# Combination of catechin, epicatechin, and rutin : Optimization of a novel complete antidiabetic formulation using a mixture design approach

Mechchate, Hamza

2021-02

Mechchate , H , Es-safi , I , Haddad , H , Bekkari , H , Grafov , A & Bousta , D 2021 , ' Combination of catechin, epicatechin, and rutin : Optimization of a novel complete antidiabetic formulation using a mixture design approach ' , Journal of Nutritional Biochemistry , vol. 88 , 108520 , pp. 1-8 . https://doi.org/10.1016/j.jnutbio.2020.108520

http://hdl.handle.net/10138/340844
https://doi.org/10.1016/j.jnutbio.2020.108520

Journal Pre-proof


# Combination of Catechin, Epicatechin, and Rutin:optimization of a novel complete antidiabetic formulation using a mixture design approach

Hamza Mechchate , Imane Es-safi , Hassan Haddad , Hicham Bekkari , Andriy Grafov , Dalila Bousta

PII: S0955-2863(20)30552-0
DOI: https://doi.org/10.1016/j.jnutbio.2020.108520
Reference: JNB 108520

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 20 May 2020
Revised date: 27 July 2020
Accepted date: 11 September 2020

Please cite this article as: Hamza Mechchate , Imane Es-safi , Hassan Haddad , Hicham Bekkari , Andriy Grafov , Dalila Bousta , Combination of Catechin, Epicatechin, and Rutin:optimization of a novel complete antidiabetic formulation using a mixture design approach, *The Journal of Nutritional Biochemistry* (2020), doi: https://doi.org/10.1016/j.jnutbio.2020.108520

# Combination of Catechin, Epicatechin, and Rutin:optimization of a novel complete antidiabetic formulation using a mixture design approach

Hamza Mechchate[a,*], Imane Es-safi[a], Hassan Haddad[b], Hicham Bekkari[a], Andriy Grafov[b], Dalila Bousta[a]

[a]Laboratory of Biotechnology, Environment, Agrifood, and Health, University of Sidi Mohamed Ben Abdellah, FSDM-Fez, Morocco.

[b]Laboratory of Inorganic chemistry, Department of chemistry, University of Helsinki, Finland.

[*]Corresponding author. E-mail address: Hamza.mechchate@usmba.ac.ma

**Comment [AUQ1]:** CE: In power edit JNB is gi in list, so I have procssed with JNEB, check for me data information.

## Highlights

- A novel antidiabetic formulation was optimized using a Mixture design approch.
- Combination of three molecules (Catechin, Epicatechin and Rutin) have been used.
- The optimal predicted mixture for the antidiabetic formulation was a binary combination of Rutin and Epicatechin (25% and 75% respectively).
- The optimum was selected according to three parameters : 1. Their hyperglycemic effect. 2. their hypoglycemic effect. 3. their Fasting Blood Glucose Area Under Curve (AUC).

## Graphical abstract

## Abstract

Nowadays, synthetic chemical antidiabetic drugs, besides their therapeutic effects, present adverse effects that could be hard to handle over time. In the last decade, studies reported new alternative molecules with more health benefits and less adverse effects.

The goal of this study is to optimize a new antidiabetic formulation using plant flavonoids: Catechin, Epicatechin, and Rutin. They are also a powerful antioxidant and anti-inflammatory molecules. A mixture design experiment will optimize their combination to obtain a new, safe multi targets

antidiabetic formulation making it a powerful combination for the management of diabetes and its complications.

To study the variation of blood glucose level (BGL) in response to the treatment over the time we performed an Oral Glucose Tolerance Test (OGTT). The BGL variations recorded as responses for the mixture design experiment. We used the molecules at a dose of 10 mg/kg.

According to the software analysis, the prediction profiler showed us the optimum combination, and the result was a binary combination between Rutin and Epicatechin (25% and 75% respectively). This combination prevented hyperglycemia and hypoglycemia, along with the best area under the curve (AUC), and after that, we validated it through a repeated oral administration on alloxan-induced diabetic mice for 28 days.

Rutin, Catechin, Epicatechin exhibit a potent antihyperglycemic activity, their synergistic combination validates a new formulation that could be a real candidate to conventional drugs.

Keywords

Diabetes, mixture design experiment, Simplex-centroid design, Catechin, Epicatechin, Rutin

*Abbreviations*: CA: Catechin; EP: Epicatechin; Ru: Rutin; AUC: Area under the curve; OGTT: Oral Glucose Tolerance Test; GV: Glycemic Variation;

## 1. Introduction

Diabetes mellitus is a chronic and complex metabolic disorder with differing underlying causes leading to hyperglycemia, which is the characteristic of the disease. Autoimmune-mediated destruction of pancreatic beta cells is the principal cause of type 1 diabetes (5% of all diabetes types) affecting the normal production of insulin, the key hormone regulating blood glucose. Specific forms of diabetes may occur by a resistance action to insulin or insulin deficiency (90% type 2 diabetes, and 5% other subtypes) [1]. The prevalence of diabetes, according to The International Diabetes

Federation, has incredibly increased from 425 million people in 2017 worldwide to over 700 million expected by 2045 [2].

Diabetes comes with different serious complications such as cardiovascular diseases and amputation, renal failure, blindness, cancer, retinopathy, neuropathy, delayed wound healing, and many others [3]. One of the main major challenges for the body to face with diabetes is oxidative stress, which is triggered under diabetic conditions through the production of free radicals via the polyol pathway, protein glycosylation and glucose auto-oxidation [4]. The reduction in the number of antioxidant levels in the body leads to cellular damage, apoptosis, and the activation and enhancement of inflammation leading to serious health complications [5].

Synthetic chemical antidiabetic drugs were developed during the years to help restore all types of dysfunctions managing the diabetic state and preventing the complications. However, the adverse effect of their prolonged administration (such as obesity, pancreatitis, gastrointestinal disorders, hypoglycemia, and hepatopathy) has changed the attention of many researchers to seek new, safer alternatives [6,7]. Recent research is now interested in flavonoids, a chemical family found in plants which presents many molecules with powerful pharmacological activities [8]. Flavonoids such as Catechin, Epicatechin, and Rutin are studied for their antidiabetic (Catechin [9,10], Epicatechin [11,12], Rutin [13]), antioxidant (Catechin [14,15], Epicatechin[16,17], Rutin [18,19]) and anti-inflammatory activities (Catechin [20,21], Epicatechin[22,23],Rutin [24,25]).

Diabetes, as a combination of multiple disorders, require adequate therapy, which makes a mixture of molecules with different molecular targets a better choice [26]. To properly study mixture and optimize the best combinations instead of the trial-and-error method and to generate the most effective ratio of each component, we need a mixture design, which is a class of response surface experiments [27].

By this modest work, we will set up a novel mixture design to study the combination of the three flavonoids to optimize the best ratio with the best beneficial effect.

## 2. Materials and Methods

### 2.1. Chemicals

Rutin hydrate (CAS Number: 207671-50-9; MW: 610,52 g/mol), (+)-Catechin hydrate (CAS Number: 225937-10-0; MW: 290.27 g/mol), (-)-Epicatechin (CAS Number: 490-46-0; MW: 290.27 g/mol), D-(+)-Glucose (CAS Number: 50-99-7), and Alloxan monohydrate (CAS Number: 2244-11-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Animals

Swiss albino mice of either sex weighing 20–25 g were used in this study. They were obtained from the animal facility of the Biology department (Faculty of Sciences Dhar El Mahraz Fez, Morocco). The animals were housed in appropriate cages and had free access to water and laboratory rodent chow. Experiment procedures were carried out under the international guidelines for the care and use of laboratory animals [28], and the protocol was approved by the institutional animal ethical committee (10/2019/LBEAS).

### 2.3. Experiment design

The single and combined effect of the three molecules was assessed using the Oral Glucose Tolerance Test (OGTT), and the ratios were determined by a Mixture design experiment based on a simplex-centroid design matrix. The results are expressed as glycemic variation (GV).

#### 2.3.1 Mixture design

Simplex-centroid design was used to study the ternary effect of the flavonoids, represented by an equilateral triangle to illustrate the distribution and repartition of the component mixtures, which varies from 1 (100% of that component) to 0 (0% of that component) (Figure 1).

Each key point of the triangle is represented as a point. 1,2,3 are at the vertices of the equilateral triangle representing 1st-degree centroids, and each of them contains only pure products. The 5,6,7 points are the binary mixtures and are represented by the sides of the triangle, and they are 2nd-degree centroids. Point 4 is a 3rd-degree centroid where all three components have the same value, and point 8 is an augmented point added to the triangle as a checkpoint.

Subsequently, data were fitted to a special cubic polynomial model using the least square regression for estimating unknown coefficients in the equation.

$$Y=b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{123}X_1X_2X_3$$

Considering Y is the response, bi is the magnitude of the effect of each component. Xi, bij are the magnitude of the interactive effect of two components, and bijk is the magnitude of the interactive effect of the three components on the response. Xi indicates the proportions of the component (i) of the mixture.

This analysis was performed using the JMP ® Pro software version 13.0.0 for windows.

**2.3.2. Oral Glucose Tolerance Test**

The goal of this test is to induce a hyperglycemic state and to evaluate the ability of the treatments to regulate glucose metabolism and to determine how quickly it is cleared from the blood during the test time. The dose of 10 mg/kg was selected because it was the lower/most effective dose among the tested doses (10, 25, 50 mg/kg).

The experimental design, as shown in table 1, consisted of 10 different groups of 5 normal mice each:

Group 1: Normal control treated orally with 0.2 ml saline
Group 2: Positive control treated orally with Glibenclamide at 1 mg/kg
Group 3: Treated orally with a single combination of RU at 10 mg/kg
Group 4: Treated orally with a single combination of CA 10 mg/kg
Group 5: Treated orally with a single combination of EP 10 mg/kg

Group 6: Treated orally with a binary combination of RU/CA (1/2:1/2) at 10 mg/kg
Group 7: Treated orally with a binary combination of RU/EP (1/2:1/2) 10 mg/kg
Group 8: Treated orally with a binary combination of CA/EP (1/2:1/2) 10 mg/kg
Group 9: Treated orally with a ternary combination of RU/CA/EP (1/3:1/3:1/3) at 10 mg/kg
Group 10: Treated orally with a ternary combination of RU/CA/EP (2/3:1/6:1/6) at 10 mg/kg

As described in figure 2, mice were fasted overnight before starting the experiment. The animals were loaded orally with a solution of D-glucose (5 g/kg) after an hour of treatments administration. Serum glucose levels were estimated post to drug administration at time 0 of the test where D-glucose was also administered and after 30, 60, and 120 min by tail vein puncture. Blood glucose level (BGL) was determined at these time intervals using the Accu-Chek blood glucose monitoring system and compatible blood glucose test strips.

**2.3.3. Responses, Responses goal, and optimum formulation**

Responses to be studied were set up based on the OGTT test result (Table 1). Three responses have been set. Response 1 is the "Antihyperglycemic response" based on the glycemic variation at T30 (30 min after glucose loading), where we have the high picks of glycemia. This response measures the effect of the treatment preventing hyperglycemia. Response 2 is the "hypoglycemic response," retrieved from the glycemic variation after 120 min of glucose loading where we have the lowest picks of glycemia and measures the effect of the treatment preventing hypoglycemia. Response 3 is the "Area under curve (AUC) response," the AUC of all formulations was calculated and added as a third response.

Response goal was set for the three responses, for the response 1 "Antihyperglycemic response" was set between 100 and 120 mg/dl, the minimum glycemia was the target. For response 2 "hypoglycemic response" was set between 50 and 70 mg/dl, a limit was defined to not fall into hypoglycemia. For response 3, "AUC response" was set between 90 and 100 mg/dl based on the

result obtained. The preferences between responses were 50% for "Antihyperglycemic response," 30% for "hypoglycemic response," and 20% for "AUC response."

The optimum formulation was calculated and deducted by the mixture design software taking into consideration the three response values and the different parameters.

**2.3.4. Optimum formulation validation**

A four weeks antidiabetic test was performed to study the effect of the predicted formulation obtained by the software.

Experimental diabetes (Mellitus) was induced with alloxan monohydrates. It was freshly prepared at the dose of 180 mg/kg and injected intraperitoneally for a 12 hour fasted animals. A solution of 0,2 ml glucose solution (4 g/L) was orally administered after the induction to prevent a hypoglycemic shock. First blood glucose measurements were taken four days after the injection, and the animals with blood glucose levels above 450 mg/dl were selected for the study. The selected mice were divided into four groups, five mice each.

Group A: Normal mice treated with 0.2 ml/day of saline
Group B: Mice with induced diabetes treated with 0.2 ml/day of saline
Group C: Mice with induced diabetes treated with 1 mg/kg/day of Glibenclamide
Group D: Mice with induced diabetes treated with 10 mg/kg/day of the optimum formulation.

During the 28 days of the study, the treatments were administered daily by intra-gastric gavage, and on the 1st, 7th, 14th, 21st, and 28th day, the fasting blood glucose (FBG) level was measured after 12h fast. Blood was collected from the tail vein, and the FBG was estimated using a glucometer (Accu-chek), whose principle is based on the glucose oxidase method.

Weekly weight measurements were taken.

**2.4. Statistical analysis**

Statistical analysis was performed using Graph Pad Prism version 8.0 for Windows. The difference between groups was assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, and values of *P* less than 0.05 were considered statistically significant. All values are expressed as mean±SD.

## 3. Results and Discussion

### 3.1. Oral Glucose Tolerance Test of the formulation

Table 1 displays the OGTT test results. The blood glucose level in all groups showed a high peak value at 30 min after glucose load and continued to drop until the end of the test. All treatments were significantly ($p<0.001$) better than normal control. The different combinations showed excellent management of the blood glucose level during the test.

For the first response "hyperglycemic response" noted at T30 (30 min after glucose load), the best result was for EP alone (100%) with a blood glucose level of 98 mg/dl followed by the combination of EP and CA (50:50) with a blood glucose level of 109 mg/dl and then the CA alone (100%) with a blood glucose level of 111 mg/dl. Those results, when compared to normal control (495 mg/dl), show how powerful the antihyperglycemic activity of those two molecules.

As for the second response, "hypoglycemic response" noted at the end of the OGTT test (T120), all groups blood glucose level were back to normal with some hypoglycemic values ( under 50 mg/dl [29,30]) observed for EP alone (100%) and CA alone (100%) which is considered a side effect.

The AUC of the blood glucose level (Figure 3) in the group treated with Glibenclamide was significantly reduced by 62% in comparison with the normal control group. For the single treatments, we noted the best reduction by EP (79%) followed by CA (76%) then RU ( 62%). For the binary result, the combination of EP and CA gives us the best reduction, with 76% followed by the combination between EP and RU (72%), CA, and RU (69%).

For the ternary combination with equal proportion (1/3/1/3/1/3), the reduction was 67%, and for the augmented point ( 2/3,1/6,1/6), it was 61%. Those results demonstrate the effectiveness and synergic effect of all combinations compared to normal and also positive control. The different activities of those molecules can explain this. Catechin decreases hemoglobin A1c level [9] and improves obesity and blood glucose control [10]. Epicatechin elevates insulin sensitivity and decreases insulin resistance [11,12]. For Rutin, it reduces carbohydrates absorption, improves glucose uptake by tissues, activates insulin secretion from β-cells, and also protects the islets of Langerhans from degenerative changes and the activation of hepatic hexokinase[13].

**3.2. Summary of fit for the response prediction models**

The coefficient of determination (R2) values indicated that the selected models provided an excellent fit to the data (Table 2). The analysis of variance (ANOVA) showed the statistical significance of the regression models.

**3.2. Effect of mixtures and validation of their interactions**

**3.2.1. Compounds effects and the fitted model**

The model coefficients were statistically significant since their p-value is lower than 0.05, except the coefficient that corresponds to binary terms $B_{12}$, $B_{13}$, $B_{23}$(Table 3). Therefore, we eliminated those coefficients from the postulated model. The most significant terms are those who represent the effects of the pure components ($B_1$, $B_2$, $B_3$) and the ternary component ($B_{123}$).

For each response a mathematical equation has been set:

"Antihyperglycemic response":

$$Y = 161.77X_1 + 115.31X_2 + 96.97X_3 + 2585.11X_1X_2X_3$$

"Hypoglycemic response":

$$Y = 63.68X_1 - 49.46X_2 + -39.12X_3 + 702.45X_1X_2X_3$$

"AUC response":

$$Y = 140.37X_1 + 95.78X_2 + 78.43X_3 + 1598X_1X_2X_3$$

**3.2.2.** ***Ternary plot and mixture profiler***

Ternary plots in figure 4 display the distribution and variability of three-part compositional data. It is a triangle with sides scaled from 0 to 1, and each side represents one of the three components. To plot a point, a line is drawn perpendicularly from the point to each leg of the triangle intersect at the component values of the point. Furthermore, the mixture Profiler visualizes and optimize the response surfaces of the experiment and shows response contours of mixture experiment models on a ternary plot.

**3.2.3. Optimal formulation prediction**

The objective of this optimization was to have a combination that fills the goal set of each response. For response 1, "Antihyperglycemic response," the goal was to reach a value between 100 and 120 mg/dl, and the minimum was the target. For response 2, "Hypoglycemic response," the goal was between 50 and 70 mg/dl, and the maximum was the target, and for response 3 "AUC response," the goal was between 90 and 100.

According to the software analysis, the prediction profiler (Figure 5) showed us the optimum combination, and the result was a binary combination between RU and EC, 25% and 75% respectively, the CA portion was discarded from the mixture as it will affect the goal set for the responses.

For this binary combination, the predicted value for response 1 "Antihyperglycemic response" was: 107 mg/dl, and for response 2, "Hypoglycemic response" was: 50 mg/dl, and for response 3 "AUC response" was: 92. We got those results with the desirability of 89%.

#### 3.2.4. optimal formulation validation

Figure 6 shows the effect of repeated oral administration of the optimum formulation on fasting blood glucose levels of Alloxan induced diabetic mice.

After seven days, the reduction reached 68% for the RU/EC (25:75) combination against 36% for Glibenclamide compared to the diabetic control group ($p<0.001$). Afterward, the treatments continued successfully to manage the diabetic status and suppression of hyperglycemia with a significant reduction ($p<0.001$) (Day 14: 71,44%; Day 21: 73,63%; Day 28: 77,73% For the optimum formulation and Glibenclamide respectively) compared to the diabetic control.

Table 4 represents the Bodyweight development of different experimental mice groups. The diabetic groups treated with the optimum formulation for four weeks showed a very significant improvement in body weight compared to the diabetic control group, which marked a gradual drop in body weight. The reduction in body weight is as observed related to hyperglycemia. Glucose builds up in the bloodstream in a lack of insulin. High blood glucose makes the kidneys work to get rid of unused sugar through the urine, causing weight loss due to dehydration and loss of calories from the sugar unused as energy [31].

Four weeks treatment with the predicted formulation resulted in spectacular management of fasting blood glucose level even better of the positive control (Glibenclamide) confirming by so the analyze done by the mixture design software, the formulation also improved the bodyweight and the overall health status of the treated mice with no toxicity sign observed during the study time.

The mixture design was a crucial part of finding the best formulation that will satisfy the goals of the study instead of an infinity of combinations that we could try. Defining and adjusting the parameters carefully will help the predicted optimized combination to be more precise and accurate.

## 4. Conclusions

This work studied the different responses of three flavonoids (Catechin, Epicatechin, Rutin) during the Oral Glucose Tolerance Test. The three molecules exhibit a potent antihyperglycemic activity as a single, binary, and ternary combinations against positive and normal control, but the factor in determining the optimum combination was the preset goal for each response. Epicatechin had the best values preventing hyperglycemia and also the best AUC but falls into hypoglycemia at the end of the test, which we consider as a side effect.

The mixture design experiment helped to optimize the best formulation that responds to the desired effect. The predicted formulation consisted of a binary combination of Rutin and Epicatechin (25:75). This combination was tested on alloxan-induced diabetic mice for 28 days and exhibited a spectacular result with no toxicity sign observed.

With their approved antidiabetic, antioxidant, and anti-inflammatory activities and multiple modes of actions, this formulation could be a safe, multi-target antidiabetic drug alternative.

**Author contributions**

Hamza Mechchate: **Conceptualization, experimental work, Writing – Original Draft**, Imane Es-safi: **Experimental work, Writing**, Hassan Hadad: **Data treatment;** Hicham Bekkari: **Conceptualization, Methodology;** Andriy Grafov: **Review & Editing** Dalila Bousta: **Conceptualization, Methodology, supervision.**

**Financing**

This study did not receive funds.

## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

**Acknowledgments**

This work is part of the European Union (EU) international staff exchange project H2020-MSCA-RISE-Marie Skłodowska- Curie Actions (MSCA), Research and Innovation Staff Exchange (RISE), Project Acronym: VAHVISTUS – Project Number: 734759.

References

[1] Mekala KC, Bertoni AG. Epidemiology of diabetes mellitus. Transplant. Bioeng. Regen. Endocr. Pancreas, Elsevier; 2020, p. 49–58. https://doi.org/10.1016/B978-0-12-814833-4.00004-6.

[2] Ma Q, Li Y, Li P, Wang M, Wang J, Tang Z, et al. Research progress in the relationship between type 2 diabetes mellitus and intestinal flora. Biomed Pharmacother 2019;117:109138. https://doi.org/10.1016/j.biopha.2019.109138.

[3] Prabhakar PK, Kumar A, Doble M. Combination therapy: A new strategy to manage diabetes and its complications. Phytomedicine 2014;21:123–30. https://doi.org/10.1016/j.phymed.2013.08.020.

[4] Zuo T, Zhu M, Xu W. Roles of Oxidative Stress in Polycystic Ovary Syndrome and Cancers. Oxid Med Cell Longev 2016;2016:1–14. https://doi.org/10.1155/2016/8589318.

[5] Wang W, Shang C, Zhang W, Jin Z, Yao F, He Y, et al. Hydroxytyrosol NO regulates oxidative stress and NO production through SIRT1 in diabetic mice and vascular endothelial cells. Phytomedicine 2019;52:206–15. https://doi.org/10.1016/j.phymed.2018.09.208.

[6] Rines AK, Sharabi K, Tavares CDJ, Puigserver P. Targeting hepatic glucose metabolism in the treatment of type 2 diabetes. Nat Rev Drug Discov 2016;15:786–804. https://doi.org/10.1038/nrd.2016.151.

[7] Mechchate H, Es-safi I, Jawhari F zahra, Bari A, Grafov A, Bousta D. Ethnobotanical survey about the management of diabetes with medicinal plants used by diabetic patient in Region of Fez-Meknes, Morocco. Ethnobot Res Appl Vol 19 2020 2020. https://doi.org/10.32859/era.19.12.1-28.

[8] Caro-Ordieres, Marín-Royo, Opazo-Ríos, Jiménez-Castilla, Moreno, Gómez-Guerrero, et al. The Coming Age of Flavonoids in the Treatment of Diabetic Complications. J Clin Med 2020;9:346. https://doi.org/10.3390/jcm9020346.

[9] Fukino Y, Ikeda A, Maruyama K, Aoki N, Okubo T, Iso H. Randomized controlled trial for an effect of green tea-extract powder supplementation on glucose abnormalities. Eur J Clin Nutr 2008;62:953–60. https://doi.org/10.1038/sj.ejcn.1602806.

[10] Nagao T, Meguro S, Hase T, Otsuka K, Komikado M, Tokimitsu I, et al. A catechin-rich beverage improves obesity and blood glucose control in patients with type 2 diabetes. Obesity 2009;17:310–7.

[11] Cremonini E, Bettaieb A, Haj FG, Fraga CG, Oteiza PI. (-)-Epicatechin improves insulin sensitivity in high fat diet-fed mice. Arch Biochem Biophys 2016;599:13–21. https://doi.org/10.1016/j.abb.2016.03.006.

[12] Josic J, Olsson AT, Wickeberg J, Lindstedt S, Hlebowicz J. Does green tea affect postprandial glucose, insulin and satiety in healthy subjects: a randomized controlled trial. Nutr J 2010;9. https://doi.org/10.1186/1475-2891-9-63.

[13] Hao H, Shao Z, Tang D, Lu Q, Chen X, Yin X, et al. Preventive effects of Rutin on the development of experimental diabetic nephropathy in rats. Life Sci 2012;91:959–67. https://doi.org/10.1016/j.lfs.2012.09.003.

[14] Basu A, Sanchez K, Leyva MJ, Wu M, Betts NM, Aston CE, et al. Green tea supplementation affects body weight, lipids, and lipid peroxidation in obese subjects with metabolic syndrome. J Am Coll Nutr 2010;29:31–40.

[15] Wein S, Schrader E, Rimbach G, Wolffram S. Oral green tea catechins transiently lower plasma glucose concentrations in female db/db mice. J Med Food 2013;16:312–7.

[16] Martín MÁ, Fernández-Millán E, Ramos S, Bravo L, Goya L. Cocoa flavonoid epicatechin protects pancreatic beta cell viability and function against oxidative stress. Mol Nutr Food Res 2014;58:447–56.

[17] SPENCER JP, SCHROETER H, KUHNLE G, SRAI SKS, TYRRELL RM, HAHN U, et al. Epicatechin and its in vivo metabolite, 3′-O-methyl Epicatechin, protect human fibroblasts from oxidative-stress-induced cell death involving caspase-3 activation. Biochem J 2001;354:493–500.

[18] Gao Z, Xu H, Chen X, Chen H. Antioxidant status and mineral contents in tissues of Rutin and baicalin fed rats. Life Sci 2003;73:1599–607.

[19] Mosoni L, Balage M, Vazeille E, Combaret L, Morand C, Zagol-Ikapitte I, et al. Antioxidant supplementation had positive effects in old rat muscle, but through better oxidative status in other organs. Nutrition 2010;26:1157–62.

[20] Widmer RJ, Freund MA, Flammer AJ, Sexton J, Lennon R, Romani A, et al. Beneficial effects of polyphenol-rich olive oil in patients with early atherosclerosis. Eur J Nutr 2013;52:1223–31.

[21] Uchiyama Y, Suzuki T, Mochizuki K, Goda T. Dietary supplementation with a low dose of (−)-epigallocatechin-3-gallate reduces pro-inflammatory responses in peripheral leukocytes of non-obese type 2 diabetic GK rats. J Nutr Sci Vitaminol (Tokyo) 2013;59:541–7.

[22] Prince PD, Lanzi CR, Toblli JE, Elesgaray R, Oteiza PI, Fraga CG, et al. Dietary (–)-epicatechin mitigates oxidative stress, NO metabolism alterations, and inflammation in renal cortex from fructose-fed rats. Free Radic Biol Med 2016;90:35–46.

[23] Morrison M, van der Heijden R, Heeringa P, Kaijzel E, Verschuren L, Blomhoff R, et al. Epicatechin attenuates atherosclerosis and exerts anti-inflammatory effects on diet-induced human-CRP and NFκB in vivo. Atherosclerosis 2014;233:149–56.

[24] Nones K, Dommels YE, Martell S, Butts C, McNabb WC, Park ZA, et al. The effects of dietary curcumin and Rutin on colonic inflammation and gene expression in multidrug resistance gene-deficient (mdr1a−/−) mice, a model of inflammatory bowel diseases. Br J Nutr 2008;101:169–81.

[25] Kim H, Kong H, Choi B, Yang Y, Kim Y, Lim MJ, et al. Metabolic and pharmacological properties of Rutin, a dietary quercetin glycoside, for treatment of inflammatory bowel disease. Pharm Res 2005;22:1499–509.

[26] Wagner H, Ulrich-Merzenich G. Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine Int J Phytother Phytopharm 2009;16:97–110. https://doi.org/10.1016/j.phymed.2008.12.018.

[27] Fadil M, Fikri-Benbrahim K, Rachiq S, Ihssane B, Lebrazi S, Chraibi M, et al. Combined treatment of Thymus vulgaris L., Rosmarinus officinalis L. and Myrtus communis L. essential oils against Salmonella typhimurium : Optimization of antibacterial activity by mixture design methodology. Eur J Pharm Biopharm 2018;126:211–20. https://doi.org/10.1016/j.ejpb.2017.06.002.

[28] National Research Council. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington (DC): National Academies Press (US); 2011.

[29] Ando H, Ushijima K, Shimba S, Fujimura A. Daily Fasting Blood Glucose Rhythm in Male Mice: A Role of the Circadian Clock in the Liver. Endocrinology 2016;157:463–9. https://doi.org/10.1210/en.2015-1376.

[30] Cryer PE. Hypoglycemia, functional brain failure, and brain death. J Clin Invest 2007;117:868–70. https://doi.org/10.1172/JCI31669.

[31] Gerich J, Penhos JC, Gutman RA, Recant L. Effect of dehydration and hyperosmolarity on glucose, free fatty acid and ketone body metabolism in the rat. Diabetes 1973;22:264–71.

Fig 1. Distribution of experimental points for the augmented Simplex-centroid design.

RU: Rutin, CA: Catechin, EP: Epicatechin

Fig 2. Oral Glucose Tolerance Test (OGTT) design and determination of the responses

Fig 3. Effect of different treatments on the total area under the curve (AUC) of blood glucose level during the OGTT test.

RU: Rutin; CA: Catechin; EP: Epicatechin ; Gli: Glibenclamide.

The results were analyzed using one-way ANOVA followed by Tukey's post hoc test, and the data are expressed as the means ± SD (n= 5 mice). ***P<0.001, compared to normal control

Fig 4. Ternary plot and mixture profiler of the different combinations of studied molecules. **A:** Ternary plot for "Antihyperglycemic response," the values express the variation of the glycemia (mg/dl) at T30 (30 min of the OGTT test). **B:** ternary plot for "Hypoglycemic response," the values express the variation of the glycemia (mg/dl) at T120 (120 min of the OGTT test). **C:** ternary plot for "AUC response" values express the area under the curve of the variation of glycemia during the OGTT test. **D:** Mixture profiler and **X** represent the optimum formulation point

Fig 5. Prediction profiler showing the best formulation with a precise proportion of the three studied molecules to get the goal of each response.
Response 1: **Antihyperglycemic**; Response 2: **Hypoglycemic;** Response 3: **AUC**

Fig 6. Effect of the predicted formulation on fasting blood glucose in Alloxan-induced diabetic mice during the experimental period of 28 days
Diab: Diabetic; Glib: Glibenclamide; Ru: Rutin; EP: Epicatechin
The results were analyzed using one-way ANOVA followed by Tukey's post hoc test, and the data are expressed as the means ± SD (n= 5 mice). *** P<0.001 compared to diabetic control

Table 1. Glycemic variation after administration of different treatments during the OGTT test

**Comment [AUQ2]:** CE: PLease check table fro input.

| Time | T0 | T30 | T60 | T90 | T120 |
|---|---|---|---|---|---|
| Treatment | | | | | |
| Normal control | 91,2±8,04 | 495,4±24,35 | 372,2±18,13 | 293,2±21,63 | 140±12,7 |
| Glibenclamide 1 mg/kg | 57 ±9,43*** | 186±12,38*** | 148,6±16,74*** | 92,4±5,94*** | 56,2±6,34*** |
| RU 10 mg/kg | 96 ± 16,26 | 167 ± 33,42*** | 133,2 ± 26,8*** | 95,8 ± 16,4*** | 67,6 ± 17,4*** |
| CA 10 mg/kg | 73 ± 7,07 | 111,4 ± 15,6*** | 78,4 ± 12,2*** | 59,8 ± 7,12*** | 48 ± 11,68*** |
| EP 10 mg/kg | 64,6 ± 12,3** | 98 ± 12,34*** | 65,4 ± 8,79*** | 56,6 ± 8,08*** | 46,4 ± 12,5*** |
| RU/CA (1/2 :1/2) 10 mg/kg | 84,8±7,25 | 132,2±19,72*** | 101,2±18,63*** | 84±11,33*** | 62,2±6,76*** |
| RU/EP (1/2 :1/2) 10 mg/kg | 67,8±5,80* | 121,2±11,3*** | 95,8±8,95*** | 78±5,33*** | 58,2±4,44*** |
| CA/EP (1/2 :1/2) 10 mg/kg | 66,6±8,26* | 109±9,72*** | 78±8,97*** | 63,2±5,58*** | 50±4,47*** |
| RU/CA/EP (1/3 :1/3 :1/3) 10 mg/kg | 77,6±25,17 | 207,2±33,84*** | 136,6±17,85*** | 106±17,41*** | 80,6±10,45*** |

| | | | | | |
|---|---|---|---|---|---|
| RU/CA/EP (2/3 :1/6 :1/6) 10 mg/kg | 85,2±7,15 | 182,8±22,21*** | 128,6±15,37*** | 99,8±13,14*** | 75,6±7,98*** |

RU: Rutin; CA: Catechin; EP: Epicatechin

The results were analyzed using one-way ANOVA followed by Tukey's post hoc test, and the data are expressed as the means ± SD (n= 5 mice). ***P<0.001, **P<0.01, *P<0.1, compared to normal control

Table 2. Summary of fit of the three responses

RMSE: root mean square error; Rsq: R-squared

*: Statistically significant probability at P<0.001

| Responses | RMSE | Rsq | P-value |
|---|---|---|---|
| **"Antihyperglycemic"** | 13,923 | 0,90 | <.0001* |
| **"Hypoglycemic** | 6,264 | 0,84 | <.0001* |
| **"AUC"** | 8,134 | 0,93 | <.0001* |

Table 3. Coefficients of each model fitted and their level of significance determined by P-value

| Responses | "Antihyperglycemic" | | "Hypoglycemic" | | "AUC" | |
|---|---|---|---|---|---|---|
| Nom | Coef. | Signif. | Coef. | Signif. | Coef | Signif. |
| **b1 (Ru)** | 161,77 | < 0.001 * | 63.68 | < 0.001 * | 140.37 | < 0.001 * |
| **b2 (CA)** | 115,31 | < 0.001 * | 49.46 | < 0.001 * | 95.78 | < 0.001 * |
| **b3 (EP)** | 96,97 | < 0.001 * | 39.12 | < 0.001 * | 78.43 | < 0.001 * |
| **b12 (RU/CA)** | 58,91 | 0.11 | 11.14 | 0.50 | -32.63 | 0,13 |
| **b13 (RU/EP)** | -32,49 | 0.34 | 25.92 | 0.1 | -9,31 | 0,64 |
| **b23 (CA/EP)** | 11,31 | 0.74 | 23.43 | 0.13 | 22.40 | 0.26 |
| **b123 (RU/CA/EP)** | 2585,11 | < 0.001 * | 702.45 | < 0.001 * | 1598 | < 0.001 * |

RU: Rutin; CA: Catechin; EP: Epicatechin

*: Statistically significant at P<0.001 probability

Table 4. Changes in the bodyweight of normal and diabetic mice during the experimental period of 28 days

| Treatment | weight | (g) |
|---|---|---|

| | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|
| Normal control | 23.4±1.8 | 24.7±1.5 | 25.2±1.4* | 26.9±1.3*** | 27.2±1.5*** |
| Diab. control | 23.8±1.6 | 21.7±2.2 | 20.1±2.7 | 19.2±2.5 | 17.7±2.4 |
| Diab. Glib 1 mg/kg | 24.1±1.6 | 23.3±1.5 | 23.3±1.6* | 23.8±1.8** | 24.1±1.7*** |
| Diab. RU/EP (25/75) 10mg/kg | 23.8±1.4 | 24.3±1.2 | 24.9±1.4* | 25.4±1.7** | 26.0±1.7*** |

Glib: Glibenclamide; Ru: Rutin; EP: Epicatechin

The results were analyzed using one-way ANOVA followed by Tukey's post hoc test, and the data are expressed as the means ± SD (n= 5 mice). * P<0.05, ** P<0.01, *** P<0.001 compared to diabetic control