Africa where the vector is prevalent. The major pathogenic tsetse transmitted trypanosome species are Trypanosomacongolense, T. vivax and T. brucei in cattle, sheep and goats and T. simiae in pigs. Animal trypanosomiasis is also encountered outside the tsetse fly belt, where the most important pathogenic trypanosome species, T. vivax and T. evansi, are transmitted mechanically by biting flies, while T. equiperdum is transmitted sexually. The principal domestic animals affected by T. evansi (*Surra*) are camels, pigs, water buffaloes and cattle. T. equiperdum causes the disease in horses and donkeys [1].Four major epidemics have occurred in recent history: one from 1896 to 1906 primarily in Uganda and the Congo Basin, two epidemics in 1920 and 1970 in several African countries, and a recent 2008 epidemic in Uganda [2].

The conventional medicines in use are toxic organic compound of arsenic, which are highly dangerous in the treatment which are only administered by injection under the supervision of a physician, as they can produce similar effects as arsenic poisoning. They are known to cause a range of side effects including convulsions, fever, rashes, bloody stools, loss of consciousness, nausea, and vomiting. Most of them are fatal in and of itself in around 8% of cases [3].

The parasites enter the human host through mucus membranes in the nose, eye, or mouth upon release from the insect vectors. Left untreated, Chagas' disease may cause dementia, megacolon, and megaesophagus, and damage to the heart muscle, and may result in death [4] [5].Acute cases are treated with nifurtimox and benznidazole, but there is currently no effective therapy for chronic cases [6].Since disease causing pathogens such as *Plasmodium falciparum, Trypanosomabrucei*are developing resistance to the conventional medicine used, an alternative approach to the drug development has been initiated and this involves the plant materials as a source [7] [8] . The parasite *Trypanosoma* has acquired resistance to most of the conventional drugs used and these drugs pose a lot of side effects which increase the risk of death. The drugs being used in treating this disease, are known to contain toxic compounds which can be fatal when administrated [9].

The discovery of these potent antitrypanosomal extracts from these plants has increased their potentials to provide lead compounds for the development of new natural drugs for effective treatment of sleeping sickness. It has also justified the claim that some medicinal plants of Nupeland possess antitrypanosomal activity and could be useful in the management of trypanosomiasis[10].In the Indian subcontinent's ancient Ayurveda system of medicine, the Punicagranatum has extensively been used as a source of traditional remedies for thousands of years. Thus Punicagranatum is considered a healthful counterbalance to a diet high in sweet-fatty (*kapha* or earth) components [11]. Ocimumtenuiflorum (sometimes spelled Tulasi), or Holy Basil, is a plant native to the Indian subcontinent. This small, aromatic sub-shrub is a cornerstone of Ayurveda (traditional Indian herbal medicine) and is revered in the Hindu religion for its many medicinal uses. Researchers evaluating its usefulness in the treatment of African trypanosomiasis found out its water extract were effective against trypanosomes [12].Madagascar or "Rosy" Catharanthusroseus is by far the most important plant medicinally, generating hundreds of millions per year in the pharmaceutical industry as a promising addition to the arsenal against childhood leukemia, Hodgkin's Disease, testicular cancer and cancerous tumors, high blood pressure, and uncontrolled bleeding, not to mention the fact that it has powerful tranquilizing properties. Unfortunately, this plant can also be poisonous if used inappropriately. Therefore, the remainder of the Rosy Catharanthusroseus section is for informational purposes only [13].

II. MATERIALS AND METHOD STUDY AREA:

Investigation of antitrypanosomal activity of traditionally used plants has been a major area of contemporary research focus. In this project, the therapeutic potentials of the crude extract of three medicinal plants namely Ocimumtenuiflorum, Punicagranatum and Catharanthusroseusare investigated. The research was carried out at the Biochemistry Department in IISc (Indian Institute of science), Bangalore, Karnataka, India.

Plant Selection:

Ocimumtenuiflorum, Punicagranatum and Catharanthusroseus plant parts (stem, seeds and leaf) were selected based on their medicinal properties and their potential in having trypanocidal activity due to the flavonoids, isoflavonoids, alkaloids and the bitter taste content in them. These plant parts were collected from rural and sub-urban areas of Bangalore.

Figure 1 Washed and cleaned plant materials

Preparation of crude extract

The leaves, stems and seeds of the plants were washed with water, cleaned and dried in the hot air oven for 48 hours at 40°-50° Celsius. The plant materials were allowed to dry completely till the condition suitable for grinding. The dried parts were collected and ground into powder separately using an electric grinder [14]. 10 g of the plant powder was weighed and macerated in 150ml of Organic solvents. Punicagranatum and Catharanthusroseus plant powder were added in 150 ml of Ethanol while Ocimumtenuiflorum powder was added in 150 ml of Ethyl Acetate. Extraction was carried out using Soxhlet apparatus and was continued until the solvent completely turned colourless. Soxhlet apparatus was washed thoroughly and cleaned before reusing it for next plant extraction. For quick extraction, solid – liquid extraction was carried out in an air tight flask which was continuously stirred and filtered using Whatman No 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at 40 °C using a rotary vacuum evaporator with the recovery of the solvent. All extracts were then stored in the refrigerator at 4°C until required.

Figure 2 Extraction using soxhlet apparatus

Figure 3 Filtrate -after solid-liquid extraction

III. EXPERIMENTAL ANIMALS

Swiss albino female mice of 6-8 weeks old were acquired from the Animal centre Facility, Indian Institute of science, Bangalore, Karnataka, India. The mice were kept in cages in the Research laboratory of the Department of Biochemistry, Indian Institute of science, Bangalore, Karnataka, India and were allowed to acclimatize for 7 days before the study. All mice were fed with commercial pellets and watered *ad libitum* throughout the duration of the study.

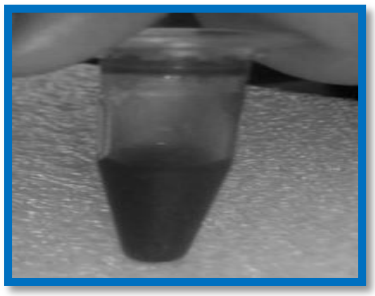
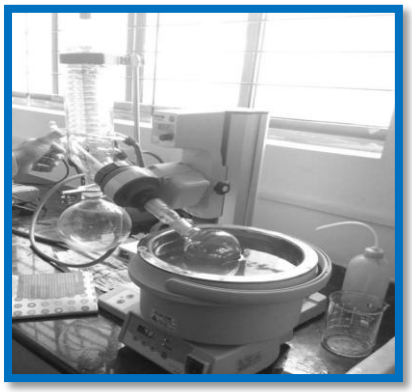


Figure 4 Rotary vacuum evaporation



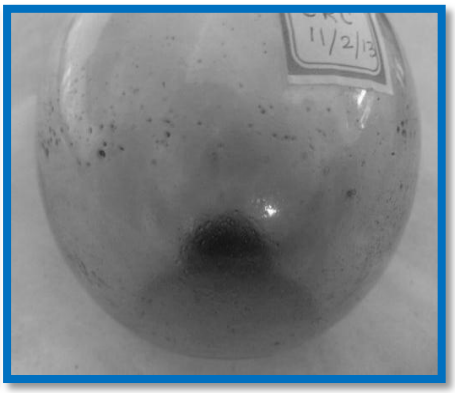


Figure 5, 6, Concentrated extract obtained and stored in a vial

Test organisms

Bloodstream trypomastigote forms of *Trypanosomaevansi* stored in glycerol with PBS-G at -20°C. On passaging, 25- 30 parasites per field, on a wet mount, were introduced intraperitonially into Swiss Albino mice of 6-8 weeks in 0.1 ml of blood. The parasitemia was checked by preparing the tail smears everyday till it reached a count of 16-32 per field. Blood from the infected mice were later obtained by cardiac puncturing and

stored in PBS-G [15]. The Phosphate Buffer Saline was prepared freshly with NaCl 8g, KCl 0.2g, Kh_2PO_4 0.2g, Na_2HPO_4 1.15g, Glucose 40g for 1L and autoclaved. On cooling, Glucose of required amount was added and the pH was set to 7.4. The Glucose in the PBS-G was the energy source required by the trypanosomes for their motility.

***In vitro* testing of the plant extracts for their anti-trypanosomal activity**

Sample preparation

The three different plant extracts were weighed into Eppendorf tubes and dissolved in 10% dimethylsulfoxide (DMSO) in PBS to produce extract solutions of concentrations 20.0 mg/ml, 10.0 mg/ml and 2.0 mg/ml. Extract solutions were prepared just before use.

Assessment of *In vitro* trypanocidal activity

Infected blood was obtained by cardiac puncturing of mice at peak parasitemia. Assessment of *In vitro* trypanocidal activity was done in duplicates in 96 well micro titer plates. 20µl of blood containing about 20-25 parasites per field was added in each of 96 well micro titer plates and was mixed with 5µl of 20.0 mg/ml, 10.0 mg/ml and 2.0 mg/ml of each of Ocimumtenuiflorum, Punicagranatum and Catharanthusroseus plant extracts accordingly. This produced effective test concentrations of 4 mg/ml, 2 mg/ml and 0.4 mg/ml, respectively. After incubation period of 5 minutes further analysis was carried out.

Cessation or drop in motility of the parasites in extract treated blood compared to that of parasite-loaded control blood without extract was taken as a measure of antitrypanosomal activity. The shorter the time of cessation of motility of the parasite, the more active the extract was considered to be (Atawodi et al., 2003). Duplicates of untreated blood and parasites suspended in 10% DMSO alone acted as controls to ensure that the effect monitored was that of the extract alone [15] [16].

After 5 min incubation in closed Eppendorf tubes maintained at 37°C, about 5µl of test mixtures mixed with equal volumes of the stain, trypan blue were placed on separate hemocytometer slides and covered with cover slips. The hemocytometer count of parasitemia according to the Rapid Matching Method was determined and recorded.

Usage of trypan blue dye to stain the parasites is scheduled just prior to the counting under hemocytometer to ensure the decrease in motility rate is only due to the testing extracts and not due to the dye used. The above process is carried out for 60 minutes with an interval of 5 minutes. Graph of the same is plotted to study the decline in parasite count per field under the microscope [17] [18] [19].

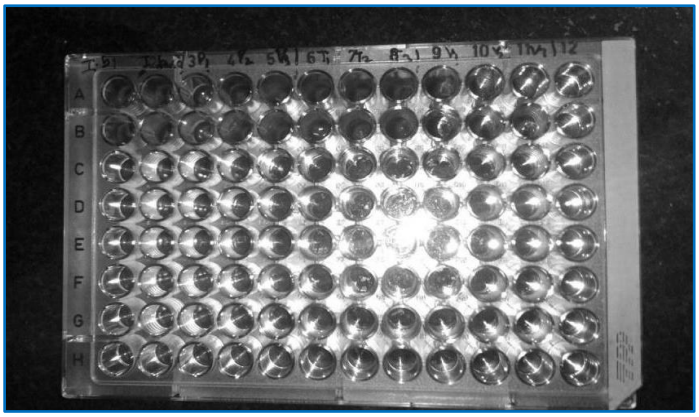


Figure 7 In vitro testing of anti -trypanosomal activity on 96 well titre plates.

On determining the ability of each extract to be antitrypanosomal, the extract of a particular concentration that has shown the highest activity was selected and the *In vitro* test was carried out again with the extract with a low initial parasitemia. The infected blood without any treatment and the infected blood with 10% DMSO were taken as controls. The parasitemia was monitored by the Rapid Matching Method. The effect of the ethanol extract of Punicagranatum leaves at different concentrations was measured for 80 minutes with an interval of 10 minutes.

Screening of the Extracts Using Thin Layer Chromatography

Thin layer chromatography is a chromatography technique used to separate mixtures. Chromatography technique was followed to screen the secondary metabolites such as for flavonoids, alkaloids and the bitter compounds in the plant which are known for their pharmacological properties. Due to the cost effectiveness, reproducibility and simplicity, thin layer chromatography was carried out.

Screening for Flavonoids

The plant material that was extracted with ethanol and ethyl acetate was filtered and used for chromatography. The adsorbent used was the silica gel $60F_{254}$ pre-coated TLC plates. The solvent system of chloroform-ethyl acetate in the ratio 60:40 was used. The plant extracts were applied onto the TLC plate and dried. The TLC plate was then placed into the chamber that was saturated with the chromatography solvents. Detection: The residual solvents were thoroughly removed from the silica gel layer with the aid of the hot air blower. Detections were made without any chemical treatment under UV-254*nm* and UV-365*nm.*

Screening for Alkaloid

The plant material that was extracted with ethanol and ethyl acetate was filtered and used for chromatography. The adsorbent used was the silica gel $60F_{254}$ pre coated TLC plates.

The solvent system of ethyl acetate-methanol-water in the ratio 100:13.5:10 was used. The plant extracts were applied onto the TLC plate and dried. The TLC plate was then placed into the chamber that was saturated with the chromatography solvents. Detection: The residual solvents were thoroughly removed from the silica gel layer with the aid of the hot air blower. Detections were made without any chemical treatment under UV-254*nm* and UV-365*nm.*

Screening for Bitter principle drugs

The plant material that was extracted with ethanol and ethyl acetate was filtered and used for chromatography. The adsorbent used was the silica gel $60F_{254}$ pre coated TLC plates. The solvent system of ethyl acetate-methanol-water in the ratio 77:15:8 was used. The plant extracts were applied onto the TLC plate and dried. The TLC plate was then placed into the chamber that was saturated with the chromatography solvents. Detection: The residual solvents were thoroughly removed from the silica gel layer with the aid of the hot air blower. Detections were made without any chemical treatment under UV-254*nm* and UV-365*nm.*

IV. RESULTS AND DISCUSSIONS

Investigation of antitrypanosomal activity of plants has been a major area of contemporary research focus. In our present studies we investigated the therapeutic potentials of the crude extract of medicinal plants, Punicagranatum, Catharanthusroseus and Ocimumtenuiflorum, which were used for the *In vitro* screening for antitrypanosomal activity against *Trypanosomaevansi* which is the etiological agent for *Surra*, one of the most serious protozoan diseases in Africa and India. On conducting the *In vitro* screening, the extracts of Catharanthusroseus and Ocimumtenuiflorum showed no significant decrease in the parasitemia whereas the Ethanol extract of Punicagranatum leaves of concentration 20mg/ml showed a remarkable decrease in the parasitemia.The Punicagranatum extract of concentration 20mg/ml was thus selected for the further analysis with low initial parasitemia. The percentage decline in the number of parasites for an interval of 60 minutes was plotted. *In vitro* Analysis of the three plant extracts in three different concentrations of 20mg/ml, 10mg/ml and 2mg/ml are shown in the table below

TIME	Infected	P20	P10	P2	DMSO
0	28	28	28	28	28
10	27.9	20.9	22	23	26
20	27.2	19.8	21.2	20.8	25.2
30	26.8	19	20.6	20.5	24.3
40	26.4	18.1	19.6	20.1	23.5
50	24.9	16.8	18.5	19.8	23.1
60	23.6	15.1	17.4	19.5	20.4

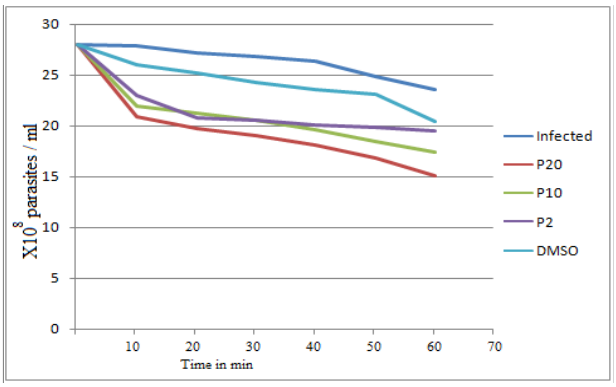


Table 1 - P20, P10 and P2 depict *in vitro* analysis of Ethanol Punicagranatum extracts with concentrations 20mg/ml, 10mg/ml and 2mg/ml respectively, showing the X10^8 parasites count/ml. Time in minutes. Graph 1 - The parasitemia in the blood treated with Ethanol Punicagranatum Extract

TIME	Infected	V20	V10	V2	DMSO
0	28	28	28	28	28
10	27.9	23	26.5	27.8	26
20	27.2	21.6	26.1	27.5	25.2
30	26.8	20	25.8	26.4	24.3
40	26.4	19.9	23	23.5	23.5
50	24.9	19.8	22	22.8	23.1
60	23.6	19.7	20.8	22.3	20.4

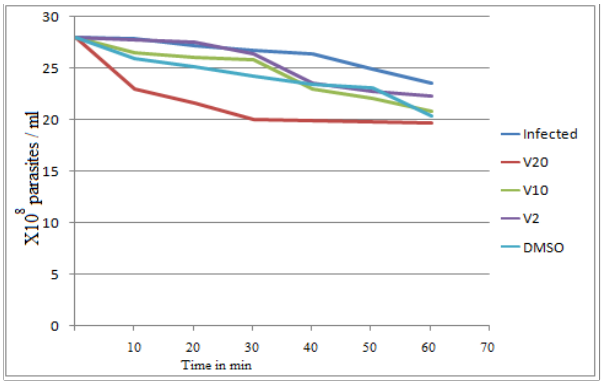


Table 2 - V20, V10 and V2 depict *in vitro* analysis of Ethanol Catharanthusroseus extracts with concentrations 20mg/ml, 10mg/ml and 2mg/ml respectively, showing the X10ˆ8 parasites count/ml. Time in minutes. Graph 2 - The parasitemia in the blood treated with Ethanol Catharanthusroseus Extract

TIME	Infected	T20	T10	T2	DMSO
0	28	28	28	28	28
10	27.9	24.5	27.6	27.1	26
20	27.2	24.2	27	26.4	25.2
30	26.8	23.8	23.9	25.9	24.3
40	26.4	23.1	23.9	23.8	23.5
50	24.9	21.6	22.3	23.2	23.1
60	23.6	20.8	20.5	22.6	20.4

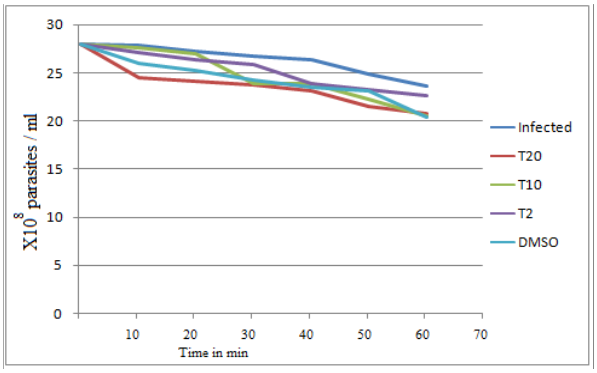


Table 3 - T20, T10 and T2 depict *in vitro* analysis of Ethyl acetate Ocimumtenuiflorum extracts with concentrations 20mg/ml, 10mg/ml and 2mg/ml respectively, showing the X10ˆ8 parasites count/ml. Time in minutes.Graph 2 - The parasitemia in the blood treated with Ethyl acetate Ocimumtenuiflorum Extract.

Screening for Flavonoids:

Pe- Ethanol Extract of Punicagranatum leaves Ve and Vm- Ethanol extracts of Catharanthusroseus LeavesT-ea- ethyl acetate extracts of Ocimumtenuiflorum leaves TLC plates observed under the Ultraviolet light of 254*nm*, Punicagranatum extract showed a thick band of flavonoids compared to the other two extracts.

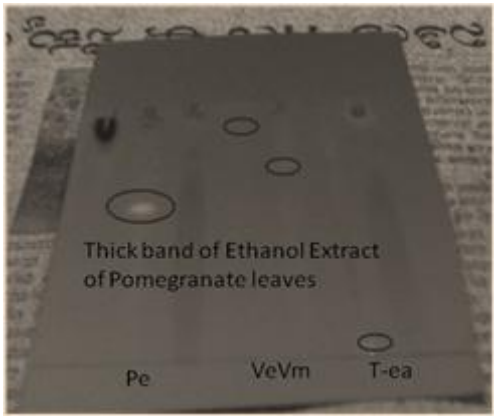


Figure 8 TLC of the plant extracts viewed under UV for Flavonoids

Screening for Alkaloid:

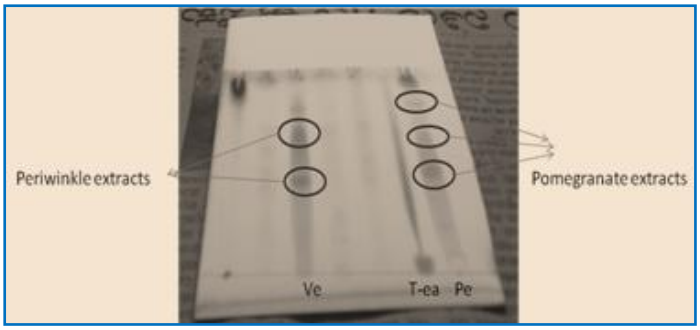


Figure 9 TLC of the plant extracts viewed under UV for Alkaloids

TLC plates observed under the Ultraviolet light of 365*nm*, Punicagranatum extract showed

Screening for Bitter compounds:

TLC plates observed under the Ultraviolet light of 254*nm*, Punicagranatum extract showed a single visible band of bitter principle compound compared to the other two extracts.

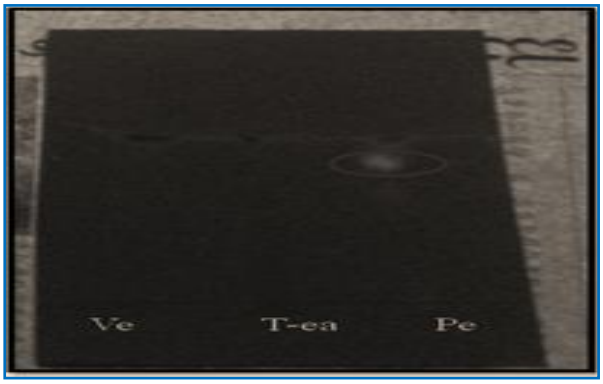


Figure 10 TLC of the plant extracts viewed under UV for bitter principle drugs

Based on the above results, the ethanol and ethyl acetate extracts of Catharanthusroseus and Ocimumtenuiflorum respectively showed no significant decline in the level of parasitemia. The graph showing the variation in the parasitemia due to the ethanol extracts of Punicagranatum leaves, we can conclude that there is a significant decline in the level of parasitemia. Hence, for further analysis, 20mg/ml concentration of the ethanol extracts of Punicagranatum was selected with lesser parasitemia count of $X10^7$ parasites per ml.

Time	Infected	P 20mg/ml	Infected + DMSO
0	9.6	9.6	9.6
10	9.5	8	9.4
20	9	6.7	8.7
30	8.5	3.9	7.6
40	7.4	2.1	6.9
50	7	1.4	6.4
60	6.5	1.1	5.4
70	6	0.5	5.2
80	5.1	0.2	3.7
90	4.5	0	3

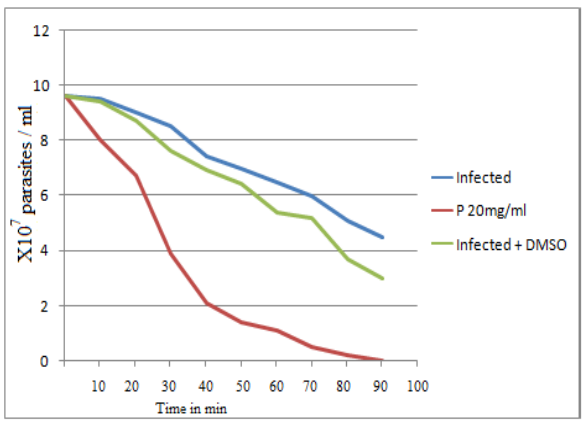


Table 4 - P20 depict *in vitro* analysis of Ethanol Punicagranatum extracts with concentrations 20mg/ml, showing the X10ˆ7 parasites count/ml. Time in minutes. Graph 1 - The parasitemia in the blood treated with Ethanol Punicagranatum Extract

Punicagranatum extract at concentrations of 20mg/ml was found to be most effective showing maximum decline in the motility of trypanosomes. Hence, the thin layer chromatography results indicate that the Punicagranatum leaves contain compounds that are high in medicinal properties like the flavonoids, alkaloids

other secondary metabolites which contribute to the trypanocidal activity. The extracts showing significant results in the *In vitro* test have to be analysed for their action against Trypanosomes *in vivo* to completely accept that the plant has the compounds that can act as a drug to treat Trypanosomiasis. On accomplishing the *in vivo* analysis, a 2D Gel Electrophoresis can be carried out to detect the variation of protein expression caused by the plant extract compared to the blood untreated with any drug. The crude extract of the plant can be separated out using a column chromatography or other separation techniques and the pure extracts obtained can be screened for their trypanocidal activity.

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