IJPSR (2023), Volume 14, Issue 2 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 04 June 2022; received in revised form, 10 August 2022; accepted, 18 November 2022; published 01 February 2023

HERBAL BASED ANTI-MICROBIAL BOOSTER FOR THE TREATMENT OF RESISTANT *MYCOBACTERIUM TUBERCULOSIS*

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Keywords:

Herbal drugs, *Vernonia amygdalina*, *Garcinia kola*, Resistant *Mycobacterium tuberculosis*

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ABSTRACT: Persistent *Mycobacterium tuberculosis* (Mtb) attributable to resistant strains has increased the burden leading to poor quality of life to the patients. Drugs of herbal origin have been identified as potential sources of new anti-tubercular agents for the treatment of this disease and emerging resistant strains of the bacterium. In this research, therefore, five herbs/plants that have been indicated in the treatment of *Mycobacterium tuberculosis* in the traditional/folklore medicine were evaluated for their potential use in the treatment of *Mycobacterium tuberculosis* infection. *Garcinia kola, Picralina nitida, Mitracarpus villosus, Vernonia amygdalina* and *Securidaca longipedunculata* were assessed for their anti-tubercular activity using standard methods. *G. kola* and *V. amygdalina* having better activity were assessed for their synergistic activity. Doses displaying *in-vivo* activity were established. The activity of the synergistic mix with Rifampicin was evaluated in a resistant Mtb strain. Two plants; *G. kola* (MIC of 125 mg/ml) and *V. amygdalina* (MIC of 250 mg/ml) performed better than others. The synergistic ratios of *V. amygdalina, G. kola* of 1:1, 2:1, 4:1 and 8:1 produced enhanced activity. An 8:1 ratio mix dose of 2500 mg/kg and above exhibited anti-tubercular activity *ex-vivo*. The plasma concentration of the hybrid mix interacted with Rifampicin to exhibit anti-tubercular activity in a resistant strain of the bacterium. *V. amygdalina* and *G. kola* in suitable ratio exerts synergistic anti-tubercular activity and interacts with Rifampicin to inhibit resistant tubercular bacilli growth.

INTRODUCTION: Tuberculosis (TB) is one of the main causes of mortality and morbidity globally. According to World Health Organization (WHO), M*ycobactrium tuberculosis* (Mtb) has infected approximately one-third of the world's population, producing more than nine million new cases and two million deaths annually; the rest of the infected people remain asymptomatic [1, 2]. The emergence of new cases and new resistant strains of the bacterium with increased virulence have made the search for new drugs and alternative natural remedy imperative.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.14(2).711-18

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.14(2).711-18

Major problems associated with current TB treatment include; the duration and complexity of treatment resulting to non-adherence to treatment leading to suboptimal response (failure and relapse), the emergence of resistance (multidrug-resistant tuberculosis MDR and extensively drug-resistant tuberculosis XDR) and continuous spread of the disease. Adverse effects in response to anti-TB drugs are common and have contributed to the problem of non-adherence [5], as well as treatment interruptions [6].

In general, tuberculosis is treated with combination of first-line drugs; isoniazid, Rifampicin, pyrazinamide and ethambutol for several months [3]. Second-line drugs for drug-resistant TB are not easily available, less effective, more toxic, and require longer use than first-line drugs [4]. Similarly, third-line drugs are not equally effective or their efficacies have not been proven and are not listed by World Health Organization [7].

There is increasing interest in using complementary and alternative medicines to treat various acute and chronic diseases [8, 9]. These stem from their affordability, availability, and with minimum/no harmful effects. Medicinal plants serve as lead for new drugs, holding potential for exploitations. Plants are rich in bioactive compounds with activities ranging from anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial to anti-viral activities [10, 11]. In particular, *V. Amygdalina*, *G. Kola*, *P. Nitida*, *M. Villosus* and *S. Longipedunculata* have been shown to possess activities against pathogenic bacteria [12-16].

More importantly, Woldetensay [17] has reported that chloroform extract of *V. amygdalina* at 10 mg/ml exert anti-tubercular activity. This is in contrast to the findings of Kahaliw *et al* [18] who contradicted such findings. There are limited information on the anti-mycobacterium tuberculosis of *Garcina kola, Picralina nitida, Mitracapus villosus* and *Securidaca longipedunculata.* In this research therefore, *Garcina kola, Picralina nitida, Mitracapus villosus, Vernonia amygdalina and Securidaca longipedunculata* were selected, evaluated and their anti-tubercular activity ascertained. The synergistic activity of the most active among them is evaluated and the optimal dosage regimen is established for the possible development of dosage forms.

MATERIALS AND METHOD:

Collection and Processing of Plant Materials: *Garcinia kola* seeds, *Picralina nitida* fruits, *Vernonia amygdalina* leaves, whole plant of *Mitracapus villosus* and bark and roots of *Securidaca longipeduculata* were collected from farms and bushes around Awka in Anambra State and Enugu metropolis Enugu State, both in Nigeria. The collected plant materials were identified, and samples kept in the herbarium of the Department of Pharmacognosy and Traditional Medicine of the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka, Anambra State Nigeria with the voucher numbers of *Garcinia kola*: PCG/474/A/051, *Vernonia amygdalina*: PCG/474/A/020, *Mitracapus villosus*: PCG/474/A/005, and *Securidaca longipeduculata*: PCG/474/A/015.

Garcina kola seeds were dried under shade and peeled. The fruits of *Picralina nitida* fruits were cut longitudinally, and the seeds were removed, dried under the shade, and peeled. Both seeds were cut into pieces and dried in a force air oven at 40 ^{o}C until dried and hard. The seeds were later milled into powder using a plate mill, sieved using a 0.8 mm sieve, and stored separately in an air-tight glass container until ready for use.

Vernonia amygdalina leaves were washed with clean water; dried under shade, and pulverized using a kitchen blender. The powders were later sieved using a 0.8 mm sieve and stored in an air-tight glass container. The whole plant of *Mitracapus villosus* and root and barks of *Securidaca longipedunculata* were sorted, washed and dried; then pulverized and stored in air-tight glass container.

Extraction of the Seeds and Leaves Powders: The powdered plants' parts were extracted by cold maceration using absolute ethanol as the solvent. A 500 g of each powdered material was measured into a large mayonnaise jar and the powder level was covered with the solvent, agitated and kept for 24 h with intermittent agitation. After that, the mixtures were filtered through a filter cloth and finally using a Whatman number 1 filter paper.

The filtrates were poured unto a pre-weighed stainless steel plate of wide surface area, and the solvent allowed to evaporate under shade. The yields were calculated from the relation:

Yield (%) = (Weight of extract/Weight of powder) x 100

The resinous extracts were packed into a wide mouthed amber glass container and stored in a refrigerator until ready for use.

Incorporation of Various Plant Extracts into Reconstituted Media: The solution of the extracts was made in DMSO. To prepare LJ medium containing 500 mg/ml extract, 50 ml of egg suspension was aseptically mixed with 30 ml of sterile LJ medium. This was supplemented with 80 ml of 1000 mg/ml plant extract to give a final concentration of 500 mg/ml extract in LJ medium.

For the preparation of LJ medium containing 250 mg/ml extract, 75 ml of egg suspension was aseptically mixed with 45 ml of sterile LJ medium. This was supplemented with 40 ml of 1000 mg/ml plant extract to give a final concentration of 250 mg/ml extract in LJ medium.

To prepare LJ medium with 125 mg/ml extract, 87.5 ml of egg suspension was aseptically mixed with 52.5 ml of sterile LJ medium. This was supplemented with 20 ml of 1000 mg/ml plant extract to give a final concentration of 125 mg/ml extract in LJ medium.

To prepare LJ medium with 62.5 mg/ml extract, 93.75 ml of egg suspension was aseptically mixed with 56.25 ml of sterile LJ medium. This was supplemented with 10ml of 1000 mg/ml plant extract to give a final concentration of 62.5 mg/ml extract in LJ medium.

To prepare LJ medium with 31.25 mg/ml extract, 96.875 ml of egg suspension was aseptically mixed with 58.125 ml of sterile LJ medium. This was supplemented with 5 ml of 1000 mg/ml plant extract to give a final concentration of 31.25 mg/ml extract in LJ medium.

Evaluation of Anti-tubercular Activity of the Extracts: Culture bottles containing different concentration of extract as prepared. In the sections above were aseptically opened under bio-safety class III hood and inoculated with a 4.1 x 10^6 Mycobacterium tubercular bacilli. Rifampicin was used as positive control while the negative control batch had no extract incorporated in the medium. The bottles were corked and incubated aerobically at 37 ^oC. They were monitored for growth for a period of 30 days.

Determination of Synergistic Activity of *Garcinia kola* and *Vernonia amygdalina*: The medium preparation was as described above. The two plant extracts that showed the highest anti tubercular activity were selected and combined according to the arrangement in **Table 2.**

For preparation of LJ medium containing 250 mg/ml of *V. amygdalina* + 125 mg/ml of *G. kola* extracts, 50 ml of egg suspension was aseptically mixed with 30ml of sterile LJ medium. This was supplemented with 80 ml of plant extract (40ml of 500 mg/ml *V. amygdalina* + 40 ml of 250 mg/ml *G kola*) to give a final concentration of 250 mg/ml *V. amygdalina* + 125mg/ml extract *G. kola* in LJ medium.

For preparation of LJ medium containing 250 mg/ml *V. amygdalina* + 62.5 mg/ml *G. kola* extract, 50ml of egg suspension was aseptically mixed with 30ml of sterile LJ medium. This was supplemented with 80ml of plant extract (40 ml of 500 mg/ml *V. amygdalina* + 40 ml of 125 mg/ml *G. kola*) to give a final concentration of 250 mg/ml *V. amygdalina* + 62.5 mg/ml *extract G. kola* in LJ medium.

For preparation of LJ medium containing 250 mg/ml of *V. amygdalina* + 31.25 mg/ml of *G.kola* extracts, 50 ml of egg suspension was aseptically mixed with 30ml of sterile LJ medium. This was supplemented with 80ml of plant extract (40 ml of 500 mg/ml *V. amygdalina* + 40 ml of 62.5 mg/ml *G. kola*) to give a final concentration of 250 mg/ml *V. amygdalina* + 31.25 mg/ml extract *G. kola* in LJ medium.

For preparation of LJ medium containing 250 mg/ml of *V. amygdalina* + 15.625 mg/ml of *G. kola* extracts, 50ml of egg suspension was aseptically mixed with 30ml of sterile LJ medium. This was supplemented with 80ml of plant extract (40ml of 500mg/ml *V. amygdalina* + 40 ml of 31.25 mg/ml *G. kola*) to give a final concentration of 250 mg/ml *V. amygdalina* + 15.625 mg/ml extract *G. kola* in LJ medium. The above-enumerated approaches

were used for the other ratio mix of the extracts and media. The synergisms of these hybrid extracts against *M. tuberculosis* were evaluated following the procedure as in section for evaluation of extracts.

***Ex-vivo* Determination of Dose Regimen:** Two groups of albino Wistar rats involving males and non-pregnant females, with 3 animals per group weighing between 126 g and 134 g were used for this study. The rats were acclimatized at room temperature for 2 weeks and fed chicken growers marsh *ad libitum* with unlimited access to water. A reconstitution of the hybrid mix of the extracts of *V. amygdalina and G. kola* at the optimal synergistic ratio (8:1) was made with DMSO. The reconstituted mixture was administered to the respective groups of animals at 2500 mg/70 kg (35.7mg/kg) and 5000 mg/70 kg (71.4 mg/kg). Blood samples were collected from the animals in each group via intra ocular puncture at 0-, 30- and 60- min after treatment. The blood samples were processed with sodium citrate as anticoagulants to obtain the plasma. A 5 mm diameter hole was bored on a solidified *M. tuberculosis*-seeded LJ agar medium (LJ medium + agar gel). The base of the holes was layered with sterile wax as sealant after which, predetermined volume of plasma was added to the holes. All these were done in a bio-containment safety cabinet. Incubation was done for 30 days. Thereafter, the diameter zones of inhibition were measured, and the mean values were determined.

Extent of Resistance of *Mycobacterium tuberculosis* Strain to Rifampicin: The *M. tuberculosis* (strain: H37Rv) seeded molten LJ agar media were dispensed into Petri dishes and allowed to solidify. A sterile borer was used to bore holes measuring 5 mm on the LJ agar medium (LJ medium + agar gel). The base of the holes was layered with sterile wax as sealant. Three drops of each of the following rifampicin concentrations were introduced into the holes; 0.4-, 0.8-, 4.0-mcg/ml and 150 mg/ml. The plates were allowed to stay 30 min for diffusion. Incubation was thereafter allowed for 30 days at 37 ^{o}C. Diameter zones of inhibition were read and recorded.

***Ex-vivo* Determination of Combined Extract-Rifampicin Activity:** The agar plates were prepared as in above. Equal drops of 0.4 µg/ml of Rifampicin made in sterile water and a reconstituted hybrid mix of extracts prepared in Subsection for *ex-vivo* determination of dose regimen were introduced into the holes. The plates were allowed to stay 30 min for diffusion. Incubation was thereafter allowed for 30 days at 37 ^{o}C. Diameter zones of inhibition were read and recorded. Rifampicin was also plated separately to serve as the control.

Statistical Analysis: The data collected were analyzed using mean and standard deviation where necessary.

RESULTS AND DISCUSSION:

Yield: The yields of the extracts are as follows: *V. amygdalina*; 1.5 %, *Picralina nitida*; 1.6 %, *Securidaca longipedunculata*; 1.8 %, *Mitracarpus villosus*; 2.9 % and *Garcinia kola*; 6.0 %. From these yield results, *Garcinia kola* has the highest yield of 6 % while *V. amygdalina* has the lowest yield of 1.5 %. Though these yields were low, these can be augmented by the good agronomic potentials of the plants. While *P. nitida* and *G. kola* are perennial plants with a large production of yearly fruits, others are annuals herbs that can be mass produced to ensure a good supply of raw/starting materials for herbal drug production.

Evaluation of Anti-tubercular Activity of the Extracts: The selected herbal plants were screened for anti-tubercular activity, and the results are shown in **Table 1**. The extract of *Garcinia kola* displayed anti-tubercular activity with a minimum inhibitory concentration (MIC) of 125 mg/ml. Next in activity was *Venonia amygdalina*, with a MIC of 250 mg/ml. This is followed by *Mitracarpus villosus*, which has a MIC of 500 mg/ml. However, other herbs like *Picralina nitida* and *Securidaca longipedunculata* did not show activity against *M. tuberculosis*. The findings hence confirm the anti-tubercular activity of *V. amygdalina* [17] and some of the indicated plants, hence, their justified use in traditional medicine.

Interestingly, these two most promising herbs with the highest anti-tubercular activity (*G. kola and V. amygdalina*), as shown in **Table 1,** are commonly used among many tribes in the South Eastern part of Nigeria as hospitality edibles and in the

preparation of a local delicacy, "bitter leaf soup", respectively. A preliminary study done with the two plants on conventional bacteria showed that the two plants' extracts exhibited synergism and provided a broad spectrum antibacterial coverage. A synergistic interaction study was carried out to ascertain whether this synergism would extend to the activity of Mycobacterium tuberculosis.

TABLE 1: ANT-TUBERCULAR ACTIVITY EVALUATION OF THE FIVE SELECTED MEDICINAL PLANTS

	Concentration (mg/ml)				
Extract	**500**	**250**	**125**	**62.5**	**31.25**
G. kola	-	-	-	+	++
P. nitida	+++	+++	+++	+++	+++
M. villosus	-	+	++	+++	+++
V. amygdalina	-	-	+	+++	+++
S. longipedunculata	+++	+++	+++	+++	+++
Rifampicin (control)	-	-	-	-	++
Negative Control	+++	+++	+++	+++	+++

Key: - = No bacteria growth, + = 1-5 bacterial colonies, ++ = 6-10 bacterial colonies, +++ = More than 10bacterial colonies.

Determination of Synergistic Activity of *Garcinia kola* and *Vernonia amygdalina*: For the synergistic studies, the MIC of 125 mg/ml and 250 mg/ml, respectively for *G. kola* and *V. amygdalina* as shown in **Table 1,** were used. A 2 fold dilutions of these MICs as shown in **Table 2,** was obtained. Table 2 shows that the synergistic ratios of *V. amygdalina*: *G. kola* of 250:125, 250:62.5, 250:31.25 and 125:125 (2:1, 4:1, 8:1and 1:1) retained activity.

An important clarification to make to enable the presentation of these herbal extracts as a dosage form is to ascertain the dosing regimen.

TABLE 2: SYNERGISTIC ANTI-TUBERCULAR ACTIVITY OF *GARCINA KIOLA* AND *VERNONIA AMYGDALINA*

V. amygdalina	***Garcina kola*** (mg/ml)			
(mg/ml)	**125**	**62.5**	**31.25**	**15.625**
250	-	-	-	+
125	-	+	++	+++
62.5	+	+++	+++	+++
31.25	+++	+++	+++	+++

Key: - = No bacteria growth, + = 1-5 bacterial colonies, ++ = 6-10 bacterial colonies, +++ = More than 10 bacterial colonies.

***Ex-vivo* Determination of Dose Regimen:** Preliminary studies were conducted with representatives of conventional bacteria to establish the concentration of orally administered hybrid mix of *G. kola* and *V. amygdalina* that will appear in the blood. Six treatment groups of three albinos Wistar rats per group weighing between 126 g and 134 g were given the following doses; 5000 mg/kg, 5000 mg/70 kg (71.4 mg/kg), 2500 mg/70 kg (35.7 mg/kg), 1000 mg/70 kg (14.3 mg/kg), 500 mg/70 kg (7.1 mg/kg) and 250 mg/70 kg (3.6 mg/kg). The choices of these dosing concentrations were based on previous studies and conventions. Dozie-Nwakile *et al* [19] employed a 5000 mg/kg dose in the study involving Kolaviron, a fraction of *G.kola* extract, in the treatment of pneumonia-like infection caused by *Streptoccocus pneumonae*. A dose of 5000 mg/kg and 2500 mg/kg are divisions of the lethal doses (LD_{50}) of the extracts [20], while doses of 1000 mg/70 kg to 250 mg/70 kg are conventional plausible dosing ranges. Blood samples collected from rats at the intervals of 0-, 0.5-, 1-, 2-, 4- and 6- hours were processed into plasma and used to inoculate the selected representative bacteria. In all the trails conducted, extracts dosing concentrations of 1000 mg/70 kg and below failed to appear in the blood, since there were no inhibitions of growth from the plasma obtained from rats dosed with such concentrations. Based on the above realization, the minimum dose of 2500 mg/70 kg and its multiple of 5000 mg/70 kg were selected and administered to the rats. The rat's plasma inoculated to the *M. tuberculosis* seeded LJ agar plates produced the inhibitions as shown in **Table 3**. The average diameter zones of such inhibitions are dose rate dependent. The implication is that higher dose rate will be preferred to achieve holistic control of tubercular activity. In this regard, a solid oral dose of 5000 mg given to an adult of 70 kg average body weight will

correspond to giving approximately seven tablets of 750 mg per tablet or ten tablets of 500 mg per tablets at once. This definitely will not encourage compliance. However, a quantity of 5000 mg of extracts can conveniently be incorporated into liquid volumes of 50- to 500- ml for a liquid drink. Presenting the preparation as a nutriceutical liquid drink will definitely entice the patient and encourage compliance. Such nutriceutical drink being able to boost the action of conventional anti-tubercular drugs will be an added advantage. Using the hybrid extract with the conventional anti-tubercular drug, will provide multiple drug armament in the treatment of *M. tuberculosis* infections to limit/prevent resistant development. Investigation into these boosting effects of the hybrid extracts toward conventional anti-tubercular drugs on resistant strain of the *Mycobacterium tuberculosis* was therefore carried out.

Extracts-Rifampicin Interaction: The plasma collected from rats administered with the hybrid extract at 30- and 60- min were mixed with the Rifampicin (150 mg/ml) in an equal ratio. The choice of these time intervals were based on the preliminary studies in which the hybrid mix of *V. amygdalina* and *G. kola* have the widest coverage of anti-bacteria activity for conventional bacteria. The mixture was then inoculated into the *M. tuberculosis* seeded LJ agar and treated as done earlier. The diameter zones of inhibition obtained are shown in **Table 3.** It is very obvious that the anti-tubercular activity of the plasma concentration of the hybrid mix of the extracts in combination with the standard drug (Rifampicin at 150 mg/ml) is still dose and time-dependent. A critical look at the data in **Table 3** reveals that, at the two dosing ranges of the hybrid extracts in combination with the Rifampicin, an improved diameter zone of inhibition was obtained compared to the hybrid extract alone.

The control (Rifampicin) alone gave an inhibition of 2 mm. This value is above that obtained when in combination with the hybrid extract. This can only be attributed to proteins binding as the plasma's inherent protein binds with Rifampicin, lowering the inhibition zone.

TABLE 3: DOSE REGIMEN OF HYBRID MIX OF *V. AMYGDALINA* AND *G. KOLA* AND IN THE PRESENCE OF RIFAMPICIN

Hybrid extract mix + Rifampicin	ZID (mm) ± SD	ZID (mm) ± SD	Hybrid extract mix
2500mg/70kg @ 0 min + Rifampicin	0.0 ± 0.0	0.0 ± 0.0	2500mg/70kg @ 0 min
2500mg/70kg @ 30 min + Rifampicin	1.0 ± 0.2	0.7 ± 0.1	2500mg/70kg @ 30 min
2500mg/70kg @ 60 min + Rifampicin	1.7 ± 0.15	0.8 ± 0.1	2500mg/70kg @ 60 min
5000mg/70kg @ 0 min + Rifampicin	0.0 ± 0.0	0.0 ± 0.0	5000mg/70kg @ 0 min
5000mg/70kg @ 30 min + Rifampicin	1.5 ± 0.1	0.8 ± 0.1	5000mg/70kg @ 30 min
5000mg/70kg @6 0 min + Rifampicin	1.8 ± 0.2	1.2 ± 0.1	5000mg/70kg @6 0 min
Control (Rifampicin) @ 150 mg/ml	2.0 ± 0.2		

Extent of Resistance of *Mycobacterium tuberculosis*: It is evident from **Table 1** that Rifampicin had activity at a concentration of 62.5 mg/ml and no activity at 31.25mg/ml. The concentration of 62.5 mg/ml is far above the plasma concentration of the drug (0.4 mcg/ml) [21].

To appreciate the extent of this resistance of the mycobacterium strain to Rifampicin, graded concentrations of Rifampicin beginning from the plasma concentration were inoculated unto *Mycobacterium tuberculosis* seeded LJ agar media. The results are shown in **Table 4** from the values obtained, the concentrations and the corresponding activity, including the activity of Rifampicin from **Table 1,** are arranged as shown in **Table 4.** There is no activity at plasma concentration of Rifampicin (0.4 mcg/ml), and the observed minimum inhibitory activity occurred at 62.5 mg/ml (62500 mcg/ml). This represents 156,250 (62500/0.4) times activity above (or 156,250 times inactivity below) the plasma concentration of Rifampicin, hence a very high resistant strain of *Mycobacterium tuberculosis*.

TABLE 4: COMPARISON OF RIFAMPICIN CONCENTRATIONS AND THEIR CORRESPONDING ACTIVITY/NON ACTIVITY

Rifampicin concentration	Sensitivity/Activity
0.4 mcg/ml	No activity
0.8 mcg/ml	No activity
4.0 mcg/ml	No activity
31.25 mg/ml	No activity
62.5 mg/ml	Activity
150 mg/ml	Activity

CONCLUSION: Three of the five plants examined showed anti-tubercular activity. Among those that showed activity, *Garcinia kola* and *Vernonia amygdalina* possess the highest activity. These two plants displayed synergistic activity at varying combination ratios. The dose level at which their hybrid mixture produces plasma concentration that gave anti-tubercular activity is very high and cannot lead to a plausible solid dosage formulation without further assessment. However, the hybrid mix can be formulated into a palatable liquid dosage drink that can be taken with the conventional anti-tubercular drug like Rifampicin for the treatment of resistant tuberculosis infections.

ACKNOWLEDGEMENT: We acknowledge Solomon Chollom (Ph.D) of the Bacteriology Unit of Diagnostics Division, National Veterinary Research Institute, Vom Jos, Plateau State, Nigeria for his assistance in carrying out some of the laboratory studies concerning *Mycobacterium tuberculosis*.

Funding: This research did not receive any specific grant from the public, commercial, or not-for-profit funding agencies.

CONFLICT OF INTEREST: The authors hereby declare no conflict of interest in this research work.

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How to cite this article:
Ezechukwu OD, Nwakile CD, Umeyor CE, Dozie-Nwakile OC, Uronnachi EM, Okezie UM, Ike CJ, Obasi JC, Okeke IJ and Oli AN: Herbal based anti-microbial booster for the treatment of resistant *Mycobacterium tuberculosis*. Int J Pharm Sci & Res 2023; 14(2): 711-18. doi: 10.13040/IJPSR.0975-8232.14(2).711-18.