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In Vivo Evaluation of Anti-Mycobacterial Activity of a Phytomedicine "MATHESIA" on *Mycobacterium ulcerans*: Influence of Alkalinity on the Activity of Antibiotics Used in the Treatment of Buruli Ulcer

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Abstract

The present study reports the *in vivo* anti-Mycobacterial Activity of a Phytomedicine "MATHESIA" on *Mycobacterium ulcerans*. It also explores the influence of ethanolamine (alkali agent) on the *in vivo* activity of some antibiotics (Isoniazid, Rifampicin, Ethambutol) used in the treatment of Buruli ulcers in DR Congo. Experiments were conducted on adult white Wistar rats of both sexes with the weights comprising between 110 - 165 g. The result obtained have shown that the phytomedicine MATHESIA (pH 10) has a good *in vivo* activity on *M. ulcerans* and the duration for wounds healing and total cicatrization was 6 days; whereas this duration was 10, 12, 14, and 16 days respectively for Rifampicin (IV, pH 10), Kibadi's solution (X, pH 10), Ethambutol (VIII, pH 10) and Isoniazid (VI, pH 10). The result also showed that the use of the ethanolamine as alkali compounded in the treatment of wounds due to Buruli ulcer along with other antibiotics reduced considerably the duration of complete healing comparatively to their solutions at pH bellow or equal to 7.

Keywords

Buruli Ulcer, *Mycobacterium ulcerans*, Mathesia, Ethanolamine, Rifampicin, Ethambutol, Isoniazid, Kibadi Solution

1. Introduction

Among the most known Mycobacterial infections in tropical countries, tuberculosis is the most widespread. Leprosy came at the second rank, followed by infection with *Mycobacterium ulcerans* called "Buruli Ulcer" most known in DR Congo as "MBASU".

If leprosy and tuberculosis result from the man-to-man transmission, the Buruli ulcer belongs to mycobacterial infections from the environment [1]. This infection prevails in marshy zones of tropical and subtropical areas of Africa, Latin America, Asia, Oceania, and Western Pacific [2]-[8].

In the Democratic Republic of Congo, mainly in Songololo territory (Kongo-Central Province), cases of the patients infected by *Mycobacterium ulcerans* were reported in many papers [5] [9] [10] [11] [12]. In their study, Kibadi, *et al.* [5] showed that 80% of infected people arrived late to the hospital when the ulcer had reached its last stage. This late arrival at the hospital is because the population allots this disease to a bad fate. So patients consult initially soothsayers and traditional healers [5] [13]. The impact of traditional treatments on Buruli ulcer in Benin was described in 1995 [14].

This disease (Buruli ulcer) was observed in many Africa countries such as Uganda [15], Ivory Coast [16], Togo [17], and Angola [18].

For the management of this disease, recent researches show that rifampicin in association with streptomycin gives the best results when the infectious lesions are at their beginning. Successes of Buruli ulcer treatment with antibiotic associations remain very limited. It is established that tissue ulceration is caused by a polyketide toxin called "Mycolactone" secreted by *M. ulcerans* [19]. Indeed, an intradermal injection of this purified toxin-induced in mice or guinea-pigs, the same skin lesions as those observed in Buruli ulcer [19] [20].

Therapeutic failures in the treatment of Buruli ulcers with antibiotic associations could be due to the inability of these to act directly on the polyketide toxin. An ideal drug must be able not only to eliminate *M. ulcerans* but also to deteriorate the structure of the toxin to stop its action on tissues. We aim in this study to evaluate the *in vivo* anti-Mycobacterial Activity of a Phytomedicine "MATHESIA" on *Mycobacterium ulcerans* which has demonstrated its positive action on multi-drug-resistant strains of *Mycobacterium tuberculosis* [21].

These authors have shown that Mathesia has a good *in vitro* activity against multi-resistant strains of *Mycobacterium tuberculosis*. In a previous study, we reported the antibacterial activity of this phytomedicine against *Streptococcus pyogenes* and *Aspergillus* sp due to the presence of Tannins, saponins, Flavonoids as well as steroids [22].

This phytomedicine is an emulsion with a basic pH > 10, because of the presence of an amino surfactant in its composition.

In this study, we have explored also the influence of ethanolamine as alkali compound on the *in vivo* activity of some selected antibiotics (Isoniazid, Rifampicin, Ethambutol) used in the treatment of Buruli ulcers in DR Congo.

2. Material and Methods

2.1. Material

Mathesia was obtained from the Industrial and Technical Group (GITCO), in Kinshasa DR Congo. It is a hydro-alcoholic emulsion of plant extracts containing the following secondary metabolites: Tannins, saponins, Flavonoids as well as steroids, and reducing sugar [22].

Kibadi's solution, a mixture of metronidazole (2 g), chloramine (2 g) and nitrofurantoin (2 g) in 1 L of distilled water, Isoniazid (INH), Rifampicin (RMP), and Ethambutol (EMB) was purchased in the pharmacy store of the University of Kinshasa hospital [13].

The *M. ulcerans* strain used in this study was isolated by the team of BU project of the Evangelic Medical Institute of Kimpese, Kongo Central, DR Congo from wounds of a patient with confirmed Buruli ulcer infection. The identity and susceptibility of this strain were confirmed at the University of Kinshasa hospital Mycobacteria laboratory on Löwenstein-Jensens medium [23].

Cyclophosphamide (an immune suppressor) powder (Cytoxan®, Bristol Myers Squibb, NY, USA) was dissolved in distilled USP water for injection to a final concentration of 20 mg/ml.

2.2. Methods

2.2.1. Preparation of *Mycobacterium ulcerans* Inoculum

M. ulcerans, strain 1615 (Trudeau Collection Strain, Lake Saranac, NY, USA), was initially grown on M7H9/OADC (Oleic acid, Albumin, Dextrose, and Catalase, pre-formulated from DIFCO) agar plates at 30˚C for 4 - 8 weeks (until yellow colonies were well-formed). Cultures in M7H9/OADC medium were brought to 15% glycerol and samples were stored at −80˚C to provide a consistent source of inocula [24]. To a plate containing *M. ulcerans* colonies M7H9/OADC medium (10 ml) was added and a sterile spreader was used to detach the cells. The suspended cells were transferred into a 600-ml T-flask containing 300 ml of M7H9/OADC medium and the flask was incubated at 30˚C for 4 - 8 weeks (until significant growth was observed). Cultures were expanded by adding 100 ml of culture to 200 ml of fresh M7H9/OADC medium [24].

2.2.2. Experimental Animals

The experiment was conducted on adult white Wistar rats of both sexes with the weights comprising between 110 - 165 g procured from the National Institute of Biomedical researches (INRB) in Kinshasa. All rats were fed with normal laboratory chow food containing 16% protein, 66% carbohydrate, 8% fats, and water. All rats were housed at a (12:12) h light and dark cycle at 25˚C and relative humidity of 60% - 70%.

2.2.3. Ethical Approval

The guidelines followed for the animal experiment were accepted by the Ethics Committee of the Public Health School of the University of Kinshasa ESP/CE/

043/2017 on 28 July 2017.

2.2.4. Experimental Design

For animal inoculation, the bacterial suspensions were centrifuged at room temperature for 20 minutes at 1800 g. The supernatant was discarded and the pellet was weighed and resuspended in sterile phosphate-buffered saline (PBS), pH 7.4. Ten-fold dilution series of this suspension were prepared and selected doses were used as inoculum. Mice were inoculated subcutaneously with 30 µl of inoculum in the left side of the tail [25] [26].

All mice used have received a total dose of 250 mg/kg of cyclophosphamide by two 0.5 mL intraperitoneal injections scheduled at day 1 (150 mg/kg) and day 4 (100 mg/kg) to suppress their immune system before to be inoculated with *M. ulcerans* [27].

Lesions appeared on the tails of mice approximately three weeks after inoculation with *M. ulcerans*. The wet bandages, after being soaked in antibiotics solutions, were applied to the lesions from that moment and were changed after every two days. A visual observation made it possible to note the evolution of the lesions with a scale from 1 to 5 which corresponds to wounds healing process phases [28]:

1) Coagulation and hemostasis phase to prevent exsanguination;

2) The early inflammatory phase which initiates molecular events, leading to an infiltration of the wound site by neutrophils to prevent infection;

3) Late inflammatory phase with macrophages present in the wound and continuation of phagocytosis process;

4) Proliferative phase with fibroblast migration and deposition of newly synthesized extracellular matrix;

5) Remodeling phase with scar tissue formation.

Animals were alienated into twelve groups and for every group, six animals were taken and the studied variables were the evolution of lesions on mice tails, and days number for wounds total healing

Groupe I (Normal control) mice served as positive control received only an injection of inoculum after cyclophosphamide administration. Group II (Normal control) mice served as negative control received an injection of inoculum without previous cyclophosphamide administration.

Group III to Group XII received also an injection of inoculum after cyclophosphamide administration. But after lesions manifested, they were treated differently with wet bandages soaked beforehand in solutions of the following antibiotics:

Group III: Rifampicin pH 6; Group IV Rifampicin along with 0.2 ml of ethanolamine with a resulting pH of 10; Group V: Isoniazid pH 6; Group VI: Isoniazid long with 0.1 ml of ethanolamine (to achieve a final pH of 10); Group VII: Ethambutol at pH 5.5; Group VIII: Ethambutol along with 0.2 ml of ethanolamine (final pH 10); Group IX: Kibadi solution pH 7; Group X: Kibadi solution along with 0.1 ml of ethanolamine (final pH 10); Group XI Mathesia at pH 10;

Group XII solution of ethanolamine in water (final pH 10).

3. Results and Discussion

3.1. Results

Main results are reported in Table 1.

Results are discussed as the action of each used antibiotics in experiments.

3.2. Discussion

3.2.1. Buruli Ulcer Development in Mice

Mice in the normal negative control (group II) have received an injection of inoculum without cyclophosphamide administration to suppress their immunity. They have not developed the Buruli ulcer even after six weeks. To avoid the interaction of the animal immune system, in this *in vivo* antibiotic study, cyclophosphamide has been used to induce neutropenia. Several works on animal infection models in mice showed that bacterial growth is inversely related to the number of granulocytes in peripheral blood. There is also a significant difference in CFU numbers at the site of infection between mice treated with cyclophosphamide (250 mg/kg) and non-neutropenic one [29] [30] [31]. Immune response and pathogenesis observed in *M. ulcerans* mouse infections are strongly influenced by the toxin mycolactone. So, mycolactone-negative bacteria are faster phagocytosed, no prominent necrosis is developing in mice [32] as observed in this study.

Table 1. Evolution of lesions on mice tails after application of wet bandages soaked in antibiotics solutions.

Group	pH	Day of treatment											
		2	4	6	8	10	12	14	16	18	20	22	24
I	7	1	1	1	1	1	1	Infection					
II	-	-	-	-	-	-	-	-	-	-	-	-	-
III	6	1	1	1	2	2	2	3	3	3	4	4	4
IV	10	1	2	3	4	5							
V	6	1	1	1	1	2	2	2	3	3	3	4	4
VI	10	1	1	2	2	3	4	4	5				
VII	5.5	1	1	1	2	2	2	3	3	3	3	4	4
VIII	10	1	1	2	2	3	4	5					
IX	7	1	1	2	2	2	3	3	4	4	4	5	
X	10	1	2	2	4	4	5						
XI	10	2	3	5									
XII	10	1	1	2	2	2	2	2	3	3	4	4	

-: no lesions nor inflammation.

Mice in group I (positive control) have received only an injection of inoculum after cyclophosphamide administration. Wounds of these mice were all infected on the 14th day of treatment because of antibiotics non-administration and the suppression of their immune system.

3.2.2. Ethanolamine Effect on Cicatrization

Mice in groups III to XII received an injection of inoculum after cyclophosphamide administration and were treated differently with wet bandages soaked beforehand in antibiotics solution after lesions appearance. Results mentioned in Table 1 showed that mice treated with antibiotics along with ethanolamine with a final pH of 10, ensure a total cicatrization of wounds caused by *M. ulcerans*. Whereas the same antibiotics with values of pH ≤ 7 did not allow a total cicatrization even after 24 days of treatment. In their study on microbial toxicity of ethanolamine, authors showed that these compounds possess an antimicrobial effect which is enhanced at high pH [33]. The antimicrobial effects of alkanolamines are greatly enhanced at high pH. Since these compounds are selectively toxic at high pH [34].

Most pathogenic bacteria produce toxins that play some important roles in diseases. *M. ulcerans* produces two polyketide-derived macrolides named mycolactone A and B. Intradermal inoculation of mycolactone A/B into guinea pigs produces lesions similar to that of Buruli ulcer in humans, demonstrating their direct correlation with Buruli ulcer [19].

Alkanolamines as alkaline compounds can induce hydrolysis of this mycolactone and then stop its action on tissues. As reported by Lin *et al.* for pharmaceuticals containing lactones, hydrolysis occurs readily due to both enzymatic and nonenzymatic processes [35]. This affects the bioavailability and the efficacy of lactone-containing drugs and pro-drugs. That's why antibiotics in mixture with ethanolamine (pH 10) stop both the action of *M. ulcerans* and its mycolactone. And so, they are more effective at pH 10 than in neutral or acidic media.

3.2.3. Antibiotics Nature Effects

Results in Table 2 show that "Mathesia at pH 10", ensures total wound cicatrization after 6 days of treatment; whereas Rifampicin, Kibadi solution, Ethambutol, and Isoniazid gave the same results after 10, 12, 14, and 16 days respectively. The good activity of Mathesia could be due to the presence of many phytochemical compounds such as alkaloids, steroids, terpenes, tannins, flavonoids, and saponins [22]. In a study on In vitro evaluation of Mycobacterial Activity of Phytomedicine Mathesia on *Mycobacterium tuberculosis*, the authors reported that the anti-mycobacterial activity of MATHESIA is certain because 32 multi-drug-resistant strains were sensitive at all concentrations (except 0.01 mg/mL). So, Mathesia can make a considerable contribution to the management of tuberculosis patients. Whereas these strains are not sensitive to antibiotics such as Rifampicin, Isoniazid, and Ethambutol [21].

Table 2. Days for wounds total healing.

N°	Antibiotic solution	Total healing after
1	Mathesia (XI, pH 10)	6 days
2	Rifampicin (IV, pH 10)	10 days
3	Kibadi (X, pH 10)	12 days
4	Ethambutol (VIII, pH 10)	14 days
5	Isoniazid (VI, pH 10)	16 days
6	Kibadi solution (IX, pH 7)	22 days

3.2.4. Rifampicin

Rifampicin is an important bactericidal agent against *Mycobacterium tuberculosis*. It inhibits bacterial protein synthesis by binding with the DNA dependent RNA polymerase enzyme of bacteria, thus preventing transcription to RNA and subsequent protein synthesis [36]. This drug has been extensively used in clinical studies as an inducer of drug-metabolizing enzymes and transporters by inducing many enzymes in the cytochrome P450 family, which are responsible for the metabolism of the majority of drugs and xenobiotics [37]. It may be, for this reason, Rifampicin is the most effective drug for the treatment of *M. ulcerans* after "MATHESIA".

3.2.5. Ethambutol

Ethambutol is another first-line drug against tuberculosis and some nonspecific mycobacteria. It is thought to be an inhibitor of metabolites essential for the survival of many bacteria. Mainly the synthesis of arabinogalactan which is an important component of the mycobacterial cell wall [38]. Ethambutol is a synthetic congener of 1,2-ethylenediamine and is used in combination with other anti-TB drugs to prevent or delay the emergence of resistant strains. On the other hand, this drug is known to be able to chelate various cations such as zinc, and copper which are required as cofactors of several enzymes important for maintaining normal body functions. In depleting levels of these cations during ethambutol treatment, It would therefore affect the normal function of various enzymes [39]. As it can be used only in combination with other anti-tuberculosis drugs, this can explain why this drug is less effective than rifampicin in the treatment of Buruli ulcer.

3.2.6. Isoniazid

Isoniazid is isonicotinyl hydrazine. It is a bactericidal agent whose mechanism of action includes inhibiting the mycolic acid synthesis in M. Tuberculosis. Mycolic acids are important components of the mycobacterial cell wall and are vital for the survival of the bacteria [40]. Isoniazid is known as a metabolism inhibitor of certain drugs, which can increase their plasma concentration to the point of toxicity. That's why it is not recommended to use it alone in affection treatment

[41]. In Buruli ulcer treatment, it takes 16 days for wounds total healing; this duration is higher than those that were observed in the treatment of Buruli ulcer with Rifampicin and ethambutol.

3.2.7. Kibadi's Solution

Kibadi's solution is a mixture of metronidazole (2 g), chloramine (2 g), and nitrofurantoin (2 g) in 1L of distilled water [42]. The author reported that local care of Buruli ulcer consisted of the application of an aqueous solution of chloramine-metronidazole-nitrofurantoin daily after debridement. Preliminary results with a follow-up of 12 months showed healing in 62 cases, recurrence in 22, and unknown outcome in 18. These findings underscore the importance of early detection and treatment with antimycobacterial [43]. In this study, the author specifies neither the pH nor the duration of treatment. It is however advisable to note that by bringing the pH to 10, the treatment duration with this solution is only 12 days.

4. Conclusions

In this work, we evaluated the *in vivo* anti-Mycobacterial Activity of a Phytomedicine "MATHESIA" on *M. ulcerans* which had demonstrated its positive action on multidrug-resistant strains of *Mycobacterium tuberculosis*. We have also explored the influence of ethanolamine as an alkali agent on the *in vivo* activity of some antibiotics (Isoniazid, Rifampicin, Ethambutol) used in the treatment of Buruli ulcers in DR Congo.

Experiments were conducted on adult white Wistar rats of either sex with the weights comprising between 110 to 165 g and fed with normal laboratory chow food containing 16% protein, 66% carbohydrate, 8% fats, and water. All rats were housed at a (12:12) h light and dark cycle at 25˚C and relative humidity 60% - 70%. Animals were alienated into twelve groups as described in the material and methods section.

The results obtained showed that MATHESIA had a good *in vivo* activity on *M. ulcerans* and the duration for wounds healing and total cicatrization was 6 days; whereas this duration was longer with others antibiotics used in this study.

The results showed also that the use of the ethanolamine as an alkali compound reduced considerably the duration for a complete cicatrization of Buruli ulcer wounds in the treatment with other antibiotics used in this study.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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