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Characterization of The Chemical Composition of White Mulberry (*Morus alba*) Leaves Extracts and Its Role in The Mutualism Between White Mulberry and Silkworms (*Bombyx mori*).

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ABSTRACT

Of the Hundreds of thousands of plant species in the world, silkworms (Bombyx mori) consume only one species, the white mulberry (Morus alba L.). This narrow specificity is still poorly understood. In this study, we compared the chemical composition of white mulberry (Morus alba L.) leaves with that of black mulberry (Morus nigra L.), used as a control. We analyzed the quantity of polyphenols, essential oils, proteins, and other substances present. The results revealed significant differences between the chemical composition of the leaf extracts from the two trees. The results of our research revealed that the yield of essential oils present in the leaves of Morus nigra (0.54%) was higher than that of the leaves of the host plant Morus alba (0.21%). The dosage of polyphenols contents showed a 72% superior value in Morus nigra leaf extracts compared to Morus alba leaf extract. In line with our results, studies have shown that high concentrations of essential oils and polyphenols present in plants play a repellent role for silkworms (Bombyx mori). The results also showed that the dosage of total chlorophyll and proteins was higher in Morus alba leaf extracts compared to Morus nigra (43% and 28% respectively). The final result was electrophoresis, which showed the presence of certain silk proteins in the leaves of Morus alba and not in those of Morus nigra, to explain the specific preferences of the silkworm. Other parameters, such as carotenoids and soluble sugars did not show any significant difference between the leaves of the two species. Thus, it appears that the silkworms are not attracted by a single molecule or substance, but rather by a structured arrangement of several substances, each playing a role in this close relationship between the white mulberry (Morus alba L.) and the silkworms (Bombyx Mori).

Keywords: *Bombyx Mori, chemical composition, essential oils, Morus sp, mutualism.*

I. INTRODUCTION

Sericulture is a traditional farming activity that involves cultivating mulberry trees to harvest their leaves to feed silkworms (*Bombyx mori* L.), reared to produce silk [1]. Silk is one of the world's oldest and most precious textiles. It is a natural animal fiber derived from the cocoons of domesticated mulberry silkworms. Silk is a structural protein that is formed, secreted, and spun into fiber [2], by many races of silkworms and several other animals capable of producing silks with similar properties, but not as good as the silk specifically produced by the white mulberry silkworm (*Bombyx mori*) [3]. Which is a monophagous, host-specific insect that feeds only on white mulberry leaves [4].

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This insect is the only living species in its family, Bombycidae, and has been domesticated for so long that it probably no longer survives in the wild [5]. According to the taxonomic classification, the mulberry tree belongs to the genus Morus in the family Moraceae, and is located in different regions of the world [6]. The mulberry tree is native to Asia but actually is cultivated in several countries. In China, there are 15 cultivated and wild species belonging to the Morus genus [7]. White mulberry (*Morus alba*), Red mulberry (*Morus rubra* L.) and black mulberry (*Morus nigra* L.) are the most common mulberry species [8].

Currently, the main source of natural silk comes from the cultivation of *Bombyx mori*, and around 150,000 tons of silk are produced each year by around fifty countries, with China and India being the largest producers [9]. Furthermore, this fiber has excellent properties such as comfort, resistance to traction, and dyeability. The sector employs around 7.63 million people [10].

Silkworm cocoons are made up of two types of silk protein: fibroin, which is a fibrous substance secreted into the lumen of the silkworm's posterior silk gland, and sericin, which unites the fibroin to form the cocoons [11]. Fibroin is made up of three entities: a high molecular weight macromolecule (FibH, 'heavy fibroin', 350 kDa), a much lighter one (FibL, 'light fibroin', 26 kDa), and a glycoprotein called P25 (30 kDa). A disulfide bridge links six heavy chain molecules to six light chain molecules and a P25 molecule is connected to the heavy and light chains by non-covalent interactions [12-14].

An article published in the Journal of Agricultural and Food Chemistry in 2015 compared the phytochemical components and antioxidant activities of white and black mulberry fruits. Results showed that black mulberry fruits contained higher levels of antioxidant compounds than white mulberry fruits [15]. Another study investigated the phytochemical screening of the stems, leaves, and fruits of *Morus alba*, *Morus nigra,* and *Morus rubra* species showed the presence of antioxidant properties in mulberry plant extract has been confirmed by the DPPH method [16].

This exclusive mutualism between the white mulberry and silkworms may be due to the particular chemical composition of the white mulberry leaves. Our study aims to compare the chemical composition of extracts from white mulberry leaves with black mulberry leaves and to determine the key substance responsible for the silkworms' choice of white mulberry leaves.

II. MATERIALS AND METHODS

1. Plant material

Nine *Morus alba* and *Morus nigra* trees were identified at different sites, leaves were collected from different parts of each tree as replicates.

- ***Murus alba***

Site 1 (31.643769, -8.020862); Site 2 (31.643805, -8.020550); Site 3 (31.644177, -8.020557); Site 4 (31.649890, -8.020408); Site 5 (31.655758, -8.014242); Site 6 (30.406470, -9.544517); Site 7 (31.646325, -8.020087); Site 8 (31.624554, -7.992515); Site 9 (31.624723, -7.993972).

- ***Murus nigra***

Site 1 (31.643906, -8.019904); Site 2 : (31.642653, -8.021289); Site 3 (30.412573, -9.552754); Site 4 (30.412448, -9.552744); Site 5 (30.407794, -9.550431); Site 6 (30.407822, -9.552492); Site 7 (30.406105, -9.551125); Site 8 (30.406511, -9.550707); Site 9 (30.407219, -9.550246).

2. Antioxidant Activity of Essential Oils

For each tree, we did 3 repetitions, and for each repetition, we took 50g of fresh leaves. The first step was the extraction of essential oils [17]. This protocol makes it possible to obtain pure essential oils by steam distillation. After 4 hours of extraction, the solution was obtained containing oil and water. Then, it was placed in a vertical separatory funnel using Hexane, followed by the addition of sodium anhydride to eliminate any excess water, finally the hexanic part was put in the Rotavapor to eliminate the hexane.

3. Determination of phenolic compounds

a. Preparation of extracts

Extraction was carried out by maceration, and for each sample, three repetitions were carried out. For each repeat, the solvent was prepared, containing (35% sterile distilled water and 70% technical methanol) 15ml water, and 35ml methanol. The total volume of solvent for each repeat was 50ml, to which 5g of plant powder was added, followed by stirring for 3 days.

b. Determination of phenolic compounds

Total polyphenols were determined by a spectrophotometric assay [18]. After 3 days, the methanol was removed by rotavapor, and the remaining material stuck to the wall of the flask was then in the separating funnel plus Hexane (3 times) for depigmented the extract.

The upper phase (Hexane and Pigments) and the lower phase were separated and the lower phase was returned to the separating funnel plus acetyl ether (3 times) to recover the polyphenols, in this decantation, the upper phase was recovered. Next, the hexanoic phase and phenolic extract were added to the rotavapor to remove the solvents, and the remaining material stuck to the wall of the flask was recovered by 1ml of absolute ethanol. First, 10 µL of the sample was added to 1.74 mL of distilled water and 0.25 mL of a 3-fold diluted solution of the Folin-Ciocalteu reagent. The resulting mixture was incubated at room temperature for 3 minutes. Next, 0.5 mL of sodium carbonate (20%) was added to the incubated mixture, followed by a second incubation for 30 minutes at a temperature of 40°C. At the end, the absorbance was read at a wavelength of 760 nm.

4. Flavonoid content

Flavonoids concentration were determined using the method described by [19,20]. To 0.25ml of the phenolic extract, 1ml of distilled water and 0.075ml of $NaNO_2$. This was followed by an initial Incubation for 6 min. Next, 0.075ml $AlCl_3$ was added, followed by a second incubation of 6 min. Finally, 1 ml NaOH was added, followed by a final incubation of 15 min. The OD was read at 510 nm. The standard range was prepared with quercetin.

5. Photosynthetic pigment content

Photosynthetic pigments were determined using the method of [21].50 mg of fresh leaves were ground in 5 ml of pure acetone (100%) and incubated for 15 minutes on ice. After centrifugation at 3000 rpm for 5 min at 4°C. The different pigments contained in the supernatant were identified at different wavelengths by spectrophotometry. Photosynthetic pigment contents are expressed in $\mu g.g^{-1}$ of fresh matter (FM).

Chlorophyll a, b and carotenoid concentration was determined according to the following formulae:

Chlorophyll a ($\mu g.ml^{-1}$) = (11.24 x OD 662 nm) - (2.04 x OD 645 nm)

Chlorophyll b ($\mu g.ml^{-1}$) = (20.13 x OD 645 nm) - (4.19 x OD662 nm)

Carotenoids ($\mu g.ml^{-1}$) = (1000 x DO 470 nm - 1.90 Chl a - 63.14 Chl b) / 214

6. Protein assay

Protein determination was carried out using the method of [22].50 mg of Mulberry leaves were ground in 2 ml of phosphate buffer pH 6.8. After centrifugation at 12500 g for 20 minutes. A quantity of 100 µl of the supernatant was placed in a tube containing 100 µl of distilled water and 2 ml of Bradford reagent. The tubes were shaken and left to stand for 5 min at 27°C. Finally, the absorbance was read at a wavelength of 595nm. Protein concentrations were determined using a calibration range of bovine serum albumin (BSA) solutions.

7. Protein identification by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein identification method [23] was used to compare the proteins of silk and mulberry leaves (White and Black), the protein extract was prepared by grinding 100 mg of leaves and cocoons in a mortar containing 2 ml of phosphate buffer (10 mM) pH 7 with 4% PVP. The crushed material was centrifuged at 9000g for 30 minutes. Next, 50 µl of the supernatant was removed and mixed with 50 µl of Laemmli buffer. The mixture was vortexed and incubated for 5 minutes in a water bath at 95°C.

8. Analysis by high-performance liquid chromatography (HPLC)

Ultra-high pressure liquid chromatography analysis was conducted using the Dionex Ultimate 3000 chromatography system (CA, USA), equipped with a quaternary pump (HPG 3400 RS), an autosampler (WPS 3000 TSL), and a column oven (TCC 3000). A Kinetex C18 reversed-phase column (250 × 4.6 mm particles, 2.6 µm). The method of [24, 25] was used for the analysis of phenolic compounds. Separation was achieved by gradient with solvent A (0.1% formic acid in water) and solvent B (100% methanol). The injection volume is 10 µl and the peaks were detected at 280 nm.

9. Soluble sugar content

The determination of soluble sugars was carried out using the method of [26].100 mg of mulberry leaves were crushed at 4°C in four ml of ethanol (80%). After centrifugation for 10 min at 5000 rpm, the supernatant was recovered for assay of soluble sugars. In test tubes, 1 ml of 5% phenol solution was added to 1 ml of extract, then 3 ml of concentrated sulphuric acid was added. After vortexing, the tubes were placed in a 25-30°C water bath for 20 min, then cooled for 6 min. Optical density was measured at 485nm. The soluble sugar content was determined by reference to a standard curve established using a glucose solution.

Statistical analyzes

For the part of the comparisons between the two mulberry species, the statistical analysis was carried out based on the 1-way ANOVA test using the SPSS version 26 program. Each value represents the average of 3 repetitions. Values with p less than 0.05 are considered significantly different.

III. RESULTS AND DISCUSSION

The results showed significant differences between *M. alba* and *M. nigra* leaves extracts. For essential oils, the yield was 0.21% for *Morus alba* and 0.54% for *Morus nigra*. According to the study by [27], experiments have revealed two types of interaction between silkworms and plant leaves. The first type concerns leave containing volatile essential oils, such as tea and soya leaves, which attract silkworms but are not consumed by them. On the other hand, the second type of leaves, which contain proteins but no essential oils, such as lettuce and fig leaves, do not attract silkworms, but they do consume them if they come into contact with them, but do not allow them to grow normally. In the case of mulberry leaves, they attract silkworms even from a distance (3 to 4 cm) and are then consumed by them. On the one hand, essential oils or volatile substances play a role in attracting silkworms but are not consumed. On the other hand, proteins and carbohydrates do not influence silkworm attraction but are consumed. In the case of mulberry leaves, they exert an attraction on silkworms even at a distance (3 to 4 cm) and are then consumed by the latter, thus possessing essential oils and specific proteins. The olfactory system of phytophagous insects plays a key role in detecting volatile substances present in leaves when they are searching for a host plant. For example, the volatile substances emitted by mulberry leaves can activate chemotaxis behavior by *Bombyx mori* [28]. Even the odors of many leaves of non-host plants (other than white mulberry) have a phagostimulant function in addition to attracting *Bombyx mori* silkworms [29,30]. This observation is not specific to silkworms alone but has also been observed in other insects. As an illustration, in one experiment they observed the presence of the whitefly (*Bemisia tabaci*), also known as the whitefly, in a field of commercial rosemary (*Rosmarinus officinalis*). This insect colonized rosemary variety '2', but was not found on variety '11'. Field bioassays and controlled laboratory tests confirmed that the essential oil composition (β-caryophyllene and limonene) differs between the two varieties, which explains why this insect is attracted to one variety and not the other [31]. The same pattern has already been observed in western flower thrips (*Frankliniella occidentalis*), which are attracted to 1,8-cineole, a major component of rosemary essential oil [32]. In a study by [33], they showed that volatile substances had a significant attractive effect on the abundance of herbivorous insects.

Our research results revealed that the quantity of essential oils present in the *Morus alba* host plant is significantly lower than that of *Morus nigra*. IC50 for antioxidant activity of essential oils from *Morus nigra* leaves was significantly lower (1.5± 0.4mg. g^{-1} DM) compared to that of *Morus alba* (11.3± 1.1 mg. g^{-1} DM). These results showed that the essential oils of black mulberry leaves exhibited higher antioxidant activity than those of white mulberry. However, despite this disparity, *M. nigra* failed to attract silkworms. This observation suggests the possibility of an attractive effect when the essential oils are present in low concentrations and a repellent effect when the concentrations are high. This is in agreement with the study conducted by [34], which showed that essential oils can have different effects on insects depending on their concentration. The results showed that essential oils can act as attractants, repellents, or even insecticides depending on the concentration used. Low concentrations of essential oils generally showed attractive activity, while repellency required higher concentrations. The same was observed by [33], who found that volatile substances, including essential oils, have the ability not only to attract insects but also to repel them.

In addition to their attractive potential, the essential oils may have a stimulating effect on the silkworms, encouraging them to consume more mulberry leaves. This increase in food intake could encourage increased silk production. A study was carried out to assess the impact of essential oils on silk production by the silkworm (*Bombyx mori*). The researchers examined the effects of fennel (*Foeniculum vulgare)* and caraway (*Carum carvi*) essential oils on the larval stages of the silkworm. The results showed that the essential oils tested have a positive effect on larval silk production if concentrations are high [35]. Mulberry leaves enriched with various essential oils and extracts of marjoram and thyme are more suitable for rearing mulberry silkworms, with a significant increase in intake and increased protein metabolism [36]. Similarly, showed that treating mulberry leaves with fenchone essential oil significantly increased the weight of the silk gland and cocoon [37].

The dosage of polyphenols showed a 72% superior value in *M. nigra* leaf extracts (47.6± 1,5 mg. g^{-1} DM) compared to *M. alba* leaf extract (13.1± 0,6mg. g^{-1} DM). The polyphenols showed a difference not only in quantity but also in quality. Indeed, analysis of the HPLC profile revealed that black mulberry contains 14 phenolic compounds, whereas white mulberry contains only 8, 5 of which are common to both species. Of these, 3 were present in similar quantities, but for the compounds identified at retention times 21:16 and 23:15, they were present in greater quantities in the black mulberry (Figure 1).

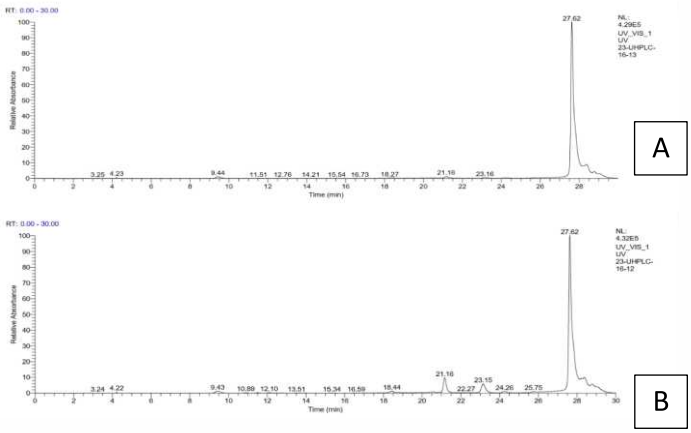

Figure 1: HPLC profiles of phenolic extracts, (A) Morus nigra (B) Morus alba

Flavonoids, which are part of the polyphenol group, showed a significantly higher concentration in *M. nigra* leaf extracts (17.4± 3,2 mg. g^{-1} DM), compared to *M. alba* leaf extracts (5.9± 1,1 mg. g^{-1} DM). Studies have shown that polyphenols present in plants play an essential part in protecting against micro-organisms, as well as acting as a repellent against various insect pests [38,39]. Accordingly, results have shown that phenolic extracts such as flavonols, hydroxy coumarins, anthocyanins, and alkyl flavones, which belong to the flavonoid group, were effective in controlling *Spodoptera frugiperda* larvae [40].

High photosynthetic activity enables the plant to increase its biomass and final yield [41]. The dosage of chlorophyll **a** and **b** showed a difference between *Morus alba* leaf extracts (5.4± 0,3 mg. $g^{-1}$and 8.7± 0,5 mg. g^{-1}FM respectively) and *Morus nigra* leaf extracts (3.9± 0,7mg. g^{-1} and 6.2± 0,4mg. g^{-1}FM respectively). On the other hand, no significant difference is observed for carotenoids between the two species. The presence of higher levels of total chlorophyll contents in the leaves of *Morus alba* compared with *Morus nigra* may explain the intense photosynthetic activity. Studies have shown that increased photosynthetic activity leads to an increase in various antioxidant enzymes, as well as a greater accumulation of proteins [42]. These results are in line with observations of high protein levels in white mulberry (5.4± 0,4mg. g^{-1}DM for *M. alba* leaf extracts and 3.9±0,2mg. g^{-1}DM for *M. nigra* leaf extracts). Similarly, the electrophoresis profile (Figure 2) showed a concordance between the proteins present in the leaves of the White Mulberry and those present in the silk of *Bombyx Mori*, making proteins the most suspected molecules in the White Mulberry - silkworm relationship, since its leaves contain proteins or subunits of silk structural proteins, which the consumption of *Morus alba* leaves is essential for silk production, this study is the first to explore the presence of a specific protein in the white mulberry, which is already present in silk.

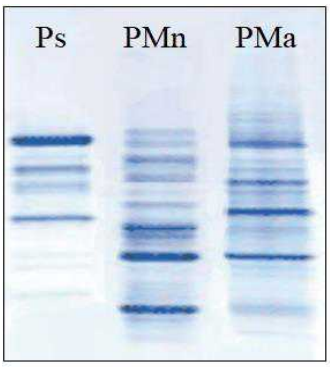

Figure 2: SDS-PAGE electrophoretic profile. [Ps] Silk proteins, [PMn] Morus nigra leaf proteins, [PMa] Morus alba leaf proteins.

Analysis of the soluble sugars contained in the two extracts revealed Analysis of the soluble sugar concentration of the two extracts showed that there was no significant difference (7.2 ±0,3mg. g^{-1}DM was recorded in the *Morus alba* leaf extract and 6.8 ±0,2mg. g^{-1}DM in the *Morus nigra* leaves) and therefore no direct influence on the silkworm's choice of white mulberry.

CONCLUSION

In conclusion, our results revealed that the exclusive consumption of white mulberry leaves by silkworms cannot be attributed to a single key molecule, but rather to a structured assembly of several substances. Each of these substances analyzed played a precise role in the complex interaction between silkworms and white mulberry. There is no doubt that the presence of silk proteins or their subunits in white mulberry leaves gives the proteins an essential and indispensable role in silk production, and therefore in the silkworm's choice of these leaves. But other compounds also play an important role, such as essential oils, which are responsible for attracting silkworms to white mulberry and repelling them from black mulberry. In addition, the polyphenols present in black mulberry have a repellent effect on silkworms, whereas their presence is negligible in *M. alba* compared with *M. nigra*. Finally, the presence of high concentrations of chlorophyll in white mulberry favored increased protein production. These results highlight the complexity of the relationship between white mulberry and silkworms, involving a range of substances and mechanisms interacting synergistically to regulate this close interaction.

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