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EVALUATION OF SINGLE AND ASSOCIATED BOTANICALS ON GLUCOSE TOLERANCE IN GLUCOSE-INDUCED HYPERGLYCEMIC MICE

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SUMMARY

Nutraceuticals can be used in addition to conventional treatments to improve glycemic control. The aim of this study was to evaluate the efficacy of *Morus alba*, *Ilex paraguariensis*, and *Chromium picolinate*, alone and in association, on glucose tolerance in glucose-induced hyperglycemic mice. Male CD1 mice were treated for 6 weeks with *Chromium picolinate* (0.8 mg/kg) (A), *Ilex paraguariensis* (1000 mg/kg) (B) and Morus alba (50 mg/kg) (C), following these combinations: A; B; C; A + B + C; A + B; A + C; B + C. The control animals were administered with the vehicle only. The oral glucose tolerance test (OGTT) was carried out in mice 4 and 6 weeks after the start of treatment with *Chromium picolinate, Ilex paraguariensis* and *Morus alba*, alone or in combination, or by the administration of vehicle (CMC 1%) in the control group, 24 h after the last daily administration.

The complete mixture A + B + C reduced the glycemic values recorded in animals 60 min after glucose administration compared to the control group values and the B + C mixture showed a significant prevention of the glycemic peak at 30 min after 4 weeks of treatment. The combination of A + B + C induced the best effect, preventing the glycemia increase at 60 min after 6 weeks of treatment. In conclusion, the nutraceutical resulted effective for use in the prevention of diabetes mellitus.

Key words
Ilex paraguariensis; Morus alba; botanicals; glucose; mice.

Impact statement
The synergic effect of a nutraceutical containing all three components *Morus alba*, *Ilex paraguariensis*, and *Chromium picolinate*, at 1000 mg of dosage, could be effective for use in the prevention of diabetes mellitus.

List of abbreviations:
OGTT: Oral Glucose Tolerance Test; AUC: area under the curve.

INTRODUCTION

The World Health Organization (WHO) estimates that more than 190 million people worldwide are affected by type 2 diabetes mellitus and this number is constantly on the rise. The pathogenesis of type 2 diabetes mel-

litus is very complex and see different actors playing a role: the main mechanisms involved are an increased peripheral insulin resistance and a decrease of beta cell function (1). Due to these mechanisms, blood glucose levels gradually rise, causing endothelial damage and increasing cardiovascular risk. In the latest years, several new drugs have been marketed for diabetes, but also phytotherapy may play a role in improving glucose metabolism and insulin resistance (2-4).

Nutraceuticals can be used in addition to conventional treatments to improve glycemic control (5, 6), or can be used to prevent type 2 diabetes mellitus, in addition to diet and physical activity, in subjects affected by dysglycemia (7).

Recently, numerous herbs, such as various Mulberry species (*Moraceae* family), showed anti-diabetic action by acting on various aspects of the pathology. White Mulberry (Morus Alba), for example, decreases body weight and adiposity (8), improves insulin resistance (9), increases glucose uptake, GLUT4 translocation and adiponectin (10), inhibits α-glucosidase activity (11), and improves endothelial function (12). On the other hand, *Ilex paraguariensis* (Yerba Maté) has some beneficial effects on glucose absorption (13), it also has hypocholesterolemic, anti-inflammatory (14), and antioxidant effects (15). Xanthines and Polyphenols, Caffeoyl derivatives, and Saponins have a role in many of the pharmacological activities of Yerba Maté (16, 17). Finally, chromium apparently has a role in maintaining proper carbohydrate and lipid metabolism in mammals. This role probably involves empowerment of insulin signaling, reduction of fat mass and increasing of lean body mass. Chromium picolinate has a greater bioavailability compared to Chromium and this formulation may explain the superior efficacy in glucose and lipid control (3). Human studies suggest that Chromium picolinate decreases insulin levels and improves glucose disposal in obese and type 2 diabetic population (18, 19).

On this basis, we evaluated the effects and synergy of a nutraceutical supplementation (Glicoset® 1000, produced by Nutrilinea S,r.l., Gallarate (VA), Italy), containing a mineral (Chromium picolinate, A) and two botanicals (*Ilex paraguariensis*, B, and *Morus alba*, C) (**table I**), in normal mice on glucose tolerance after an Oral Glucose Tolerance Test (OGTT). In particular, the aim of this study was to evaluate the anti-hyperglycemic effect of three substances: A, B, and C, alone and in combination, on glycemic profile in a mice model.

MATERIALS AND METHODS

Study design

In this study we valuated the anti-hyperglycemic effect of A, B and C, alone and in association, in a sample of mice subjected to a

Table I. Composition of the nutraceutical supplement (Glicoset® 1000).

Ingredients	Daily intake:
Chromium picolinate	100 mcg (250% RDD)
Ilex paraguariensis	1000 mg
Morus alba 2% I-deoxinojirimcina	Of which 1 mg DNJ
Silicon dioxide	q.s.
Magnesium stearate	q.s.
Dicalcium Posphate	q.s.
Microcrystalline cellulose	q.s.
E172	q.s.

RDD: Recommended Daily Dose; DNJ: I-deoxinojirimcina; q.s.: quantum sufficit.

load of glucose. The following combinations were evaluated: A; B; C; A + B + C; A + B; A + C; B + C.

Animals

Male CD1 mice (Envigo, Varese) that weighed about 20-25 g at the start of the experiment were used, housed in the Laboratory Animal Stable Center of the University of Florence (Ce.S.A.L.). The animals were placed in cages of 26 cm x 41cm, in environments with a temperature of 23 ± 1 °C with a 12-hour circadian cycle and fed according to the standard diet and ad libitum water.

All treatments were carried out following the Directives 2010/63/EU of the European Parliament and of the Council of the European Union (September 22, 2010) regarding the protection of animals used for scientific purposes. The ethical policy of the University of Florence conforms to the National Institutes of Health Guide for the care and use of laboratory animals (NIH Publication n. 85-23, revised 1996; University of Florence Assurance n. A5278-01). Formal approval for conducting the experiments was given by the university council. The experiments were carried out according to the ARRIVE guidelines (20) trying as much as possible to minimize the suffering of the animals and their number.

Administration of the mixture

The mixture, consisting of Chromium picolinate (0.8 mg/kg), *Ilex paraguariensis* (1000 mg/kg) and *Morus alba* (50 mg/kg), was suspended in a 1% carboxymethylcellulose (CMC) solution and administered orally daily for 6 consecutive weeks. The control animals were administered with the vehicle only.

Oral glucose tolerance test

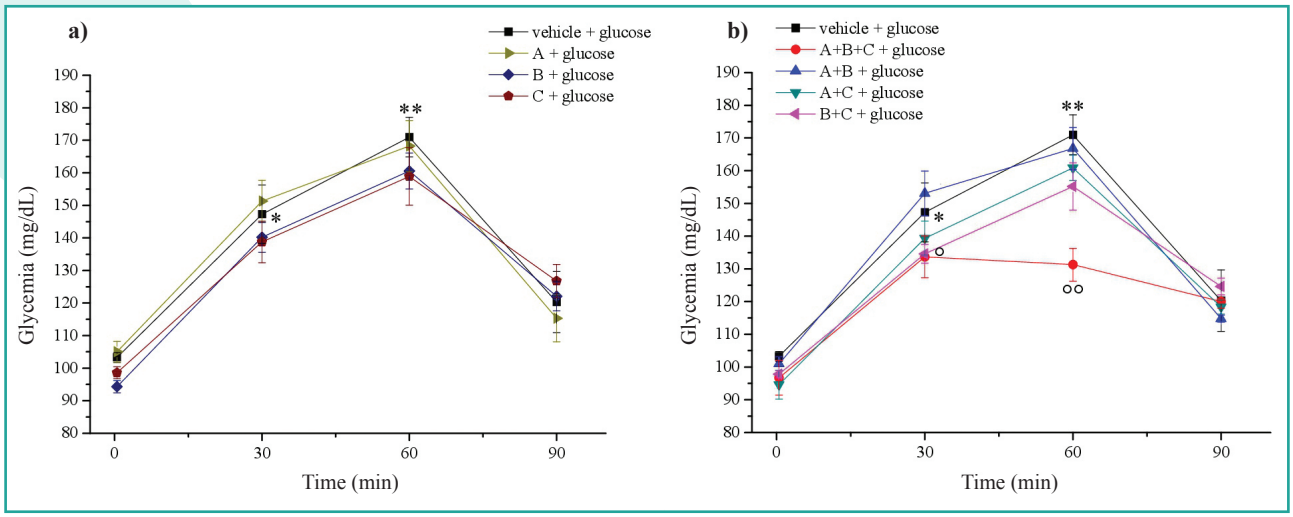
The OGTT was carried out in mice 4 and 6 weeks after the start of treatment with the mixture consisting of Chromium picolinate, *Ilex paraguariensis* and *Morus alba* or by the administration of vehicle (CMC 1%) in the control group, 24 h after the last daily administration. Glucose (3 g/kg) was solubilized in water and administered orally after fasting the animals for 4 h; blood glucose values were measured at minute 0, 30, 60 and 90 by blood sampling from the caudal vein and analysis with the Accu-Check Aviva planar sensor based on the glucose oxidase method.

Statistical analysis

All experimental results were expressed as mean ± standard error (M ± SE). Each group of treatment was represented by 10 mice. A one-way analysis of variance (one-way ANOVA) was conducted, followed by the Bonferroni test to verify the significance between two averages. Residual analysis showed that ANOVA residuals followed a normal distribution. The analysis of variance and the Bonferroni test were performed with the statistical program Origin 9.1. Differences with p value < 0.05 were considered significant.

RESULTS

The animals were treated daily for 6 consecutive weeks orally using, alone or in combination, 0.8 mg/kg of A, 1000 mg/kg of B and 50 mg/kg of C. On weeks 4 and 6, we evaluated the effectiveness of these substances in protecting animals from the glycemic peak induced by a glucose load (3 g/kg per os; performed after a 4 h fast). The results were compared with those of a group of vehicle-treated animals. The results obtained were reported in **figures 1 and 2**. On week 4 of treatment, the acute administration of glucose 3 g/kg significantly increased the glycemic values in the control animals after 30 min (147.3 ± 9.0 mg/dL *vs.* 103.3 ± 1.5 mg/dL), this increase peaked 60 min (171.0 ± 6.1 mg/dL) after the administration of sugar (**figure 1 a**). Daily treatment with the complete mixture A + B + C significantly reduced the glycemic values recorded in animals 60 min after glucose administration (131.3 ± 5.0 mg/dL) compared to the control group values (**figure 1 b**). The B + C



Figures 1 a, b. Evaluation of glycemia levels after 4 weeks of treatment. Each value was expressed as mean ± S.E.M. of 10 mice. * P < 0.05 and ** P < 0.01 *vs* values recorded at time 0 (0 min) in the same group; ° P < 0.05 and °° P < 0.01 *vs.* vehicle-treated group.

mixture showed a significant prevention of the glycemic peak at 30 min (134.6 ± 2.9 mg/dL) (**figure 1 b**).

The experiments were repeated after 6 weeks of treatment with the mixture. The glucose load induced a significant increase after 30 and 60 min after administration (144.8 ± 7.7 mg/dL and 180.5 ± 4.6 mg/dL, respectively *vs* the control group -93.5 ± 6.1 mg/dL) (**figure 2**). The repeated treatment with the complete mixture A + B + C induced the best effect, significantly preventing the glycemia increase at 60 min (138.5 ± 4.3 mg/dL) (**figure 2 b**). At 60 min also the mixtures B + C (154.6 ± 4.7 mg/dL), A + B (158.4 ± 6.1 mg/dL) (**figure 2 b**) and the product A (166.4 ± 3.3 mg/dL; **figure 2 a**) gave significant lower results. To note, neither after 4 weeks nor after 6 weeks none the tested formulations modified the basal glycemic threshold measured at time 0 before the glucose intake.

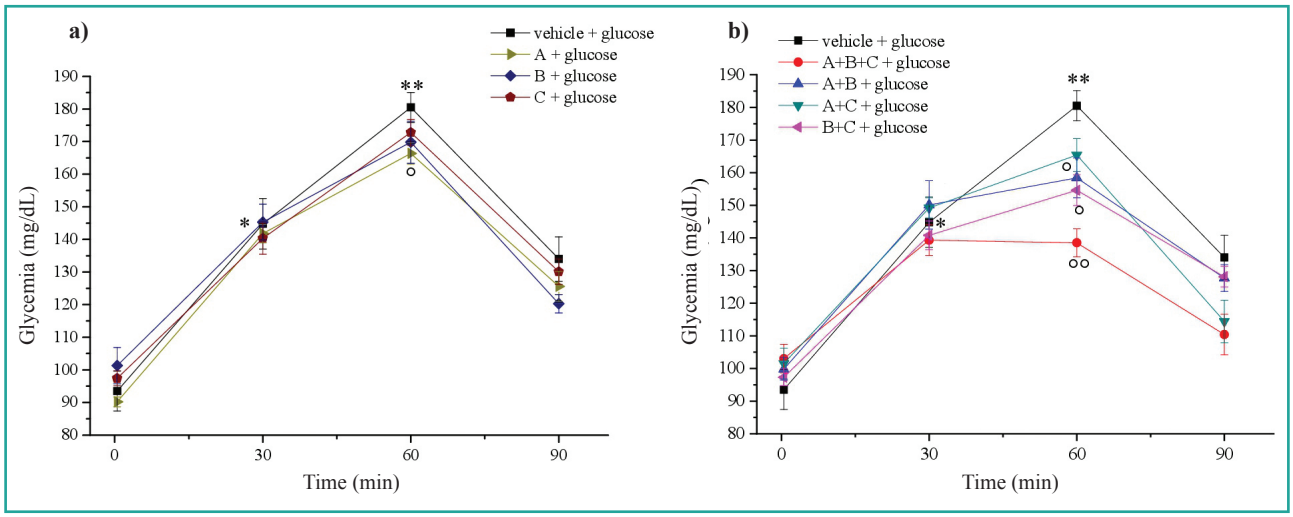
DISCUSSION

In this study, it has been demonstrated that Chromium picolinate (one of the three components) reduced glycemia values (-14.1 mg/dL, -7.8%) 60 min after the oral glucose load at 6 week of treatment.

Mita et al showed that everyday consumption of Chromium picolinate, at the dose of 2 mg/kg or 10 mg/kg for 12 weeks and of 10 mg/kg for 4 weeks in obese diabetic mice, significantly decreased blood glucose levels at 120 and 180 min after OGTT compared to control when the higher dosage was utilized for a 12-week period (21). It has been also reported that, in diabetic rats subjected to glucose tolerance test, Chromium picolinate daily intake at the dose of 1 and 10 mg/kg for 32 and 16 weeks, respectively, ameliorated glucose tolerance similarly for both amounts used. However, the lowest dose of Chromium picolinate after 6 weeks produced reductions in the relative changes in glucose area under the curve (AUC) that were resulted significant only at the end of the treatment period for both dosages (22).

The consumption, separately, of *Ilex paraguariensis* and *Morus alba*, the two other compounds contained in nutraceutical used in our study, slightly reduced glycemic values at 60 min following OGTT after 4 and 6 weeks of treatment. However, though the lowering did not attain statistical significance, it represents a trend towards reduction.

Mate tea is an infusion derived from *Ilex paraguariensis* leaves. Hussein *et al.* reported that, in obese diabetic mice subjected to intraperitone-



Figures 2 a, b. Evaluation of glycemia levels after 6 weeks of treatment. Each value was expressed as mean ± S.E.M. of 10 mice. * P < 0.05 and ** P < 0.01 *vs.* values recorded at time 0 (0 min) in the same group; ° P < 0.05 and °° P < 0.01 *vs.* vehicle-treated group.

al glucose tolerance test, daily consumption of mate aqueous extract at the dose of 100 mg/kg for 7 weeks significantly decreased blood glucose levels at 60 (-69.2 mg/dL, -13.0%) and 120 min (-93.0 mg/dL, -22.1%) after sugar load compared to untreated obese diabetic mice utilized as controls (16). In addition, Pereira *et al*. investigated the acute effect of two fractions (ethyl acetate (EtOAc) and n-butanol (n-BuOH)) of native *Ilex paraguariensis* and two infusions (green and roasted mate) of commercial Ilex paraguariensis in rats after OGTT. The authors observed that 200 mg/kg of EtOAc fraction significantly reduced glycemia at 15 (-48.6 mg/dL, -28.5%), 30 (-54.0 mg/dL, -28.2%) and 60 min (-36.0 mg/dL, -21.2%) after oral sugar administration, respect to hyperglycemic rats adopted as controls. The n-BuOH fraction (200 mg/kg) showed a significant blood glucose lowering 15 (-28.0 mg/dL, -16.4%), 30 (-46.5 mg/dL, -24.3%) and 60 min (-28.8 mg/dL, -17.0%) following OGTT compared to hyperglycemic controls, respectively. The n-BuOH and EtOAc fractions at the dose of 100 mg/kg reduced likewise glycemia (-19.5 mg/dL, -11.4% for n-BuOH and -19.3 mg/dL, -11.4% for EtOAc) at 15 and 60 min respectively compared to hyperglycemic control group. The green mate infusion (200 mg/mL) decreased blood glucose levels at 15 (-41.4 mg/dL, -24.3%), 30 (-35.6 mg/dL, -18.6%) and 60 min (-30.1 mg/dL, -17.7%) after OGTT, respect to hyperglycemic controls, and this dose has resulted more effective than 50 and 100 mg/mL in improving glucose tolerance. As regard roasted mate infusion, the best sugar-lowering effect was obtained with the dose of 100 mg/mL, compared to 50 and 200 mg/mL, and this dosage led to a glycemia reduction of -27.9 mg/dL (-16.3%) at 15 min and of -26.4 mg/dL (-13.8%) at 30 min after oral glucose administration respect to hyperglycemic control group (23).

The consumption of two polysaccharides extracted from *Morus alba* fruit, generally known as White Mulberry, in murine model of type 2 diabetes mellitus has been also evaluated. The two fractions have significantly decreased blood glucose levels at 180 min after oral sugar administration and the OGTT-AUC values (158.71 and 157.53, respectively) compared to that of untreated diabetic rats (176.83) following 7 weeks of supplementation (24). Another study reported that, in type 2 diabetes mellitus rats subjected to oral glucose tolerance test, the extract of Mulberry leaf (Folium Mori) at the daily dose of 2 g/kg b.w. determined a significant lowering in the AUC of OGTT after 4 weeks of treatment compared to normal controls and untreated diabetic rats (25).

In agreement to the literature data, our results highlight that mostly Chromium picolinate, and to a lesser extent Ilex paraguariensis and *Morus alba*, individually, are able to ameliorate glucose tolerance thanks to their glucose-lowering activity. As regard the associations of different nutraceutical compounds, we observed that the combination of Chromium picolinate, *Ilex paraguariensis* and *Morus alba* (A + B + C) caused, at 60 min afterwards OGTT, a significant blood glucose lowering of -39.7 mg/dL (-23.2%) and -42.0 mg/dL (-23.3%) after 4 and 6 weeks of treatment respectively. Also the association of *Ilex paraguariensis* and *Morus alba* (B + C) has decreased glycemia levels at 30 (-12.7 mg/dL, -8.6%) and 60 min (-25.9 mg/dL, -14.3%) following oral glucose administration after 4 and 6 weeks of supplementation, respectively. Moreover, Chromium picolinate plus Ilex paraguariensis (A + B) 60 min after oral sugar load has reduced blood glucose values (-22.1 mg/dL, -12.2%) at 6 weeks of treatment.

Kan et al reported that, in a murine model of insulin resistance and type 2 diabetes mellitus, the administration of a mixture containing Mulberry leaf (120 mg), Fenugreek seed (88 mg) and American ginseng (300 mg) extracts at 3 different doses (42.33, 84.66 and 169.33 mg/kg b.w.) has significantly decreased glycemia levels at 30 and 120 min after OGTT with a significant AUC reduction respect to control. Moreover, the lowering induced by the three dosages was similar (26).

In our study, the combination of Chromium picolinate, Ilex paraguariensis and *Morus alba* (A + B + C) was found to be the most effective in reducing glycemia following OGTT at the end of treatment period. Since Chromium picolinate, alone, exhibited the best anti-hyperglycemic effect after OGTT at 6 week and each compound of our supplement has its own mechanism of anti-hyperglycemic action, although still unclear, the higher effect of Chromium picolinate, *Ilex paraguariensis* and *Morus alba* mixture on postprandial glycemia than that of the single compounds as well as the other associations has proved that the improvement of glucose tolerance is due to the ability of the different hypoglycemic agents to synergize among them.

CONCLUSIONS

Our study showed the synergic effect of the three components (A + B + C) of the proposed nutraceutical, suggesting that a nutraceutical containing all three components, at 1000 mg of dosage, could be effective for use in the prevention of diabetes mellitus.

ETHICS

Fundings

There were no institutional or private fundings for this article.

Conflict of interests

Stefania Murzilli and Arianna Vanelli are employed by Nutrilinea srl, Varese, Italy. The other authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Authors' contributions

Design and conduction of the study: GD and CG; data collection: LDCM; data interpretation and manuscript writing: GD, PM and ADA. All authors read and approved the final version of the manuscript.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article.

Ethical approval

All treatments were carried out following the Directives 2010/63 / EU of the European Par-

liament and of the Council of the European Union (September 22, 2010) regarding the protection of animals used for scientific purposes. The ethical policy of the University of Florence conforms to the National Institutes of Health Guide for the care and use of laboratory animals (NIH Publication n. 85-23, revised 1996; University of Florence Assurance n. A5278-01).

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