ANTIOXIDANT AND ENZYME INHIBITORY EFFECTS OF *MORUS SP.* EXTRACTS

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ANTIOXIDANT AND ENZYME INHIBITORY EFFECTS OF *MORUS SP*. EXTRACTS (Abstract): *Morus* leaves water extracts are used for a long time for its anti-diabetic, anti-inflammatory, and cardiovascular disease-treating properties. Our research's **objective** was to evaluate the hypoglycemic and antioxidant effects of *Morus* leaves extracts obtained by using different solvents. **Materials and methods**: The extracts from *Morus nigra* and *Morus alba* leaves were obtained by ethanol-water extraction, respectively hot water extraction. We assess the enzyme inhibition (α-amylase and α-glucosidase) ability of extracts from *Morus sp*. leaves, and we correlate these effects with polyphenols and flavonoids content. **Results**: Total polyphenols content ranged between 89.4 mg gallic acid equivalents/g ethanol-water extract of *Morus nigra* leaves and 46.81 mg gallic acid equivalents/g aqueous extract of *Morus alba* leaves. Ethanol-water extracts contain more polyphenols and flavonoids than aqueous extracts. The most important effect on alpha-amylase was presented by the ethanol-water extract from *Morus alba* leaves (EC50 88.26±0.52 µg/mL) and on alpha-glucosidase by the ethanol-water extract from *Morus nigra* leaves (EC50 69.86±0.03 µg/mL mL). From the point of view of antioxidant effects, the extracts inhibit lipoxygenase, with the maximum effect in the case of the ethanol-water extract from *Morus alba* leaves (EC50 23.75±1.77 µg/mL). The ethanol-water extracts have a greater capacity to chelate the ferrous ion compared with aqueous extracts, the most active being the ethanol-water extract from the *Morus nigra* (EC50 213.32±0.26 µg/mL). **Conclusion**: The conducted study highlights the ability of the analyzed extracts to control the enzymes involved in the digestion of carbohydrates and to reduce oxidative stress. **Keywords:** *MORUS ALBA*, *MORUS NIGRA*, ANTIOXIDANT ACTIVITY, ANTIDIABETICS.

Diabetes mellitus is the most common endocrine disease affecting over 537 million adults worldwide in 2021 with a predicted rise to 643 million by the end of the decade (1). Type II (T2DM) is the major form of diabetes, accounting for about 90% of the cases. Diabetes is caused by impaired insulin secretion, insulin resistance or a combination between the two which leads to a dysregulated metabolism of the

carbohydrates and lipids (2). Chronic hyperglycemia induces oxidative stress, affects many biological structures, and represents the most important cause for diabetes consequences such as peripheral neuropathy, diabetic retinopathy, diabetic nephropathy, or diabetic ketoacidosis.

The management of diabetes involves, among lifestyle changes, administration of oral hypoglycemic substances which are associated with side effects such as hypoglycemia, weight gain (insulin, sulphonyl urea), lactic acidosis (biguanide), and gastrointestinal disturbances. Therefore, herbal medicines present an attractive alternative in the management of diabetes as they are generally accepted to present less adverse effects compared to modern conventional pharmaceutics (2). Moreover, considering that more than three quarters of adults living with diabetes are in low- and mid-income countries both the patients and the national healthcare institutions could benefit for herbal alternative medicines as they are generally less expensive than synthetic pharmaceutics (1, 2). In the recent years, the number of studies on using plants as a source of diverse biologically active substances with anti-inflammatory, diabetic, cardiovascular diseases, anti-cancer, and antioxidant effects have increased (3, 4). The bioactive substances from plants, such as: polyphenols, phenolic acids, flavonoids, flavonols, diterpenes, tannins, phytosterols, fatty acid esters, phenylpropanoids, alkaloids, glycosides, etc., have attracted significant interest in medicinal chemistry and natural product research (5, 6).

Polyphenols are divided into four main classes: flavonoids, phenolic alcohols, stiblins, lignans and represent the biologically active substances found in plan-based diets. The structure and proprieties of these compounds depend on several factors, such as plant cultivar/genotype, growing environment conditions, soil characteristics, harvest time, and storage conditions. These substances come from plants, such as fruits, vegetables, cereals, and coffee, and are an integral part of the human diet. Polyphenols are also recognized as a preventative for degenerative illnesses (7).

Polyphenols and flavonoids, by their hydroxyl groups can scavenge free radicals, release hydrogen that will neutralize different oxidants and also, they have the ability to interact with different enzymes and to control activity of these (7). Depending on the source and combination, they may exhibit diverse properties such as specific enzyme inhibition activity.

α-Amylase and α-glucosidase are two important enzymes responsible for catabolizing starch, glycogen and disaccharides to glucose that will be absorbed into the blood. Inhibition of these enzymes is often employed for controlling blood glucose level by decreasing the rate of intestinal carbohydrate metabolism.

Oxidative stress participates in the onset and worsening of diabetes. The identification of compounds that have both antioxidant effects and the ability to control the enzymes involved in the digestion of carbohydrates represents an advantage for the patient with diabetes because the number of drugs administered to him is reduced.

Morus alba, *Morus nigra* and *Morus rubra* are woody plant from the Moraceae family that has been cultivated worldwide in sericulture from ancient times. They have been used in Chinese medicine for several purposes such as antifever, diuretics, protection of the liver, improvement of eyesight, and to prevent cardiovascular diseases. *Morus sp*. main active compo-

nents and the compositions of phenol, alkaloid, and flavonoid, have been thoroughly investigated for their noteworthy characteristics (8). Mulberry fruits are used as a natural treatment to reduce the blood pressure, to treat fever or to prevent liver and kidney damage (9, 10, 11). The antithrombotic, anti-obesity, antiinflammatory, anti-carcinogenic and neuroprotective effects of mulberry fruit flavonoids are registered in the Pharmacopoeia of the People's Republic of China (12).

Morus sp. leaves polyphenols are functional compounds possessing various beneficial effects against cardio metabolic diseases, including anti-hyperglycemic, antihyperlipidemic, anti-obesity, antihypertensive, anti-oxidative, anti-inflammatory, anti-atherosclerotic, and cardioprotective effects (4). Leaves of *M. nigra* are commonly used by women during menopause as a replacement for the conventional hormonal substitute therapy, with a similar effect to the estrogens (13). Roots, bark, stem twigs, and fruits of *Morus sp.* possess valuable bioactive ingredients that can be explored in pharmaceutical, health care, cosmetic and food industries (8).

The aim of our study was the assessment of antioxidant and enzyme inhibition (α-amylase and α-glucosidase) ability of extracts from *Morus sp*. leaves and to correlate these effects with polyphenols and flavonoids content.

MATERIAL AND METHODS

Chemicals and reagents

Reagents: Folin-Ciocalteu's Reagent, gallic acid, sodium nitrite, aluminum chloride, acarbose, alpha-glucosidase, alpha-amylase, 4-nitrophenyl α-D-glucopyranoside (pNPG), sodium hydroxide, acetate buffer (pH 5.25), ferrous sulphate, ferrozine, potassium ferricyanide, trichloroacetic acid, ferric chloride, 3,5-dinitrosalicylic acid were purchased from Sigma Aldrich (Germany). Dimethyl sulphoxide (DMSO), methanol, ethanol, hydrochloric acid were purchased from Merck (Germany). The water used in this study was ultrapure water generated with Milli-Q PLUS (Millipore Corporation).

Preparation and analysis of the extracts

Morus alba and *Morus nigra* leaves were collected from their natural habitat in Iasi (Coordinates 47°9′24″N 27°29′10″E), Romania, in May 2022. Vegetal samples were air-dried and were ground with an IKAA1 analytical mill. Voucher specimens are deposited at the Department of Pharmacognosy, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy from Iasi, Romania.

Preparation of aqueous extract: 10 g of dried and powdered leaves were extracted twice with 150 mL boiling distilled water and was maintained 20 minutes to 80° C. After cooling the extract was filtered on Whatman filter paper no.1 and the combined extracts were lyophilized in order to obtained lyophilizate that was used for analysis (14).

Preparation of the ethanol-water extract: 2 g of dried and powdered leaves were extracted with 2 x 50 mL ethanol-water 70:30 on water bath, at reflux, for 45 minutes. The extracts were filtered and completed to 100 mL using the same solvent. The extracts were dried using a rotary evaporator.

Dried extracts were solved in dimethyl sulfoxide (for ethanol-water extract) or water (for lyophilizate extract) in order to obtain solutions with concentrations between 0.0097 and 20 mg/ml that have been

used for the tests (14). We obtained and analyzed four extracts: MAE (*Morus alba* ethanol-water extract), MAA (*Morus alba* aqueous extract), MNE (*Morus nigra* ethanol-water extract), MNA (*Morus nigra* aqueous extract).

Determination of total phenols content: 1 ml diluted solutions of extracts were mixed with 1 mL of Folin-Ciocâlteu's Reagent, allowed to stand for 5 minutes at 25ºC before adding solution of sodium carbonate 20%. For 120 minutes, the samples were kept in the dark, before measuring the absorbance at 750 nm. Gallic acid was used as standard. The results were expressed as gallic acid equivalents (GAE) (mg/1 g dry extract) (15).

Determination of flavonoids content: 0.25 mL diluted extract were mixed with 1.525 mL distilled water, 0.075 mL solution sodium nitrite 5%- and 0.15-mL solution aluminum chloride 10%. After 5 minutes 0.5 mL solution sodium hydroxide 1M was added and the absorbance of solution was determined at 510 nm against sample blank. The results were expressed as rutoside equivalents (mg RE/1 g dried extract) (16).

Antioxidants and enzyme inhibition tests

Antioxidant activity of the extracts was determined by iron chelating test, ferric ions reducing test, lipoxygenase inhibition.

Iron chelating test: 0.2 mL diluted extracts, 0.74 mL 0.1 M acetate buffer (pH 5.25), 0.02 mL 2 mM ferrous sulphate solution and 0.04 mL of 5 mM ferrozine solution were mixed. After 10 in the dark medium the absorbance of the solutions was determined at 532 nm. The iron chelating activity was determined using the following formula: Activity % = 100 × (A_C – A_S)/(A_C), where A_C is the absorbance of the control solution (without chelating compounds) and A_S is the absorbance of the sample solution. Rutoside was used as positive control. The assay was conducted in triplicate. The EC_{50} was calculated for each extract that present a chelating activity over 50% and is expressed as mg sample/mL final solution (17).

Ferric ions reducing test: 0.5 mL diluted extracts, 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide solution 1% were mixed and maintained at 50ºC for 20 minutes. After cooling the solutions were mixed with trichloroacetic acid solution 10%, centrifuged at 3,000 rpm, and an aliquot of the upper layer was mixed with distilled water and ferric chloride solution 1%. The absorbance of solutions was determined at 700 nm. Rutoside was used as positive control (18).

Lipoxygenase inhibition test: 0.05 mL diluted extracts were mixed with 0.05 mL lipoxygenase buffer solution (pH 9) and kept for 10 minutes at room temperature. Afterwards, 2 mL linoleic acid buffer solution 0.16 mM (pH 9) were added. The absorbance was determined at 234 nm for 120 seconds. The results were calculated using the following formula: % activity = (A_E – A_{ES}) x 100/A_E, where A_E is the difference between the absorbance of the enzyme solution alone after 90 seconds and the absorbance of the same solution after 30 seconds and A_{ES} is the difference between the absorbance of the enzyme solution mixed with the sample after 90 seconds and the absorbance of the same solution after 30 seconds (19). Rutoside was used as positive control.

The capacity of extracts to control carbohydrates digestion was evaluated by alpha-amylase and alpha-glucosidase inhibition tests.

Alpha-amylase inhibition test: 0.04 mL diluted extracts were mixed with 20 mM phosphate buffer pH 6.7, 0.08 mL alpha-amylase solution (2 UI/mL), 0.2 mL 0.5% starch solution in 20 mM phosphate buffer pH 6.7 and were maintained at 37°C for 10 minutes. The reaction was stopped with 0.32 mL 96 mM 3,5-dinitrosalicylic acid and the mixture was maintained at 100°C for 15 minutes. After cooling was added distilled water and the absorbance of each solution was measured at 540 nm. The percentage of alpha-amylase inhibition was calculated as follows: $(1–A_C/A_S) \times 100$, where A_C is the absorbance of control and A_S is the absorbance of the sample. Acarbose was used as positive control (20).

Alpha-glucosidase inhibition test: 0.1 mL diluted extracts were mixed with alpha-glucosidase (1 U/mL), phosphate buffer, pH 7.0, and were maintained at 37°C for 5 minutes. After that 0.25 mL p-nitrophenyl-alpha-D-glucopyranoside solution was added and the mixture was maintained again at 37°C for 10 minutes. The absorbance of the final solution was determined at 405 nm. The percent of alpha-glucosidase inhibition was calculated as follows: $(1–A_C/A_S) \times 100$, where A_C is the absorbance of control and A_S is the absorbance of samples containing extracts. The inhibitory concentration of the extract required to inhibit the activity of the enzyme by 50% (EC_{50}) was calculated by regression analysis. Acarbose was used as positive control (21).

For each test, the concentration of the extract required to obtain a positive effect over 50% (EC_{50}) was calculated by regression analysis and expressed as µg sample/mL final solution.

Statistical analysis: all determination were conducted in triplicate and the results are expressed as mean ± standard deviation. Statistical analysis of the data was performed using ANOVA test.

RESULTS

Radojkovi and co-workers investigated the effects of various extraction parameters on the extraction yield of phenolic compounds and the antioxidant activity of the extracts from *Morus nigra* leaves using Response Surface Methodology (RSM). It was shown that three factors, namely ethanol concentration, temperature and liquid/solid ratio have the most significant influence (22). Starting with the data collected from this specialty field, we have chosen to do extractions using 70% ethanol and boiling distilled water.

Morus sp. extracts can be obtained through different extraction processes such as: pressurized liquid technique, supercritical-fluid, microware-assisted, ultrasound-assisted, in the solid phase, but the solid-liquid extraction is the most advantageous compared to other methods due to the reduced processing and the convenience of operation (23, 24, 25). These methods often use toxic solvents and may require an evaporation or concentration step. Large amounts of solvents are needed, which could be hazardous to the human health and the environment, difficult and time-consuming to completely remove. Furthermore, the possibility of thermal degradation of the bioactive ingredients cannot be neglected due to the high temperatures of the solvents during the prolonged extraction time. Considering these conditions and also the fact that in traditional medicine are used the aqueous extracts from the *Morus sp*. leaves, we decided to realize and analyze the aqueous extracts resulted after concentration through lyophilization.

Chemical composition of extracts

The total phenolic contents of extracts from *Morus alba* and *Morus nigra* leaves ranged from 46.80 to 95.20 mg GAE/g (tab. I). Ethanol-water extracts obtained from *Morus* leaves contain more polyphenols and flavonoids than aqueous extracts. This is explained by the ability of ethanol-water solution to extracts some lipophilic compounds from the vegetal samples. In traditional medicine *Morus* leaves aqueous extracts are used for diabetic patients and the ratio between vegetal material and water is lower than that was used by us to prepare the aqueous extracts.

These results were in accordance with those reported by other researchers that also found higher total polyphenolic content for *M. nigra* than for *M. alba* (26).

The variation of phenolic compounds in the leaves extracts depends on many factors, such as degree of maturity at harvest, genetic differences and environmental conditions but also the solvent type, extraction time, temperature in the different extraction procedures and analytical methods used in each work (7).

Suriyaprom and coworkers observed that the aqueous extracts of *Morus* leaves exhibited the high antioxidant and antibacterial activity, which was associated with a higher phenolic and anthocyanin content (27).

TABLE I.

Total phenolic and flavonoids content of *Morus sp*. extracts

No	Sample	Polyphenol (mg GAE/g)	Flavonoids (mg RE/g)
1.	MAA	46.81 ± 0.01	3.25 ± 0.01[a]
2.	MNA	61.31 ± 0.02	7.28 ± 0.01
3.	MAE	62.53 ± 0.02	7.16 ± 0.005[a]
4.	MNE	89.46 ± 0.07	11.22 ± 0.01

MAE - *Morus alba* ethanol-water extract; MAA - *Morus alba* aqueous extract.
MNE - *Morus nigra* ethanol-water extract; MNA - *Morus nigra* aqueous extract;
[a]p <0.0001 (ethanol-water extract *vs.* aqueous extract)

Yazdankhah and co-workers investigated total phenol content, antioxidant activity and anthocyanin components of ethanol-water *Morus nigra* extract and they noticed that *Morus nigra* extract had a good antioxidant potential and the main detected anthocyanin by HPLC method was cyanidin-3-glucoside (28).

The assessment of antioxidant activity

Iron 2+ is essential for human body but at the same time, depending on concentration could be involved in toxic processes. This ion participates in toxic conversion of hydrogen peroxide and superoxide anion to hydroxyl radical that affects proteins, nucleic acids and lipids by oxidation (29). Uncontrolled oxidative processes induce different pathological conditions and increase the risk for cardiovascular disease, diabetes, inflammatory state, and cancer. Natural compounds that have the ability to block iron 2+ will control oxidative processes and will reduce the intensity of oxidative stress.

Morus sp. extracts have the ability to block iron ions depending on extract concentration. Ethanol-water extracts from *Morus nigra* are seven times more potent than lyophilized extracts from the same species. For Morus alba extracts, the ethanol-water are four times more potent (fig. 1).

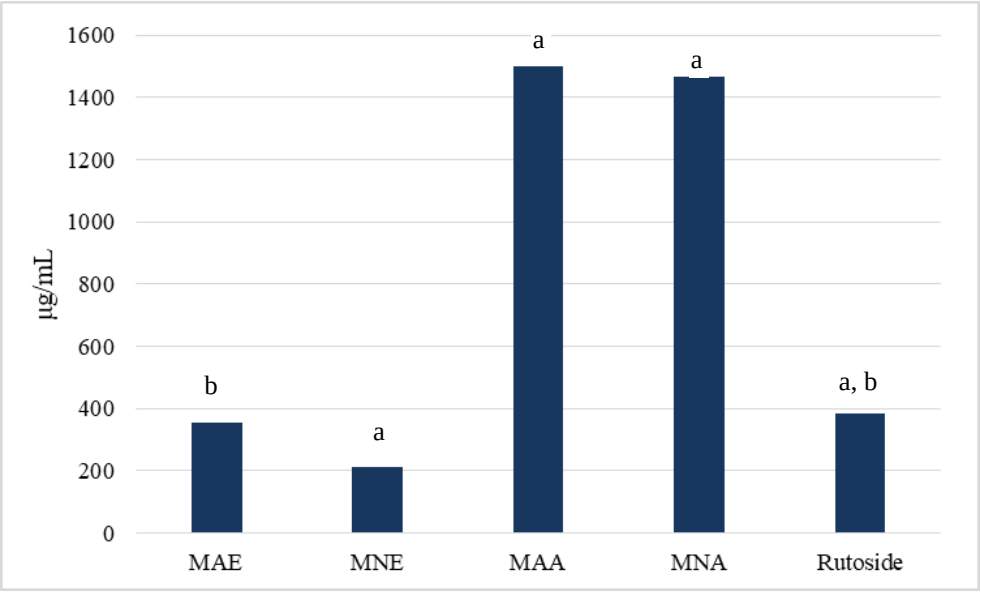

Fig. 1. The variation of the EC_{50} values of the *Morus sp*. extracts in the iron chelating test MAE - *Morus alba* ethanol-water extract; MAA - *Morus alba* aqueous extract; MNE - *Morus nigra* ethanol-water extract; MNA - *Morus nigra* aqueous extract; sample *vs.* positive control - a: p<0.0001 extremely statistically significant; b: p < 0.05 statistically significant

Compared to the Rutoside used as a positive control, the ethanol-water extracts have a greater capacity to chelate the ferrous ion (fig. 2), which suggests the involvement in this process of other compounds present in the plant extracts (30).

Iron 3+ is another form of iron that participates in redox processes in the human body. The high ratio Fe^{3+}/Fe^{2+} induced oxidative stress (31). Compounds with hydroxyl groups have the ability to transform Fe^{3+} to Fe^{2+} and finally to chelate Fe^{2+}.

15-lipoxygenase is a non-heme iron enzyme that catalyze the oxidation of unsaturated fatty acids to peroxides involved in physiological and pathological processes. High rate of its activity is correlated with inflammation and uncontrolled degradation of lipids and membrane structures.

The activity of enzyme can be modified until it is blocked by compounds that reduce Fe^{3+} to Fe^{2+}, chelate Fe^{2+} or modify the three-dimensional structure of the enzyme. Organic compounds such as polyphenols and flavonoids have the ability to release protons and electrons that modify the enzyme activity (32).

All extracts influence the enzyme activity in a concentration-dependent manner (fig. 3). The less active is *Morus alba* aqueous extract with maximum inhibition of 47.91±0.88%.

Morus alba ethanol-water extract has the most important effect on lipoxygenase inhibition test, but this extract doesn't contain the most important quantities of polyphenols or flavonoids. This effect sustains the necessity to establish the chemical composition of extracts and to evaluate the interactions between the most important groups of active compounds and enzyme.

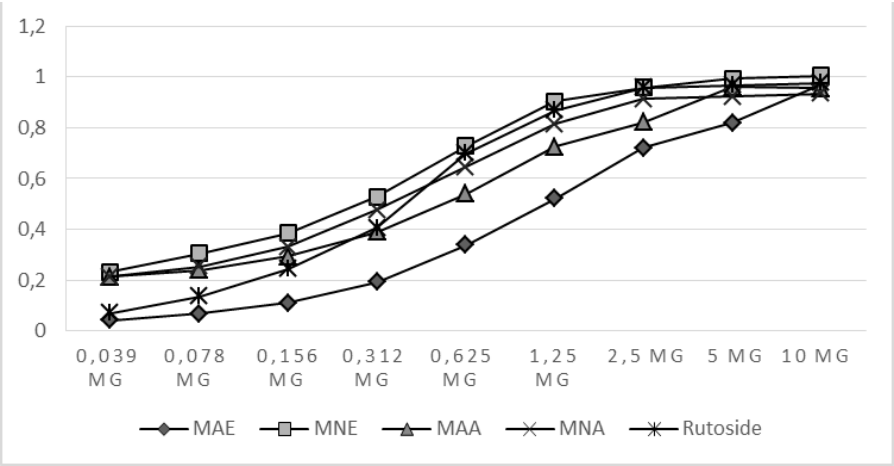

Fig. 2. The variation of absorbance for the ferric ions reducing test
MAE - *Morus alba* ethanol-water extract; MAA - *Morus alba* aqueous extract; MNE - *Morus nigra* ethanol-water extract; MNA - *Morus nigra* aqueous extract

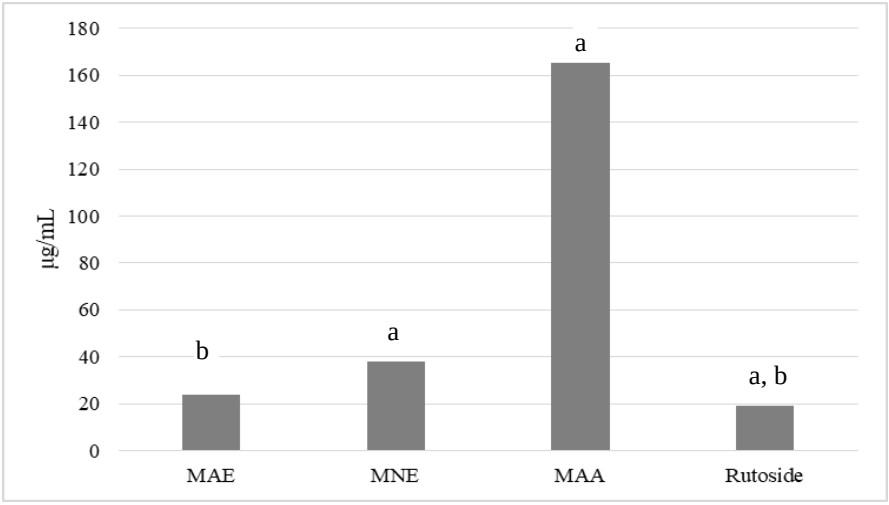

Fig. 3. The variation of the EC_{50} values of the *Morus sp.* extracts in the lipoxygenase inhibition test. MAE - *Morus alba* ethanol-water extract; MAA - *Morus alba* aqueous extract; MNE - *Morus nigra* ethanol-water extract; MNA - *Morus nigra* aqueous extract; a - $p<0.0001$ extremely statistically significant; b - $p < 0.05$ statistically significant

The ability to control carbohydrates digestion

Alpha-amylase and alpha-glucosidase are the most important digestive enzymes for carbohydrates transformations in the small intestine. All compounds that have the ability to control these enzymes will control the value of postprandial hyperglycemia by lowering carbohydrates hydrolysis and glucose absorption.

Long-term hyperglycemia is difficult to control, and this phenomenon is correlated with high risk for diabetes and obesity. Some studies suggest the necessity to con-

trol these enzymes in order to control diabetes onset (33).

The values of EC_{50} determined for alpha-amylase and alpha-glucosidase inhibition tests are presented in figures 4 and 5.

All extracts reduce alpha amylase activity with more important effects for ethanol-water extracts compared to aqueous ones. The EC50 values determined for the ethanol-water extracts are approximately three times lower compared to those for acarbose.

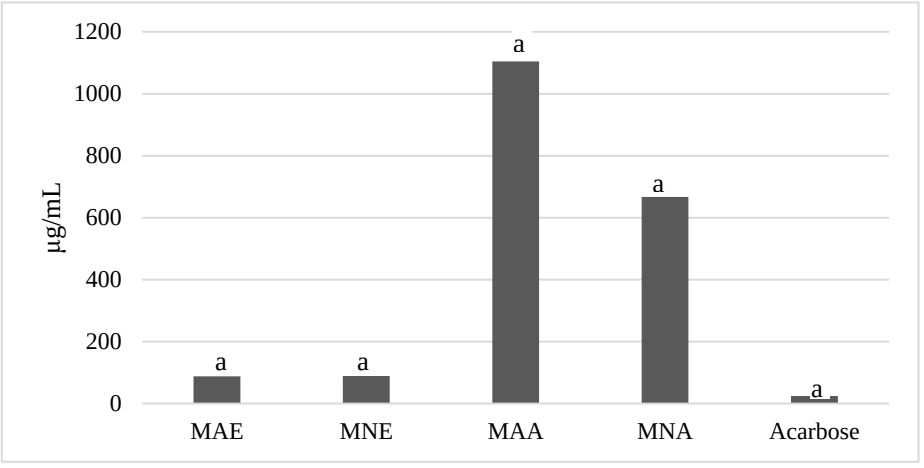

Fig. 4. The variation of the EC_{50} values of the *Morus sp.* extracts in alpha-amylase inhibition test. MAE - *Morus alba* ethanol-water extract; MAA - *Morus alba* aqueous extract; MNE - *Morus nigra* ethanol-water extract; MNA - *Morus nigra* aqueous extract; a - p<0.0001 extremely statistically significant

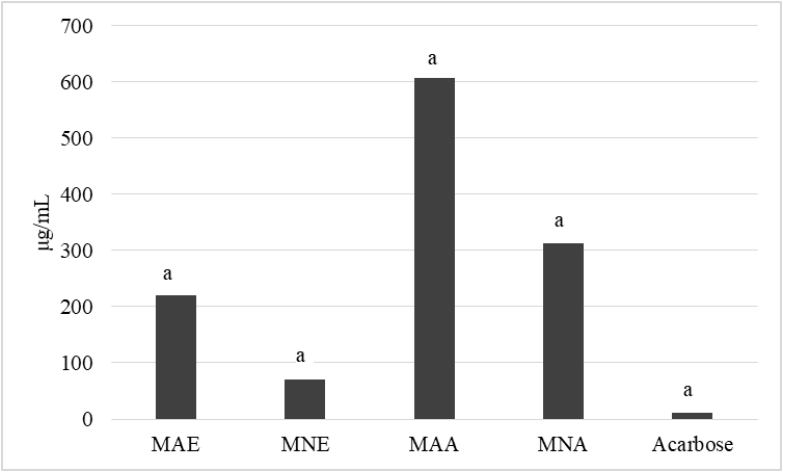

Fig. 5. The variation of the EC_{50} values of the *Morus sp.* extracts in alpha-glucosidase inhibition test. MAE - *Morus alba* ethanol-water extract; MAA - *Morus alba* aqueous extract; MNE - *Morus nigra* ethanol-water extract; MNA - *Morus nigra* aqueous extract; a - p<0.0001 extremely statistically significant

DISCUSSION

The extracts control alpha-glucosidase activity in special manner, the effect is not so important until concentration below 0.625 mg/mL and reach almost total inhibition at concentration above 10 mg/mL for ethanol-water extracts.

In the alpha-amylase inhibition test, the ethanol-water extract from both species have similar effects. It also observed that the effect of the same extracts is different in the alpha-glucosidase inhibition test with a stronger effect for extracts from *Morus nigra*. The enzyme inhibition effects are correlated with polyphenols content. Extracts from the two species can inhibit enzymes differently, so Eruygur and colleagues observed a more intense action for *Morus alba* in the alpha-glucosidase inhibition test and for *Morus nigra* in the alpha-amylase inhibition test (34).

Morus sp. extracts control digestive enzymes like acarbose that is one of the most important drugs used for this effect. The use of acarbose is limited by therapeutic compliance due to side effects such as flatulence, diarrhea, bloating and liver toxicity. This side effects are induced by the aggressive action of acarbose on enzymes, for this reason moderate inhibitors are more acceptable by the patients (35).

Another interaction between active compounds from extracts is with starch that is the substrate for alpha-amylase. Plant extracts rich on polyphenolic compounds modify the physicochemical properties of starch molecule and reduce it digestibility on the small intestine (36). Polyphenols interact with starch structure and realize hydrogen bonds with hydroxyl groups of this (37).

Depends on concentration isolated polyphenol carboxylic acids such as gallic acid can increase starch transformation by reducing the value of pH. The use of these acids in pharmaceutical forms is limited by this effect (38).

Chlorogenic acid, rutin, isoquercitrin, and quercitrin are present in *Morus alba* leaves and have been shown to have hypoglycemic properties and an ameliorating effect on diabetic nephropathy, insulin resistance, and dyslipidemia in rats. In extensive work, Hunyadi and his collaborators have demonstrated that chlorogenic acid and rutin play a major role in the in vivo anti-diabetic activity of *Morus alba* leaf extract (39). The preliminary analysis by HPLC highlights the presence of polyphenols in our samples, thus in future research the quantification of these compounds and the highlighting of their biological implications will be conducted

The obtained results allow the establishment of correlations between the extraction conditions, the composition of the extracts and their biological actions. The results also support the use of extracts for in vivo studies, with the aim of highlighting their ability to control blood glucose levels.

CONCLUSIONS

Morus leaves contain a variety of polyphenols that have antioxidant effects and free radical scavenging *in vitro*.

The analyzed extracts have an antioxidant activity and capacity of controlling the enzymes involved in the digestion of the carbohydrates, but in order to raise the therapeutically efficiency and also to reach optimal effects by administrating lower quantities of vegetal extracts, it’s necessary to fraction these extracts and an advanced separation of the compounds with important biological effects.

CONFLICT OF INTEREST AND FUNDING

The authors declare that there is no conflict of interest, and they received no specific funding regarding this scientific research.

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