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Research Article
Toxicological Evaluation of Polyherbal Medicines used for the Treatment of Tuberculosis in Eastern Cape, South Africa

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Abstract

Background: Polyherbal remedies are widely used for the treatment and management of various diseases in developing countries. These remedies often contain active pharmacological compounds, thus, the evaluation of herbal remedies used for the treatment of tuberculosis in the Eastern Cape province for their toxicity is of great importance. **Materials and Methods:** Nine polyherbal medicines used for the treatment of tuberculosis were assayed for their toxicity using hatchability success and larval mortality of brine shrimp (*Artemia salina* Leach). These remedies were liquid preparations and coded according to their respective place of collection, viz., King Williams Town site A, King Williams Town site B, King Williams Town site C, Hogsback first site, Hogsback second site, Hogsback third site, East London, Alice and Fort Beaufort. **Results:** The percentage hatchability success of 44.42, 42.96 and 39.70% were observed in cysts incubated with herbal preparations from King Williams Town site A, Hogsback first site and Hogsback third site, respectively. The hatching success in these remedies was significantly higher than the positive control (nystatin) and the negative control (sea water) at $p<0.05$. The herbal preparations from King Williams Town site A and East London exhibited significantly more inhibitory hatchability effects with minimum inhibitory concentration values of 2.4 and 2.8 mg mL^{-1}, respectively. The mortality of *A. salina* nauplii incubated in Alice, King Williams Town site B and King Williams Town site C remedies was significantly higher than when larvae was incubated in both controls. Based on Meyer's index, the LD_{50} of each polyherbal medicine was between 2.9-4.0 mg mL^{-1}, the LD_{50} values greater than 1 mg mL^{-1}. **Conclusion:** The polyherbal remedies evaluated in this study are considered non-toxic and are therefore safe for the patients. However, further *in vivo* toxicity tests are required to validate the safe use of these polyherbal remedies.

Key words: Polyherbal, *Artemia salina*, hatchability assay, lethality test, tuberculosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Polyherbal medicines are extensively used in many developing countries for the prevention and treatment of various diseases such as diabetics, wound infection and tuberculosis. These preparations are freely hawked in South Africa and are prevalent in other African societies. They are readily available and affordable. Polyherbal preparations contain a mixture of three or more medicinal plants. The rationale behind the combination of these herbs could not be justified by the traditional healers as it was a practise that they found to be effective. A review of studies into medicinal plants used to treat various diseases revealed that 80% of the people in South Africa make use of herbal remedies for the treatment of ailments at some stage in their life[1].

Despite the widespread use of polyherbal remedies for the treatment of several illnesses, little is known about their toxicity and safety. The evaluation of the toxic action of these remedies is important in order to acquire their maximum benefits to humans, even though they have been proven to be efficacious in pharmacological studies or by clinical evaluation[2]. The reports of patients experiencing adverse effects such as diarrhoea, abdominal pain, ulcer, dizziness, loss of appetite, abortion of pregnancy and stroke caused by these remedies are on the rise[3]. Other dangerous effects include heart attacks, heart-rate irregularities, liver toxicity, seizures, psychoses and death[4,5]. Hence, the need to analyse the physiological effects of polyherbal remedies is imperative.

This toxicity test aims at establishing the therapeutic index, LD_{50}. The greater the index the safer the remedies, the smaller this margin the more chances of producing unwanted effects[6]. In the literature, there is no information on the toxicological study of polyherbal medicines in South Africa to authenticate their usage and guarantee the safety of the users. Thus, the objective of the current study was designed to investigate the toxicity of polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape province using brine shrimp assay.

MATERIALS AND METHODS

Collection of polyherbal medicines: Polyherbal medicines evaluated in this study were purchased from hawkers and healers in five communities namely, Alice, Fort Beaufort, Hogsback, King Williams Town and East London, all within the Amathole district municipality of the Eastern Cape province, South Africa. Each of the liquid remedy was already prepared by the seller and packaged in a 2 L container. They were transported to Medicinal Plants and Economic Development Research Centre, University of Fort Hare for analysis. Each medicine was labelled and coded according to their respective place of collection; namely: King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB).

Sample preparation: Each remedy was filtered using Buchner funnel and Whatman No. 1 filter paper. The filtrate obtained was frozen at -40°C and freeze dried for 48 h using a freeze dryer (Vir Tis benchtop K, Vir Tis Co., Gardiner, NY). The resulting sample was packaged in a clean container. For brine shrimps experiment, each sample was re-suspended in sea water to yield a 20 mg mL^{-1} stock solution.

Assay procedure: The assay was carried out using modified method of Otang *et al.*[7]. Five petri dishes containing 30 mL of filtered sea water each was prepared and a two-fold dilution was set up to yield a series of concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg mL^{-1}) of the polyherbal medicines. A positive control was prepared in test tubes containing nystatin in seawater (30 µL mL^{-1}), while petri dishes containing sea water only served as the blank control.

Brine shrimp (*Artemia salina*) hatchability assay: The *Artemia salina* cysts (Sera, Heidelberg, Germany) were obtained from an aquaculture shop in East London, South Africa. These were used to evaluate the hatchability success of *A. salina* cysts against different concentrations of the polyherbal medicines[8]. Briefly, different concentrations (0.0625-1 mg mL^{-1}) of the herbal remedies and positive control were prepared in sea water. The *A. salina* cysts were stocked at a density of 15 individuals per petri dish containing 30 mL of the incubation medium at varying concentrations. The plates were partly covered and incubated at 28°C under constant illumination in a digital incubator (MRC Laboratory equipment, model LE-509) and aeration. Thereafter, the petri dishes were examined with the aid of a hand lens against a white background that allowed the nauplii to be separated from the shells. The number of free nauplii in each petri dish was counted after every 12 h for 72 h. The percentage of hatchability success was calculated by comparing the number of hatched nauplii in a chosen concentration with the total number of cysts stocked[9]. The minimum concentration of the polyherbal medicines (or control drug) that inhibited the hatching of the cysts was taken as the MIC. All the assays were carried out in three replicates.

Brine shrimp lethality assay: This was carried out in order to determine the toxic level of the polyherbal preparations. About 1 g of the shrimp cysts were introduced into 1 L sea water. The beaker was partly covered and incubated at 28°C under constant illumination in a digital incubator and aeration. After 36 h of hatching, the phototropic nauplii were collected and an aliquot (0.15 mL) containing 15 nauplii was pipette into each petri dish for each remedy solution and controls. The numbers of surviving larvae in each petri dish were counted after every 12 h. The setup was allowed to remain for 72 h under constant illumination. Finally, the percentage of deaths was calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation[9].

The percentage of mortality was calculated as follows:

$$\text{Mortality (\%)} = \frac{\text{(Total nauplii-Alive nauplii)}}{\text{Total nauplii}} \times 100\%$$

Determination of MIC 50 and LD_{50}: The percentage hatchability success and mortality data obtained from the five concentrations of each polyherbal remedy was used to construct a dose-response curve. The Minimum Inhibitory Concentration (MIC 50) was determined as the concentration of the polyherbal remedy/control drug that inhibited hatching of 50% of the cysts. The LD_{50} was taken as the concentration required for producing 50% mortality[10]. The LD_{50} values were determined from the best-fit line obtained by linear regression analysis of the percentage lethality versus the concentration.

Statistical analysis: Statistical analysis was performed on MINITAB version 12 for windows (Minitab Inc., Pennsyvania, USA). One-way analysis of variance was used to test for the effect of concentration and time of exposure of the herbal remedies on the hatchability success of the cysts and mortality of the larvae in comparison to controls[11]. The p-value less than 0.05 were considered significantly different.

RESULTS AND DISCUSSION

Brine shrimp hatchability assay: The percentage hatchability success of *Artemia salina* cysts incubated with different polyherbal medicines are shown in Fig. 1. High hatchability successes of 44.42, 42.96 and 39.70% were observed in the cysts incubated with polyherbal remedies from KWTa, HBfs and HBts, respectively and were significantly higher than the controls. On the other hand, the hatching success of brine shrimps in KWTc, HBss, FB and EL remedies were higher than those incubated in nystatin but lower than those in the sea water. The herbal preparations from AL and KWTb exhibited more inhibitory effects with hatchability successes of 26.59 and 20.37%, respectively, which were lower than the controls. The inhibitory effects of the remedies on hatchability were expressed as MIC values (Table 1). The remedies from KWTa and EL exhibited more inhibitory hatchability effects with MIC values of 2.4 and 2.8 mg mL^{-1}, respectively. However, KWTb, FB and HBts preparations had the highest value of 3.9, 3.8 and 3.9 mg mL^{-1}, respectively, indicating that they could be less toxic among the 9 remedies (Table 1).

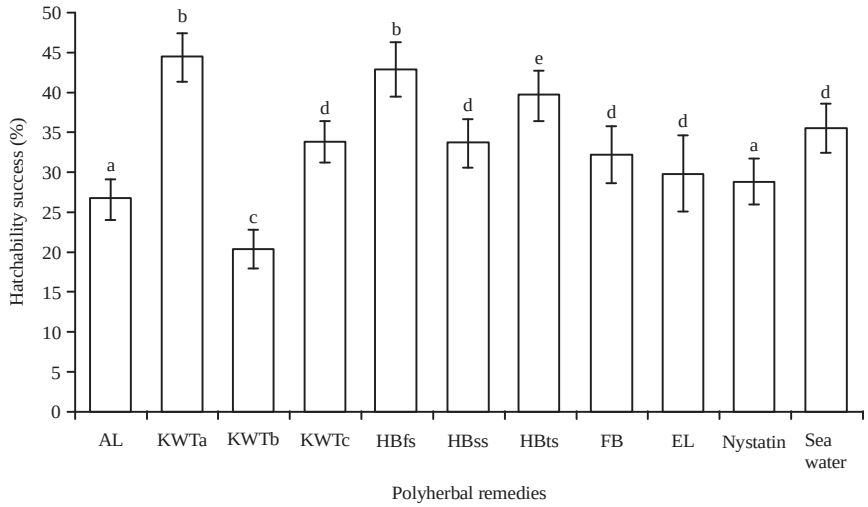

Fig. 1: Percentage hatchability success of *A. salina* cysts incubated in different polyherbal medicines. Means are values of five concentrations for each remedy ±SD. Bars with different letters are significantly different ($p<0.05$)

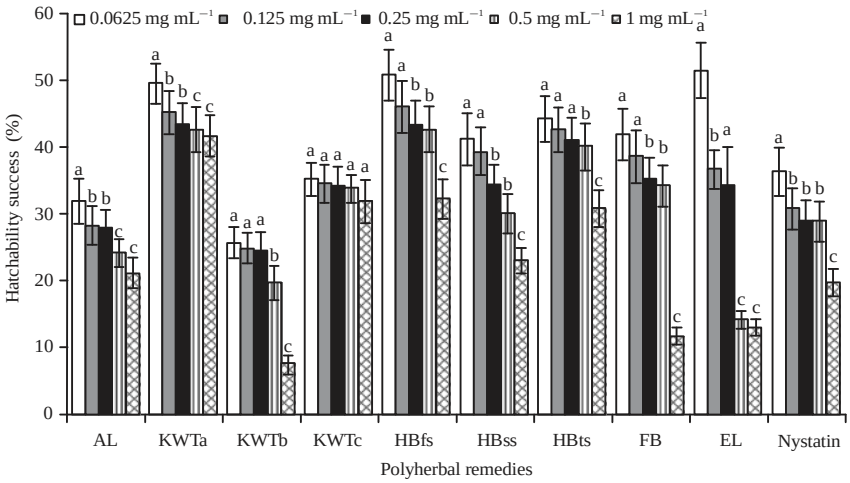

Fig. 2: Percentage hatchability success of *A. salina* cysts incubated in different concentrations of polyherbal medicines. Bars with different letters are significantly different

Table 1: Hatchability and lethality of *A. salina* incubated in different concentrations of polyherbal medicines

	Hatchability assay		Lethality assay	
Remedies	MIC 50 (mg mL^{-1})	R^2 (%)	LD_{50} (mg mL^{-1})	R^2 (%)
AL	3.0±0.87^a	96	3.0±0.03^a	99
KWTa	2.4±0.97^b	88	3.1±0.00^b	97
KWTb	3.9±0.23^c	74	3.8±0.68^c	80
KWTc	3.5±0.99^d	89	2.9±0.64^d	91
HBfs	3.4±0.72^e	88	3.4±0.15^e	95
HBss	3.3±0.56^f	96	3.2±0.04^f	96
HBts	3.8±0.83^g	78	3.4±0.55^g	92
FB	3.9±0.15^h	74	3.7±0.00^b	84
EL	2.8±0.04^i	93	4.0±0.01^h	70
Nystatin	3.3±0.68^j	86	3.5±0.37^i	92

Data are Means±SD of three replicates. Means with different superscript in the same column are significantly different ($p<0.05$), R^2 (%) is the coefficient of determination of the regression equation

Effect of polyherbal medicines concentration on hatchability success: The hatchability success of *A. salina* cysts decreased significantly with increasing concentrations of the polyherbal medicines (Fig. 2). The herbal preparations from EL, HBfs, KWTa and HBts had the highest hatchability success in the lowest concentration. In AL and HBss remedies, the hatching success decreased with increase in concentrations of the treatments. However, the inhibition of the hatching success in FB preparation was significantly decreased at 1 mg mL^{-1}. With increasing concentration from 0.0625 mg mL^{-1}, the hatchability success of the cysts incubated with KWTb remedy elicited more inhibitory effect on the hatching success at 1 mg mL^{-1} (Fig. 2). Thus, the decrease observed in the hatchability success of *A. salina* cysts as the concentrations increased could be as a result of the relative concentration of toxic metabolites present in the remedies as the concentration increases. However, none of the preparations exhibited total inhibition at 1 mg mL^{-1}, this might be due to the cysts possessing a resistant cyst stage which makes it tolerant to wide range of salinities[12].

Effect of exposure time on hatchability success: The effect of exposure time on hatchability obtained in this study revealed that the sensitivity of *A. salina* to polyherbal therapies was strongly dependent on exposure period (Fig. 3). The lowest hatchability success of the cysts was observed at 12 h of exposure in all the remedies. Higher hatchability success in incubations of KWTa, KWTc, HBfs, HBss, HBts and EL remedies at 24 h exposure were significantly higher than the controls. At 36 h of exposure, the hatchability successes of 49.78, 54 and 51.11% was observed in KWTa, HBfs and HBts medicines, respectively. The hatching success of *A. salina* cysts into nauplii in incubations of AL, HBfs, HBss, HBts, FB and EL remedies at 48 h of exposure ranged from 39.56-59.56%. The optimal hatching of cysts to yield a large number of nauplii is achieved with 48 h of exposure[13]. In AL, KWTb and EL remedies, the hatchability success was lower when compared with both controls at 60 h of exposure. Likewise, the hatching success of all the remedies except KWTa was lower than in the sea water at 72 h of exposure. *Artemia* is highly vulnerable to toxins and chemical metabolites at the early developmental stages[14], this could have led to the very low hatchability success of the cysts observed at 12 h of exposure. Thus, the resistant cysts stage of *A. salina* to higher salinities makes the hatchability assay less desirable assay than the lethality test for the preliminary screening of herbal remedies' toxicity test. The use of freshly hatched nauplii has been used to circumvent the toxic tolerant stage of *A. salina* cysts and this increases the sensitivity of the lethality assay[11,7].

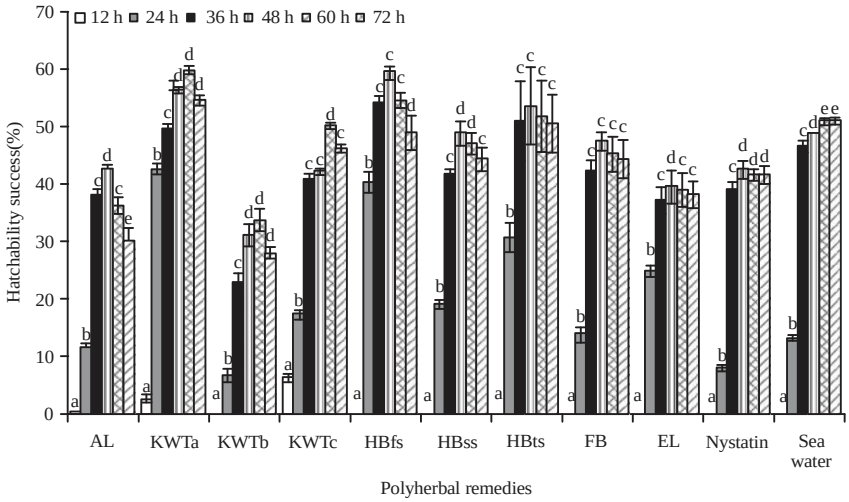

Fig. 3: Percentage hatchability success of *A. salina* cysts incubated at different durations in the polyherbal remedies. Bars with different letters are significantly different

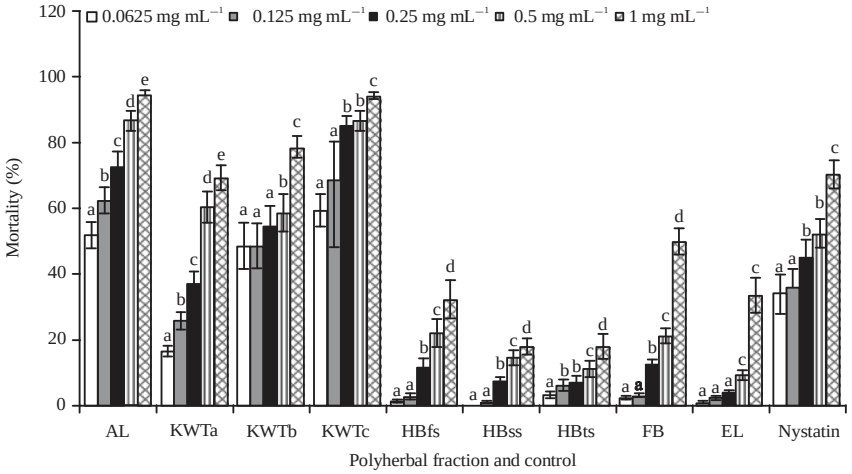

Fig. 4: Percentage mortality of *A. salina* nauplii incubated in different concentrations of the polyherbal medicines. Bars marked with different letters are significantly different

Effect of varying concentrations on brine shrimp mortality: The effect of varying concentrations of polyherbal remedies on the mortality of larvae is shown in Fig. 4. The degree of mortality of nauplii in all the remedies was in concentration dependent fashion. The percentage mortality of larvae in HBfs, HBss, HBts, EL and FB remedies was significantly lower than the positive control. Maximum mortalities of 94.63, 78.72 and 94.67% occurred at the highest concentrations of 1 mg mL^{-1} in incubations of AL, KWTb and KWTc, respectively, which was significantly higher than the positive control. It could be deduced that the polyherbal remedies have both toxicological and pharmacological activities based on the dosage administered.

Effect of exposure time on brine shrimp mortality: The effect of remedies on the larvae over a period of time was carried out to determine the sensitivity of the larvae to toxic secondary metabolites present in the remedies. The mortality of nauplii incubated in all the remedies increased exponentially with time (Fig. 5). The observed result was depended on the length of incubation period as earlier reported by Otang *et al.*[7]. Exposure of the larvae for lesser periods (<36 h) in HBfs, HBss, HBts and EL remedies did not induce mortality when compared with the controls. However, increase in mortality was observed in AL, KWTa, KWTb and KWTc remedies which was significantly higher than the controls, except at 72 h in nystatin. Brine shrimp nauplii attain the second and third instars of their life cycle within 48 h of exposure, thus revealed

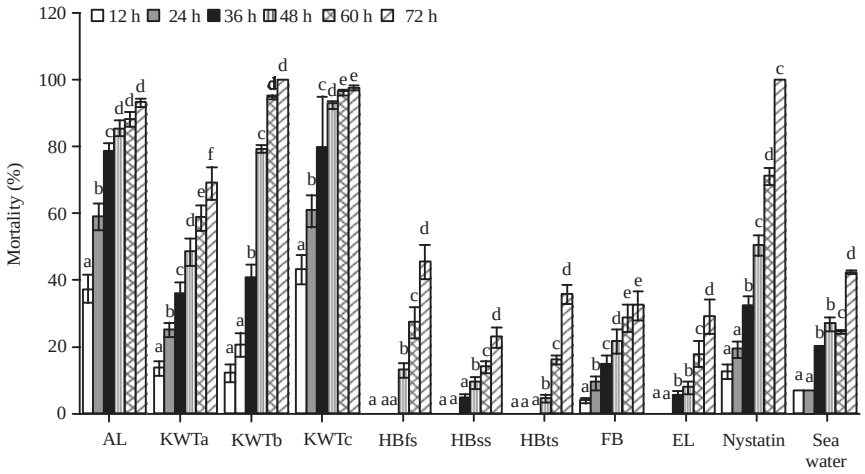

Fig. 5: Percentage mortality of *A. salina* nauplii incubated at different durations in the polyherbal remedies. Bars with different letters are significantly different

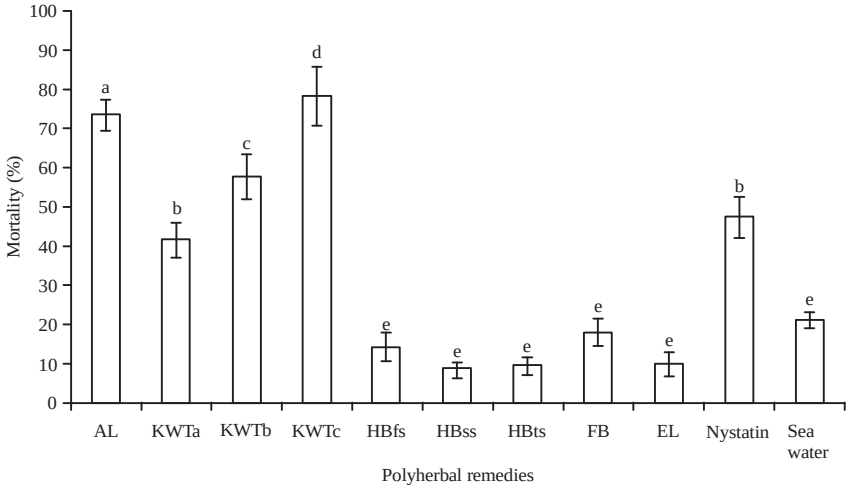

Fig. 6: Percentage mortality of *A. salina nauplii* incubated in different polyherbal medicines. Means are values of five concentrations for each polyherbal remedy±SD. Bars marked with different letters are significantly different

their greatest sensitivity to toxins at this time[15]. However, these findings revealed that the maximum sensitivity of the nauplii to the remedies was attained at 72 h of exposure. This could probably be due to the presence of nutritive metabolites in the remedies. Thus, the survival of high number brine shrimp nauplii in all the remedies after 60 h of exposure could be explained by the presence of non-toxic metabolites in the polyherbal medicines.

Brine shrimp lethality assay: Brine shrimp lethality results and LD_{50} values obtained are shown in Fig. 6 and Table 1, respectively. The larvae mortality of A. salina nauplii incubated in AL, KWTb and KWTc remedies was significantly higher than when larvae are incubated in both controls. However, lower larvae mortality was observed in the remedies from HBfs, HBss, HBts, FB and EL. Also, the number of larvae mortality was reduced in KWTa remedy than in nystatin but higher than those in sea water. While, EL remedy exhibited more lethality effects with LD value of 4.0, the lowest lethality effects was observed in KWTc remedy (Table 1).

In evaluating herbal preparations for toxicity, the LD_{50} values are commonly expressed either by comparison with Meyer's or to Clarkson's toxicity index. The extracts with LD_{50} less than 1000 μg mL^{-1} are considered as toxic, while extracts with LD_{50} greater than 1000 μg mL^{-1} are considered as non-toxic[13]. Clarkson classified cytoxicity as non-toxic when LD_{50} is above 1000 μg mL^{-1}, low toxic when the LD_{50} is between 500 and 1000 μg mL^{-1}, medium toxic when the LD_{50}

is between 100-500 μg mL^{-1}, while extracts with LD_{50} of 0-100 μg mL^{-1} are highly toxic[16]. According to these benchmarks, the LD_{50} of the nine polyherbal medicines varied between 3.0-4.0 mg mL^{-1}, these estimated LD_{50} values are greater than 1000 μg mL^{-1} (Fig. 6, Table 1). Thus, based on the criterion of toxicity, these remedies are considered non-toxic.

CONCLUSION

The findings from this study have shown that polyherbal remedies used for the treatment of tuberculosis in the study area exhibited non-toxic level i.e., LD_{50} values greater than 1 mg mL^{-1} with brine shrimp toxicity assays. Thus, they are considered safe for the patients. However, further *in vivo* toxicity test are required to validate the use of these polyherbal remedies.

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