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"Antitrypanosomal Activity of *Mucuna pruriens* Seed Crude Oil Extract: In Vitro and In Vivo Insights from Balb/c Mice"

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Abstract.
Background: *Trypanosoma evansi, the causative agent of surra, is a significant pathogen affecting livestock, leading to substantial economic losses. While Suramin is a standard antitrypanosomal drug, concerns about drug resistance and cytotoxicity have prompted the search for alternative treatments. Mucuna pruriens Seed Crude Oil Extract (SCOEMP) has emerged as a potential antitrypanosomal agent due to its bioactive compounds.*

Aim: *This study aimed to evaluate the antitrypanosomal efficacy of varying concentrations of SCOEMP in vitro and in vivo, comparing its effectiveness and cytotoxicity with Suramin.*

Method: *The study involved in vitro testing of SCOEMP at concentrations of 11.6mg/ml, 23.2mg/ml, and 46mg/ml, with Suramin at 3.5g/kg as a control. The antitrypanosomal activity was measured against a positive control (untreated group). In vivo studies involved the administration of SCOEMP at 100mg/ml and 400mg/ml post-infection, with parasitemia levels recorded over time. Cytotoxicity and selective indices were also assessed.*

Results: *In vitro results showed statistically significant antitrypanosomal activity across all concentrations of SCOEMP (3.56±1.12, 2.28±0.49, and 1.51±0.44, $p < 0.0001$), indicating dose-dependent efficacy. Suramin exhibited strong antitrypanosomal activity (0.553±0.48), but with a higher cytotoxicity IC50 (26.4±1.46). SCOEMP demonstrated relative non-toxicity, with a cytotoxicity value exceeding 100 and selective indices of (>28.0, >43.8, and >66.1). In vivo, SCOEMP showed a statistically significant reduction in logarithmic parasite density at both 100mg/ml and 400mg/ml, suggesting its effectiveness in reducing parasitemia.*

Conclusion: *The results indicate that Mucuna pruriens Seed Crude Oil Extract has promising antitrypanosomal properties in vitro and in vivo, with lower cytotoxicity compared to Suramin. These findings support further research into SCOEMP as an alternative treatment for Trypanosoma evansi infections, with a focus on optimizing doses and exploring its mechanisms of action in clinical settings.*
Keywords: *Antitrypanosomal Activity;Mucuna Pruriens Seed Crude Oil Extract;Trypanosoma Evansi;Cytotoxicity and Selective Index;In Vitro and In Vivo Analysis;Suramin Alternative Therapy*

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I. Introduction:

Trypanosoma evansi is a protozoan parasite and a causative agent of surra, a debilitating disease affecting a range of animals, including horses, camels, dogs, and livestock (Ereqat et al, 2020). This parasite has a broad distribution, predominantly in tropical and subtropical regions, posing significant economic and health challenges in agriculture and animal husbandry(Fiorin et al, 2023). Although primarily an animal pathogen, *T. evansi* has also been reported in humans, underlining its importance in both veterinary and medical contexts(Boushaki et al, 2019). This essay explores the infection dynamics, epidemiology, life cycle, transmission, laboratory diagnosis, and treatment options for *T. evansi*.

Infection with *T. evansi* results in surra, a disease characterized by intermittent fever, weight loss, anemia, and progressive weakness. If untreated, surra can lead to severe complications, including neurological disorders and death. The impact of surra on animal health and productivity is significant, leading to substantial economic losses in regions where it is endemic(Cuypers et al, 2017).

Trypanosoma evansi has a widespread distribution across Asia, Africa, and South America. It is particularly prevalent in areas with extensive livestock rearing and poor veterinary infrastructure. The

epidemiology of *T. evansi* is influenced by various factors, including the presence of suitable vector species, animal movement, and climatic conditions. The parasite is known to affect a broad host range, contributing to its transmission and persistence in different environments(Kamidi et al, 2017).

Unlike other trypanosomes, *T. evansi* does not rely on the tsetse fly for transmission. Instead, it is mechanically transmitted by biting insects, such as horseflies and stable flies. This mode of transmission gives *T. evansi* a unique life cycle, where the parasite circulates in the blood of its host and can be transmitted to new hosts through insect bites. Once in the bloodstream, *T. evansi* undergoes rapid multiplication, contributing to the spread of infection(Kamidi et al, 2017).

The primary mode of transmission for *T. evansi* is mechanical, involving blood-feeding insects like tabanids (horseflies) and stomoxys (stable flies). These vectors acquire the parasite from infected hosts and can transmit it to new hosts during subsequent blood meals (Mirshekar et al, 2017). The mechanical transmission does not require a complex developmental stage in the vector, as seen with other trypanosomes, which makes *T. evansi* highly adaptable to different environments and capable of spreading quickly(Dkhil et al, 2022).

Diagnosing *T. evansi* infection involves a combination of clinical assessment, microscopic examination, and molecular techniques. Microscopy is used to detect the presence of the parasite in blood smears, but this method has limitations due to intermittent parasitemia (Selim et al, 2022). Molecular methods, such as polymerase chain reaction (PCR), offer greater sensitivity and specificity, allowing for early detection and accurate diagnosis. Serological tests, like ELISA, are also used to detect antibodies against *T. evansi* in animal sera(Al-Kharusi et al, 2022).

Treatment of *T. evansi* infection primarily involves antiparasitic drugs. The most commonly used drugs include suramin and diminazene aceturate, which are effective in reducing parasitemia. However, drug resistance is an emerging concern, necessitating the development of new therapeutic strategies. In addition to drug treatment, control measures such as vector control, animal management, and quarantine practices are crucial to prevent the spread of infection (Al-Kharusi et al, 2022).

Orthodox treatment methods for *T. evansi* focus on using antiparasitic drugs to manage and eradicate the infection. However, several challenges are associated with these treatments, including drug resistance, adverse effects, and accessibility issues (Mazzeti et al, 2021). Two commonly used drugs are suramin and diminazene aceturate, both drugs have proven effective in treating *T. evansi* infections, but they require careful administration and monitoring due to potential side effects.

Mucuna pruriens seed crude oil contains a complex mixture of bioactive compounds, including fatty acids, alkaloids, flavonoids, and other secondary metabolites. These compounds are known to possess a range of biological activities, such as anti-inflammatory, antioxidant, and antiparasitic effects (Okou et al, 2023). The diverse composition of the oil suggests that it could exert multiple effects on trypanosomal parasites, offering a multi-faceted approach to combating infections like surra(Okou et al, 2023).

Recent studies have investigated the antitrypanosomal properties of *Mucuna pruriens* seed crude oil. The proposed mechanism of action involves interfering with the parasite's cellular processes, such as disrupting membrane integrity, inhibiting key enzymes, or causing oxidative stress, leading to the parasite's death. Additionally, some bioactive compounds in the oil may enhance the host's immune response, helping to combat the infection more effectively (Rezaeilaal et al, 2024).

In vitro studies have shown that *Mucuna pruriens* seed crude oil can inhibit the growth of *Trypanosoma evansi*, indicating its potential as an antitrypanosomal agent. In vivo studies involving animal models, such as Balb/c mice, have also demonstrated the oil's efficacy in reducing parasitemia and mitigating the symptoms associated with surra. These findings suggest that *Mucuna pruriens* seed crude oil could be a viable alternative or complementary treatment for *T. evansi* infections.

II. MATERIALS AND METHOD

Experimental Animal:

60 Mice for antiparasitic and toxicity Studies in vivo and 12 immune Modulatory and antiparasitic In-vitro Studies. The mice were acclimatized for seven days in the Animal Facility located in the Larkin Building within the Faculty of Health Science at the University of Benin. During this period, the mice were housed in well-ventilated cages and provided with daily cage cleaning and a constant supply of food and water at a room temperature of 37°C. The mice will be fed with commercially prepared vital food to maintain uniform nutrition.

Acute Toxicity Studies:

The acute toxicity studies on Mucuna pruriens seed crude oil extract-SCOEMP were estimated using 6 mice subdivided into 3 groups of 2 each using standard methods of Ibeh *et al*., (2000). To each group administered different concentrations of SCOEMP, (100mg, 200mg and 400mg), the mice were observed physically for signs of toxicity (Stretching, respiratory distress, paw-licking, changes in posture and responsiveness and finally,

mortality) for the first four hours and the number of deaths per group was recorded in a 24hour observation (Alelign et al, 2020).

In-vivo inoculation of Mice with Live *T.evansi*

A fresh culture of *T. evansi* was obtained from Honest Laboratories, United Kingdom and maintained in vitro according to the standard protocol. Healthy male mice 6-8 weeks old and with an average weight of 23g were used for the experiment. The mice's tail was cleaned and disinfected with 70% ethanol afterwards the mice were injected intravenously with approximately 1 x 10^6 *T. evansi* parasites suspended in 1 mL of PBS. Mice were observed for any signs of illness or discomfort and provided appropriate care and treatment as needed. After 14 days, blood samples were collected from the rat by tail vein puncture using heparinized capillary tubes (Baldissera et al, 2017).

Determination of Parasitaemia

A thin blood smear was prepared using a microscope slide and it was air-dry afterwards, the smear was fixed by immersing it in 100% methanol for 2-3 minutes. Slides were stained with Giemsa stain for 30-60 minutes and rinsed with distilled water (Isaiah et al, 2021). Afterwards, parasite identification and density were determined under a light microscope at 1000x magnification to confirm the presence of *T. evansi* parasites.

In vitro Determination of Anti Trypanosomal Activity of Seed Crude oil Extract of Mucuna pruriens

The evaluation of antitrypanosomal activity for extracts, fractions, and the active compound followed the methodology outlined by Otoguro et al. (2008), with certain adjustments made to the sample concentrations. To create the stock solution, 2.9mg of the test samples were dissolved in 250 µl of absolute ethanol (EtOH), resulting in an 11.6 mg/ml concentration. Subsequently, the stock solution was further diluted with distilled deionized water (ddH2O) to generate seven distinct final concentrations, ranging from 0.03 to 6.25 µg/ml and 0.001 to 0.100 µg/ml, respectively.

In a formal tone, the extensively recognized trypanocidal medication, Suramin (acquired from Sigma-Aldrich, USA), served as the positive control in an in vitro antitrypanosomal assay. Precise measurements of the drug were taken and subsequently dissolved in 100% dimethyl sulfoxide (DMSO, sourced from Sigma-Aldrich, USA) to produce a stock solution with a concentration of 40 mg/ml. Following this, the stock solution underwent further dilution in a round-bottom 96-well plate to create six concentrations, ranging from 0.195 to 6.25 µg/ml.

Human Cell Culture

Initiating the process of reviving the HepG2- human liver cells (American Tissue Collection culture ATCC), we carefully retrieved the cryogenic vial containing them from the liquid nitrogen tank. We gently thawed the cells in a 37°C water bath, enabling them to transition gradually from their frozen state. Afterwards, we transferred the revitalized cells to a nurturing environment, which consisted of 5 ml of Dulbecco's Modified Eagle's Medium (DMEM) sourced from Sigma-Aldrich, USA, as detailed in Appendix 1.7. This medium was supplemented with essential nutrients, including 5% (v/v) Fetal Bovine Serum (FBS) from PAA, Austria, and protective antibiotics, 1 U/ml penicillin and 50 µg/ml streptomycin, also from PAA, Austria. The T-25 cm2 tissue culture flasks provided the ideal surface for the growth and development of the cells. The next phase of the experiment was conducted in a humidified incubator where a controlled atmosphere of 5% CO2 at 37°C was maintained, as outlined by Siti Syarifah et al. in 2011. During this period, the HepG2-cell lines thrived and formed healthy adherent cells that congregated to create a single-layer culture within the flasks.

Cytotoxicity assay

To assess the cytotoxicity of the test substance on healthy liver cells, we passed the HepG2-cells in suspension through centrifugation. After centrifugation, we obtained a cell pellet, which we then counted using the Neubauer haemocytometer chamber. We then plated a 100 µl cell suspension in 96-well plates, following the protocol outlined by Jean-Robert et al., (2009), ensuring a density of 4.0 × 10^4 cells per well. The plates were incubated for 24 hours in a humidified incubator at 37°C, allowing the cells to attach securely to the plate.

After concluding the initial incubation period, we replaced the old media with 90 µl of 10% supplemented DMEM. This was followed by the introduction of our test samples, which were carefully diluted to concentrations of 0.1, 0.39, 1.56, 6.31, 25.0, and 100 µg/ml. We also included negative solvent controls, where cells were treated with 5% (v/v) ethanol and 25% (v/v) DMSO but excluded the test samples. In addition, control-blank wells containing sterile MiliQ water were part of our experimental design. 3.5g/kg body weight suramin served as our positive control, introduced at concentrations ranging from 0.1 to 100 ηg/ml. The setup was then left to incubate for 72 hours in a CO_2 incubator.

Cell Viability Study

To evaluate cell viability, we used the Alamar Blue assay (Räz et al., 1997) and extended the incubation period to 72 hours. The impact of our test samples on cell health was assessed by determining the IC_{50} values. A fluorescence plate reader (Mecure Zenit GP200, ENGLAND) was used for analysis, with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The resulting data underwent thorough processing using the Excel program to derive the IC_{50} values. Our commitment to rigorous research practices ensured that the assay was performed in triplicates and was part of at least three independent experiments, presenting the IC_{50} values as a mean ± standard deviation (Otuguro et al., 2008).

Selective Idex Assay:

In our research, the Selectivity Index (SI) served as a crucial metric for assessing the relative toxicities of the test compounds in contrast to normal mammalian cells. This index is determined by calculating the ratio of the IC50 value obtained from the cytotoxicity assay on normal cell lines to the IC50 value from the antitrypanosomal assay (Otuguro et al., 2008)

Statistical Analysis

GraphPad Prism 8.4.3 was used for the analysis of data acquired, and a combination of Parametric and non-parametric ANOVA (Paired One-way and Two-way analysis of variance) was adopted after data was passed through a normality test (Shapiro Wilk and D'Agostino & Pearson test) to determine their levels of distribution. P values are indicated as $P<0.05$ as significant and $P>0.05$ as non-significant. Post Hoc analysis was carried out using the Dunns multiple comparison test.

III. Results:

The purpose of this in vitro study was to evaluate the anti-trypanosomal effectiveness of Mucuna pruriens Seed Crude Oil Extract (SCOEMP) at varying concentrations and compare its efficacy against the established treatment control, Suramin 3.5g/kg, as well as an untreated group serving as the positive control. The study aimed to identify the optimal concentration of SCOEMP for antitrypanosomal activity and to assess whether it could be a viable alternative or complement to Suramin.

Comparative analysis of the in vitro anti-trypanosomal effectiveness of various concentrations (11.6mg/ml, 23.2mg/ml, and 46mg/ml) of *Mucuna Pruriens* Seed Crude Oil Extract (SCOEMP) and Suramin 3.5g/kg in contrast to the untreated group (positive control) as seen in Figure 4.1. Our study demonstrates statistically significant antitrypanosomal efficacy across all concentration levels of SCOEMP in vitro (3.56±1.12, 2.28±0.49, and 1.51±0.44, P=<0.0001). Moreover, the treatment control (Suramin 3.5g/kg) exhibits considerable antitrypanosomal activity when compared to the untreated group (positive control) (Figure 4.1).

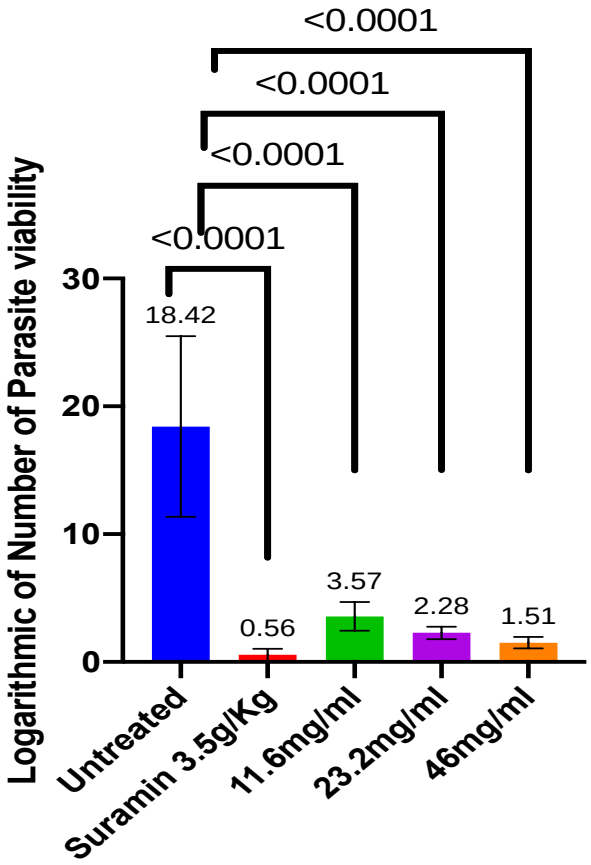

Figure 4.1: "In vitro Comparative Analysis of Antitrypanosomal Efficacy*: Mucuna Pruriens* Seed Crude Oil Extract (SCOEMP) vs. Suramin 3.5g/kg": Comparative analysis of the in vitro antitrypanosomal effectiveness of different concentrations (11.6mg/ml, 23.2mg/ml, and 46mg/ml) of Mucuna Pruriens Seed Crude Oil Extract (SCOEMP) and Suramin 3.5g/kg, contrasted against the untreated group (positive control). The study reveals statistically significant antitrypanosomal efficacy across all SCOEMP concentration levels in vitro (3.56±1.12, 2.28±0.49, and 1.51±0.44, P=<0.0001). Additionally, the treatment control (Suramin 3.5g/kg) demonstrates noteworthy antitrypanosomal activity when compared to the untreated group. n=6, P<0.05= significant, P>0.05=non-significant

Upon administration of 100mg/ml SCOEMP post-infection, minimal antitrypanosomal activity was observed on Day 0. However, as the observational period advanced, a discernible regression in logarithmic parasite density was noted on days 2, 4, and 8 (6.20±0.83, 5.91±0.63, and 5.75±0.53, respectively) in comparison to Day 0 (7.20±0.74). This observed trend achieved statistical significance ($p<0.05$). In the comparative analysis across days 2, 4, and 8, although a decreasing trend in trypanosomal activity was apparent, no statistical significance was observed ($p>0.05$) (Figure 4.2).

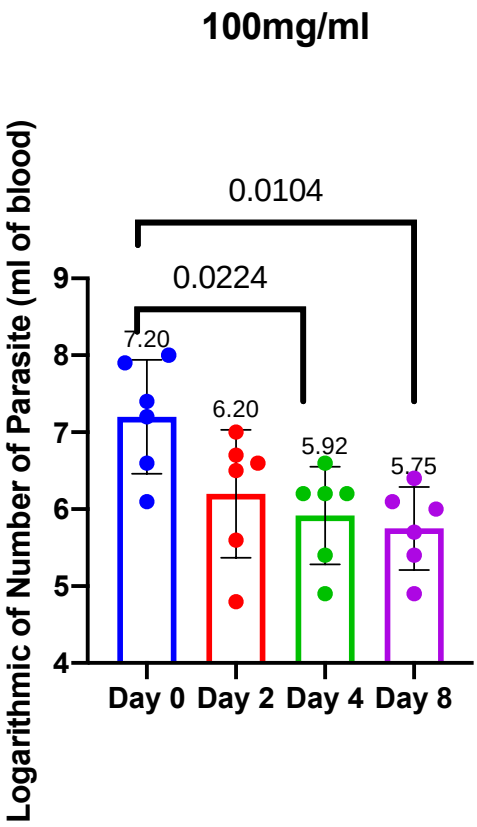

Figure 4.2: **Antitrypanosomal Effect of 100mg/ml SCOEMP on Balb/C Mice Post-Infection**. The bar graphs illustrate the evaluation of the antitrypanosomal effect of 100mg/ml of *Mucuna pruriens* Seed Crude Oil Extract (SCOEMP) on Balb/C mice post-infection, involving a comparative analysis across different exposure time points (days 0, 2, 4, and 8). Upon administration of 100mg/ml SCOEMP post-infection, minimal antitrypanosomal activity was observed on Day 0. However, as the observational period advanced, a discernible regression in logarithmic parasite density was noted on days 2, 4, and 8 (6.20±0.83, 5.91±0.63, and 5.75±0.53, respectively) in comparison to Day 0 (7.20±0.74). This observed trend achieved statistical significance ($p<0.05$). In the comparative analysis across days 2, 4, and 8, although a decreasing trend in trypanosomal activity was apparent, no statistical significance was observed ($p>0.05$). P,< 0.05= significant, p>0.05= Non-significant, n=6.

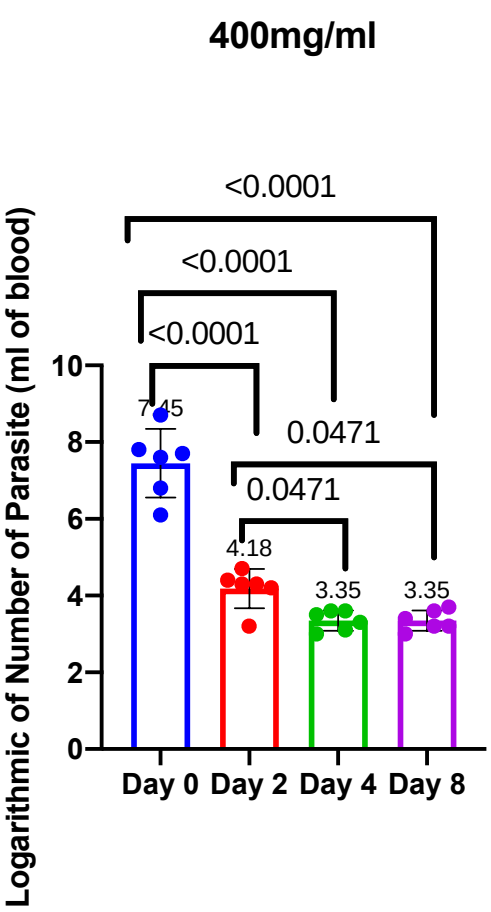

Figure 4.3: **Antitrypanosomal Effect of 400mg/ml SCOEMP on Balb/C Mice Post-Infection**. The bar graph illustrates the evaluation of the antitrypanosomal effect of 400mg/ml of *Mucuna pruriens* Seed Crude Oil Extract (SCOEMP) on Balb/C mice post-infection, involving a comparative analysis across different exposure time points (days 0, 2, 4, and 8). After administering 400mg/ml SCOEMP post-infection, minimal antitrypanosomal activity was observed on Day 0. However, as the observation period progressed, a noticeable decrease in logarithmic parasite density was observed on days 2, 4, and 8 (4.18±0.51, 3.35±0.25, and 3.35±0.26, respectively) compared to Day 0 (7.45±0.89). This trend was statistically significant ($p<0.05$). P,< 0.05= significant, $p>0.05$= Non-significant, n=6.

After administering 400mg/ml SCOEMP post-infection, minimal antitrypanosomal activity was observed on Day 0. However, as the observation period progressed, a noticeable decrease in logarithmic parasite density was observed on days 2, 4, and 8 (4.18±0.51, 3.35±0.25, and 3.35±0.26, respectively) compared to Day 0 (7.45±0.89). This trend was statistically significant ($p<0.05$). In the comparison of days 2, 4, and 8, although there was a declining trend in trypanosomal activity, the peak antitrypanosomal activity was observed on days 4 and 8 with a statistically significant trend ($p<0.05$) (Figure 4.3)

The outcomes of these comparative analyses are systematically presented in Table 4.1. Our findings elucidate that varying doses of SCOEMP, specifically at concentration gradients of 11.6 mg/ml, 23.2 mg/ml, and 46 mg/ml, manifest a discernible antitrypanosomal activity, registering mean values of (3.56±1.11, 2.283±0.49, and 1.513±10.44), respectively. In contrast, Suramin, administered at 3.5 g/kg, exhibits a notably heightened antitrypanosomal efficacy with a mean value of 0.553±0.48. The cytotoxicity assessment of SCOEMP at diverse concentrations in vitro consistently demonstrates a rate exceeding 100, indicative of its relative non-toxicity to cells. Furthermore, the selective indices associated with these concentrations are delineated as (>28.0, >43.8, and >66.1), thereby signifying a moderate selective index for the parasite and a diminished level of toxicity towards host cells. This empirical exploration not only contributes to a nuanced understanding of the antitrypanosomal effects of SCOEMP but also underscores its potential as a therapeutic agent, showcasing a favourable balance between efficacy against the parasite and cytocompatibility with host cells.

Table 4.1: Comparative In vitro Anti-Trypanosomal activity, Cytotoxicity and Selective index of dose gradient of Seed crude oil extract derived from Mucuna pruriens-SCOEMP

Extract Dose Concentrations	Anti-Trypanosomal Activity	Cytotoxicity IC_{50}µg/ml	Selective Index (SI)
SCOEMP 11.6mg/ml	*3.567±1.11	>100	>28.0
SCOEMP 23.2mg/ml	*2.283±0.49	>100	>43.8
SCOEMP, 46mg/ml	*1.513±0.44	>100	>66.1
Suramin 3.5g/kg	**0.553±0.48	26.4±1.46	47.3

Key: ** High Antrypanosomal activity
*Moderate Antitrypanosomal activity, n=6

Selective	SI ≥100
Moderately Selective	10 ≤ SI <100
Not Selective	≤10

IV. Discussion/Conclusion:

This study aimed to assess the in vitro and in vivo antitrypanosomal activity of *Mucuna pruriens* Seed Crude Oil Extract (SCOEMP) across various concentrations and compare its efficacy against suramin, a standard antitrypanosomal drug. The results from the in vitro study, as outlined in Table 4.1, demonstrate a statistically significant reduction in parasitemia across all tested concentrations of SCOEMP, indicating its potential as an effective antitrypanosomal agent. Notably, the study found that higher concentrations of SCOEMP (23.2mg/ml and 46mg/ml) exhibited a more substantial antitrypanosomal effect, with lower parasitemia values (2.283 ± 0.49 and 1.513 ± 0.44, respectively), compared to the positive control.

When compared to Suramin 3.5g/kg, which displayed strong antitrypanosomal activity (0.553 ± 0.48), the antitrypanosomal efficacy of SCOEMP is noteworthy. However, the data suggest that Suramin has greater antitrypanosomal activity this is in tandem with previous study (Abdullahi et al, 2023; Zoltner et al, 2020). Nonetheless, the observed cytotoxicity assessment revealed a significant advantage for SCOEMP, as its cytotoxicity values exceeded 100 across all tested concentrations, indicating relative non-toxicity to host cells. This contrasts with Suramin, which showed a cytotoxicity IC50 of 26.4 ± 1.46, suggesting higher toxicity risks. These findings point to a favourable balance between antitrypanosomal activity and safety for SCOEMP (Amjad et al, 2022).

The in vivo results further support the antitrypanosomal potential of SCOEMP. At a concentration of 100mg/ml, there was a significant reduction in logarithmic parasite density over time, demonstrating an observable decline in parasitemia from day 0 (7.20 ± 0.74) to days 2, 4, and 8 (6.20 ± 0.83, 5.91 ± 0.63, and 5.75 ± 0.53, respectively). The in vivo study also demonstrated that higher concentrations of SCOEMP (400mg/ml) yielded even more pronounced antitrypanosomal activity, with logarithmic parasite density decreasing from day 0 (7.45 ± 0.89) to days 2, 4, and 8 (4.18 ± 0.51, 3.35 ± 0.25, and 3.35 ± 0.26, respectively). This suggests a dose-response relationship, indicating that higher concentrations of SCOEMP result in greater reductions in parasite density(Novaes et al, 2018).

In comparing the selective index, SCOEMP demonstrated higher values (>28.0, >43.8, and >66.1) compared to Suramin (47.3). This indicates a moderate to high selectivity for the parasite, suggesting that SCOEMP has lower toxicity toward host cells, which is a desirable characteristic for therapeutic agents. This observation implies that SCOEMP has the potential to be a safer alternative to conventional antitrypanosomal drugs, with a balance between efficacy and cytocompatibility (Shanmugavel & Krishnamoorthy, 2021).

The results from this study provide a solid foundation for further exploration of SCOEMP as an antitrypanosomal agent. The significant reduction in parasitemia in vitro, coupled with the observed trends in vivo, suggests that SCOEMP could play a role in treating Trypanosoma evansi infections(Kamkaen et al, 2022). However, further research is needed to understand the specific mechanisms of action, optimize dosages, and evaluate the long-term safety of SCOEMP in clinical settings(Shanmugavel & Krishnamoorthy, 2021).

In conclusion, this study highlights the antitrypanosomal potential of Mucuna pruriens Seed Crude Oil Extract. It demonstrates a statistically significant reduction in parasitemia in vitro, with a favourable cytotoxicity

profile and moderate to high selective indices. These findings warrant additional investigation into SCOEMP's efficacy, safety, and application in treating Trypanosoma evansi infections in vivo and clinical practice.

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