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The background of the cover features a person's hands holding a glowing, semi-transparent globe of the Earth. The globe is surrounded by various digital data visualizations, including bar charts, line graphs, and network diagrams. The overall color scheme is a mix of dark blue, purple, and white, creating a high-tech, futuristic atmosphere.

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# Oligoelements, formulation, accelerated aging tests and anti-fatigue activity of two mulberries products based on Antananarivo local specimens

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**ABSTRACT:** While the benefits of mulberries are widely acknowledged, there is still a scarcity of commercial products derived from the leaves of this plant that specifically cater to health and wellness. The main objective of this study was to enhance the value of leaves from local specimens of mulberries in Antananarivo, which is scientifically called as *Morus alba*. The analysis of oligoelement components in leaves was carried out using Polarized Energy Dispersive X-ray Fluorescence (PEDXRF). Two formulations were developed, one in teabag form and the other as a pot of mulberry granules. Their potential for long-term serviceability was evaluated through accelerated aging tests. Modified String test and Forced swimming endurance test were used to determine the effect of all formula on Short-term and relatively long-term physical exercises in mice. Each mulberry teabag and pot of mulberry granules contains approximately 0.054 and 0.324 g of calcium, respectively. These products display a potential long-term serviceability and exhibit an anti-fatigue activity in mice. The study results indicate that the developed mulberry products present a promising alternative for combating fatigue and preventing certain diseases associated with oligoelement deficiencies. More clinical studies are needed to ascertain its optimal dosages and to evaluate their long-term toxicity.

**KEYWORDS:** *Morus alba*, Oligoelements, formulation, aging tests, anti-Fatigue.

## I. INTRODUCTION

Fatigue, a situation in which the capacity for work is diminished and efficiency of accomplishment reduced, can be classified as physical or mental, depending on its cause. Because fatigues play an important role in worker's productivity, searching for plant extracts with good anti-fatigue activity may lead to a commercially useful therapeutic agent.

*Morus alba* L. is commonly known as Mulberry or White-Mulberry, and is generally used as a food source [1]. Mulberry trees can be propagated by seeds, cuttings, or graftings however, there is limited commercial formulations based on these plant [1].

The main objectives of the current study were twofold: firstly, to enhance the value of leaves from *Morus alba*, particularly by formulating products based on local specimens in Antananarivo and predicting their potential long-term serviceability; and secondly, to explore the oligoelement compositions and anti-fatigue activity of these formulations.

## II. RELATED WORK

Physical fatigue, a complex physiological process, is associated with person's health and extreme exercise [2] [3]. Accumulation of excess metabolite and depletion of energy is the key reasons for this type of fatigue [2] [3] [4]. Compile information on various aspects of *Morus alba* L. including a plant used, phytochemicals and detailed pharmacological information were reported by Ramandeep S. and collaborator in 2013 [1].

Exercise endurance is an important variable in assessing anti-fatigue effects [5]. Generally, anti-fatigue activity is investigated by using short-term and long-term physical exercise tests. Forced swimming endurance test is considered to be the most effective method to assess endurance capacity for exercise performance and known to give a good reproducibility [5].

### III. METHODOLOGY

#### 1. Chemicals and materials

Three specimens of mulberry (*Morus alba* L.) were collected on May 2023 at Sabotsy Namehana (large leaves), Nanisana (large leaves) and Ampasapito (small leaves) from Antananarivo town (region of Analamanga, Madagascar). The plant name has been checked with <http://www.theplantlist.org> and specimens were identified by the botanist at the Botanical and Zoological Park of Tsimbazaza (PBZT).

Fresh leaves were washed, air-dried at 36°C till dryness and then crushed. The air-dried and crushed leaves were stored in a drying cabinet before the experiment. During the sample processing, necessary measures were taken in order to avoid any loss or contamination of metals.

Syrup was prepared at room temperature by adding 180 g of sugar in 100 ml of distilled water and the solution was stirred till a large proportion of sugar had dissolved. Ethanol, sugar and distilled water were purchased from the local market.

#### 2. X-ray Fluorescence-Analyses

Elemental analysis was conducted at the X-ray Fluorescence and Environment Department of the INSTN-Madagascar laboratory at the, University of Antananarivo, Madagascar.

Air-dried leaves were finely powdered and sifted through a 150 µm sieve. Subsequently, 3 g of the sieved powder per pellet were manually pressed using a pressing machine and subjected to analysis through polarized energy dispersive X-ray fluorescence (PEDXRF) with a SPECTRO-XEPOS instrument. Measurement parameters, as outlined in Table 1 [6], were established for precision. Data acquisition was performed using SPECTRO X-Lab Pro software [7].

Table 1: Measurement conditions with the SPECTRO-XEPOS instrument

Secondary excitation	Tube voltage/intensity	Elements	Times of measurement
Molybdenum (Mo)	40kV/0.88mA	Fe, Ni, Co, Cu, Zn	300 s
Bragg Cristal (HOPG)	17.5kV/2 mA	Na, Mg, Al	300 s
Cobalt (Co)	35kV/1 mA	K, Ca, Mn, Ti, V	300 s

#### 3. Formulation of mulberry products and packaging

Two distinct formulations were developed using mulberry leaves, one in teabag form and the other as granules.

**Teabags:** Two grams (2 g) of dry leaf powder were weighed (KERN 770; max: 60/210 g, d=0.01/0.1 mg; min: 0,001 g, e: 1 mg; Germany) and then put directly into a teabag. The identical procedures were iterated until the desired quantity of mulberry teabags was achieved.

**Granules:** One hundred grams (100 g) of leaf powder was macerated in 400 ml of 90° ethanol for 6 days then filtered. The filtrate was evaporated at 40°C using a rotavapor (BÜCHI type KRvr 65/45) to obtain a semi-solid hydroalcoholic extract. Hydroethanolic extract (16.23 g), sugar (254 g) and 0.3 ml of syrup were mixed in a mortar at room temperature. Successively, mixture was passed through an iron grater, dried at 45°C for 1 hour and then pounded and sieved. Regular granules were then obtained and packaged in a pot (48 g/pot).

#### 4. Accelerated aging tests and sensory evaluations

Accelerated aging tests as described previously by WATTS and its collaborators in 2007 and reported by Randriatiana in 2017 were used.

Samples were aged in a laboratory oven at 40°C (MEMMERT 854 Schwabach, t°= 40 - 220°C; Western Germany) or at ambient temperatures (+/-20°C). Organoleptic and physicochemical evolutions were appreciated at the beginning (0 day), then 5, 10 and 15 days during the aging experiment period [8] [9]. Visual (color), olfactory (odour), taste (sweetness) and tactile (friability) hedonic scores were assigned by 6 humans volunteer as following: Average scores (/20) were calculated using Microsoft Office Excel 2013 software.



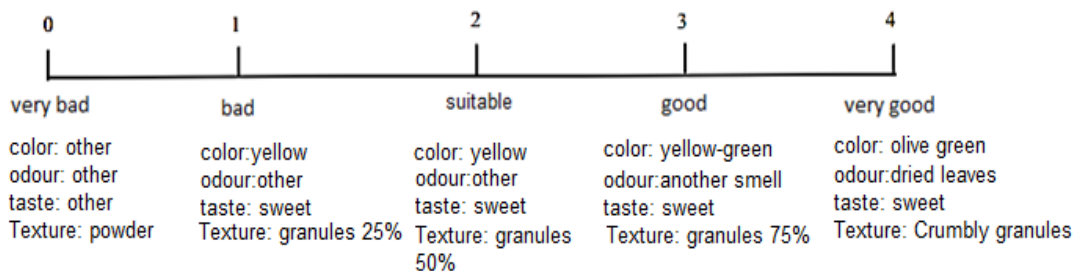


Fig. 1: scores

### 5. Evaluation of anti-fatigue activities in mice

All procedures were approved by the Animal Ethical Committee of the Faculty of Sciences, University of Antananarivo —Madagascar.

SWISS mice of both sexes, weighing between 25 – 30 g were obtained from the animal facility at the IMRA (Institut Malgache de Recherche Appliquée).

Adaptive trainings were made for 5 minutes once per week. Fifteen hours (15 h) prior to the exhaustion experiment, Mice (15 mice) were fasted and randomly divided into the following five groups (3mice/group): Group 1: control-mice treated with distilled water; Group 2–3: reference-mice treated with 1.6 g/kg of sugar or 5 mg/kg of caffeine respectively; Group 4–5: mice treated with 1.6 g/kg of granules or 1.6 g/kg of teabag respectively. All drugs were solubilized in distilled water. The anti-fatigue activity was evaluated directly by measuring the increase in exercise tolerance and endurance capacity in mice.

**Modified String test:** String test or Grip strength test as described previously was adapted to assess short-term physical exercise tolerances in mice [10]. Metal bar (2 mm diameter × 38 cm long) were fixed by a metallic support column at each end. The bars are 60 cm above the floor and special surface was placed at the base to cushion any mice that fall off. Distilled water or drugs were administered orally (10 ml/kg) and hind legs of all tested mice were tied. Thirty minutes (30 min) after the gavage treatment, mice were suspended on the horizontal bar and the endurance times were recorded. Mice were regarded as being exhausted when they fall off.

**Forced Swimming Endurance Test (FSET):** The test was conducted as described previously [5] [11] [12] [13] [14]. Briefly, the swimming exercise was carried out in an acrylic plastic pool (65 × 50 × 50 cm), filled with 30 cm depth of water at 25±1°C. Distilled water or the corresponding agents were administered orally (10 ml/kg) to mice. Thirty minutes (30 min) after the gavage treatment, the FSET tests were made and the endurance times were noted. Records were made from the beginning of the test until exhaustion. Mice were regarded as being exhausted when they were underwater for 8 s.

### 6. Statistical analysis

Experiments were conducted in duplicate (n=2), triplicate (n=3), or more (n=6). The results are expressed as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD). Homoscedasticity tests were performed using Fischer-Snedecor’s F-test, and mean comparisons were conducted using Student’ s t-test. Microsoft Office Excel 2013 was employed, and statistical significance was considered at  $p \leq 0.05$ , with high significance denoted at  $p \leq 0.01$ . ANOVA and post hoc tests were additionally employed to confirm specific hypotheses.

## IV. RESULTS AND DISCUSSION

### 1. Oligoelements from the leaves of mulberry specimens

Table-2 illustrates that all selected specimens predominantly contain essential oligoelements such as Na, K, Mg, Al, Ca, and Fe. Calcium and potassium emerge as the most abundant among the detected oligoelements, both exceeding 2%. Notably, Nanisana exhibits the highest concentration of calcium, while Ampasapito showcases the maximum concentration of potassium (refer to Figure 2). Additionally, other oligoelements, including Vanadium, Cobalt, Nickel, Titanium, Zinc, Copper, and Manganese, are also discernible in the specimens (refer to Table-3).

Analysis of mulberry leaves reveals notable dissimilarities among specimens. Subsequent statistical analysis confirms the heteroscedasticity (F-Test,  $p < 0.05$ ) in the calcium components between the large leaf specimen from Sabotsy Namehana and the small leaf specimen collected at Ampasapito. These specimens grow several kilometers apart. On the other hand, difference of variance is not significant (F-Test,  $p > 0.05$ ) among large and small leaf specimens which grow less than 1 kilometer apart - case of the Nanisana specimen and that of Ampasapito. However, significant difference is obtained by comparing the average calcium of these two specimens (t-test,  $p < 0.05$ ). These findings



suggest that variations in oligoelement components are contingent upon both the soil type and the specific variety of *Morus alba* under consideration. Other authors have reported similar results [1]. In addition, specimens with large leaves would be richer in calcium.

Table 2: oligoelements from the leaves of selected specimens with concentration more than 0.03%

Elements	means (in %, n=2)	SD
Sodium	0,068	0,024
Magnesium	0,170	0,039
Aluminum	0,084	0,029
Potassium	2,016	0,080
Calcium	2,569	0,128
iron	0,044	0,018

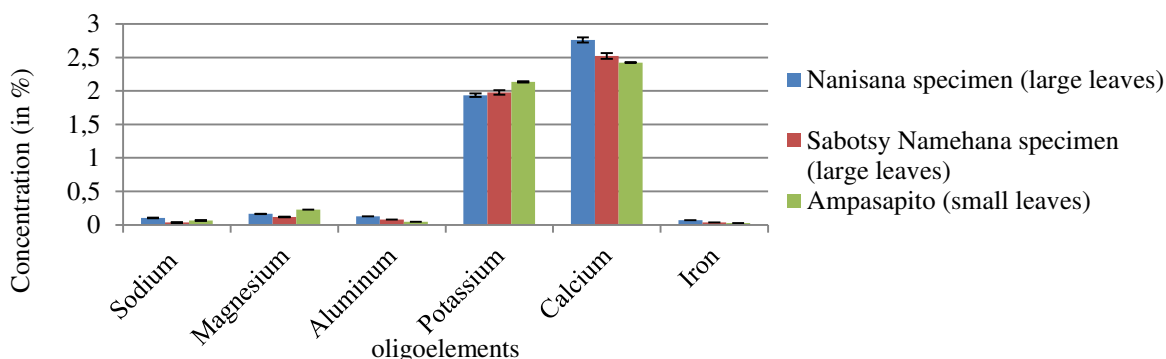


Fig. 2: Concentrations of major oligoelements of the leaves of *Morus alba* selected specimens (in % ± SD, n=2). Data are presented as Mean ± SD, n=2.

Table 3: oligoelements from the leaves of selected specimens with concentration less than 136 mg/kg

Elements	Mean ± SD (in mg/kg, n=2)		
	Nanisana (large leaves)	Sabotsy Namehana (large leaves)	Ampasapito (small leaves)
Titanium	1.05 ± 1.05	28.60 ± 0.10	26.55 ± 4.35
Vanadium	< 1.00	< 1.00	< 1.00
Manganese	84.10 ± 1.40	38.85 ± 0.55	135.5 ± 0.40
Cobalt	< 3.00	< 3.00	< 3.00
Nickel	2.25 ± 0.15	2.20 ± 0.00	1.40 ± 0.00
Copper	10.55 ± 0.85	9.90 ± 0.30	7.90 ± 0.30
Zinc	51 ± 1.30	69.75 ± 1.05	26.55 ± 4.35

**2. Formulation and packaging**

Seven (7) dry leaves weigh approximately 3.6 g. Exactly 16.23 g of hydroethanolic extract is obtained by macerating 100 g of air-dried and powdered leaves, i.e. a yield of 16.23%.

Each teabag contains, approximately 0.054 g of calcium, amongst other. Its organoleptic and physicochemical characteristics are reported in table 4. Its primary (5x7 cm<sup>2</sup>) and secondary packaging (8x5x7 cm<sup>3</sup>) are presented in figure 3 (a, b, c).

Each pot of granules (figure 3: d, e) corresponds to 6 teabags and includes approximately 0.324 g of calcium (i.e. 0.32 g of hydroethanolic extract of *Morus alba* per 8 g of granules). Its recommended indication and instructions for use are appended in table 4.

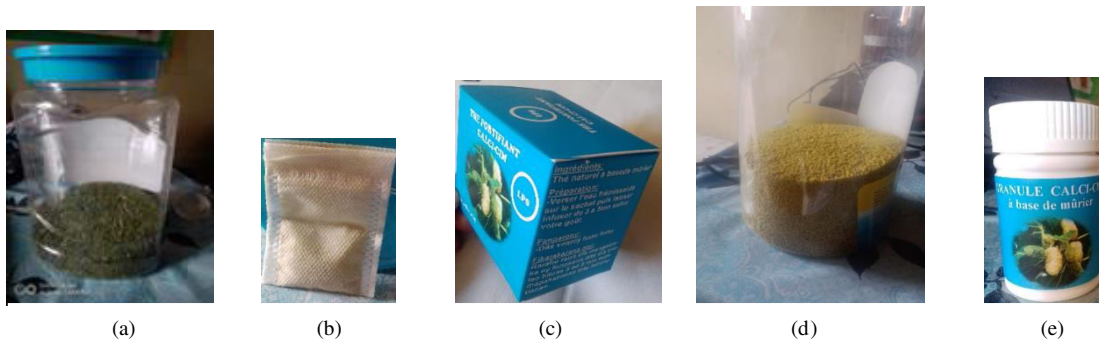


Fig. 3: mulberry products. (a): dried and crushed leaves; (b): Primary packaging with 2 g of dried and crushed leaves (5x7 cm<sup>2</sup>); (c): secondary packaging which contains 10 teabags (8x5x7 cm<sup>3</sup>). (d): mulberry granules; (e): Primary packaging which contains 48 g of granules.

Tableau 4: Description and proprieties of mulberry based products

Product name	mulberry granules	mulberry teabags
Odour	dried leaves	dry leaves
Taste	sweet	bland
Colour	yellow	olive green
Texture	crumbly granules	-
Administration	oral suspension	1 bag x 2 per day (morning and noonday) during 3 days
Preparation	dilute in lukewarm water	3-5 minutes in hot water
Indications	reduces the risk of osteoporosis; fortifying, dental disease treatment; not recommended for children under 5 years old	reduces the risk of osteoporosis; fortifying, dental disease treatment; not recommended for children under 5 years old

### 3. Accelerated aging tests

Evolutions of organoleptic and physicochemical scores of mulberry granules during the aging tests are presented in the following figure (figure 4). As shown, scores are greater than 18.5/20 under all the studied aging conditions and no significant modification are recorded ( $p > 0.05$ ,  $n=6$ ). However, noticeable discoloration of products that are aged at 40°C appears on the 15th day. Except for color change, no variations of physical or organoleptic properties are observed. At the end of the aging period, the mulberry granules demonstrated satisfying organoleptic and physicochemical properties. However, for the sake of brevity, only the data on loss of color are obtained. This color change may be interpreted as an important sign of degradation of leaf pigments. Indeed, the leaves of *Morus alba* contain pigments such as chlorophyll (green), carotene (yellow and orange) and xanthophyll (red). Among all, chlorophyll breaks down more quickly, revealing the yellow and orange of the carotenoids [1].

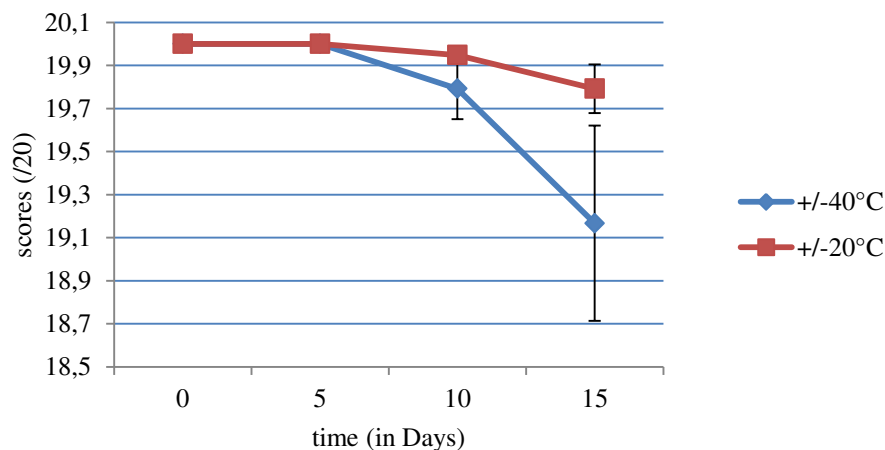


Fig. 4: Evolutions of organoleptic and physicochemical scores of mulberry granules during the aging tests. Data are presented as Mean ± SEM, n=6.

#### 4. Anti-fatigue activity of teabag extracts and mulberry granules

Performances of mice during the anti-fatigue evaluations were presented in Figure 5.

The mice treated with distilled water (control) stayed suspended on the horizontal bar during  $30.34 \pm 3.72$  seconds. In contrast, mulberry teabag extracts or granules – treated mice remained suspended on the horizontal bar during  $228.67 \pm 28.35$  and  $107.67 \pm 4.36$  seconds, respectively. Difference observed are statistically significant when compared to control (t-test,  $P < 0.05$ ,  $n=3$ ). Similarly, the caffeine (5 mg/kg) group showed significant differences (t-test,  $p < 0.05$ ,  $n=3$ ) compared with the control.

The performances of mice during the Forced Swimming Endurance Test (FSET) were also presented in Figure 5. As shown, swimming time of all treatment and reference groups were longer than that of the control group (distilled water) (t-test,  $n=3$ ,  $P < 0.05$ ). Compared with that of the control group, the endurance time periods of teabag extracts and granules – treated groups are 1,173 (t-test,  $n=3$ ,  $P < 0.05$ ) and 2,303 fold (t-test,  $n=3$ ,  $P < 0.01$ ) longer, respectively.

Thus, results may show that, the exercise tolerances of mice are elevated after mulberry teabag extracts or granules treatments. Indeed, endurance times of all treatment groups are remarkably longer than that of the control group ( $P < 0.05$ ). All these results suggest that the mulberry teabags and granules had anti-fatigue activities against short-term and relatively long-term physical exercises. Among the tested products, the mulberry granules exhibited the highest activity in Forced Swimming Endurance Test and it's probably due to the association of sugar and oligoelements in this formula.

Tying hind legs of all tested mice during the “string test” experiment make it possible to evaluate anti-fatigue activity, although it has been usually applied to assess muscular strength or neuromuscular function. Experimental data obtained demonstrated that caffeine used as reference drug during this study also show a significant effect and its valid this modified method. Indeed, the effect of caffeine on endurance performance time is largely described in literature [10] [15] [16] [17].

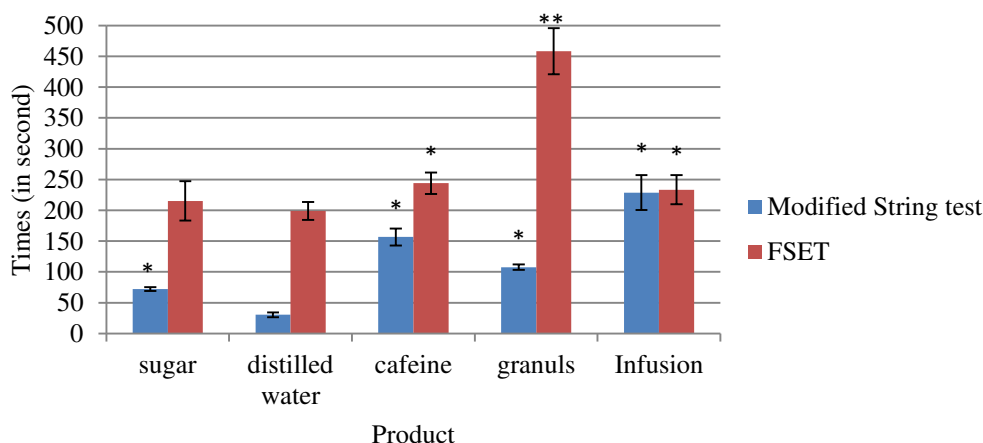


Fig. 5: Effect of distilled water (control), sugar (1.6 g/kg) or 5 mg/kg of caffeine and 1.6 g/kg of granules or teabag (10 ml/kg), on the Performances of mice. FSET: Forced Swimming Endurance Test. Data are presented as mean  $\pm$  S.E.M ( $n=3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$  compared to vehicle – treated group (t-test).

#### V. CONCLUSION

The present study aims to elucidate the oligoelement components found in the leaves of Antananarivo local mulberries and formulate two distinct products. Variations in oligoelement components are influenced by both soil type and the specific variety under consideration. Notably, the Large Leaf Mulberry from Nanisana emerged as the richest in calcium among all studied specimens.

Products derived from the leaves of Antananarivo local mulberries exhibited exceptional anti-fatigue activity and demonstrated potential long-term serviceability. This study contributes to the valorization of *Morus alba* leaves, suggesting that mulberry products offer a promising alternative for combating fatigue and preventing diseases linked to oligoelement deficiencies. Further clinical studies are necessary to establish optimal dosages and ensure safety in human consumption.

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