Food & Function

Cretan tea (Origanum dictamnus L.) as a functional beverage: investigation on antiglycative and carbonyl trapping activities

Journal:	*Food & Function*
Manuscript ID	FO-ART-12-2017-001930.R1
Article Type:	Paper
Date Submitted by the Author:	n/a
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Food & Function

2015 Impact Factor: 2.676
www.rsc.org/foodfunction

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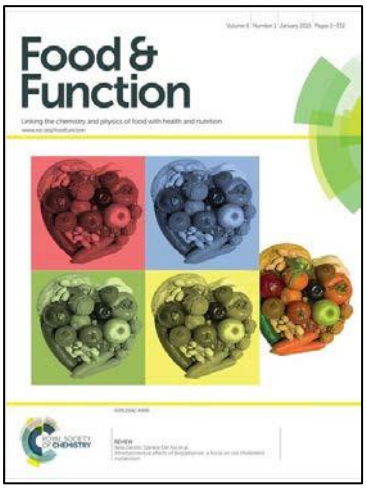

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Best wishes

Philippa Hughes
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RESPONSE TO REVIEWERS' COMMENTS:

We revised our manuscript according to the comments and suggestions of Reviewers. We highlighted in yellow the additions and/or changes in the revised manuscript

REVIEWER REPORT(S):
Referee: 1

Comments to the Author
General comment:
The manuscript entitled Cretan tea (Origanum dictamnus L.) as a functional beverage: investigation on antiglycative and carbonyl trapping activities is very interesting and the data presented here is of relevance to the field of functional food composition.
The work is well structured and well-presented and the issue has been addressed with sufficient scientific rigour, in my opinion this manuscript can be considered for publication with only a few minor revisions.

Specific Comments:
-The antiglycative activity is investigated with different in vitro test, the authors the authors should better explain the reason.

We thank the Reviewer for the comment. We better explain the reason in the text (Introduction section: lines 87-93).

-In the description of RP-HPLC-DAD-ESI- MS the authors reported the mass fragment with amu and Da. I suggest using only a measurement unit.

We thank the Reviewer for the comment. We used only Da as measurement unit (see lines 267, 268, 270, 275, 296, 302)
- I suggest to number the figures and table in the order of text presentation
We thank the Reviewer for the comment. We changed the order of the Figures in the text

Referee: 2

Comments to the Author
Manuscript entitled "Cretan tea (Origanum dictamnus L.) as a functional beverage: investigation on antiglycative and carbonyl trapping activities" by the authors Mariarosa Maietta, Raffaella Colombo, Federica Corana, Adele Papetti was aimed to investigate usefulness of herbal tea in the reduction of non-enzymatic protein glycation and in the scavenging of free radicals.
In my opinion, the Manuscript presented in this form has scientific merit and should be accepted for the publication in Food & Function after minor revisions. In the following some recommendations and comments are given.

Introduction
Page 5 …and several polyphenols were characterized in the aqueous extract (hydroxycinnamic acids and catechins) 17, 25-28 and in different polar solvent extracts (phenolic acids and flavonoids).11, 14,16, 18, 23, 28,29
Authors should be more specific when citing published results. For example, cite ref no 17 for

hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids. Other possibility is to cite a review on the work done in this field.

We thank the Reviewer for the comment. We better specified references related to each class of polyphenols (see page 4, lines 73-75 .

Section 2.4.
Page 8 … (or positive control AG) or phosphate buffer (blank). Unclear sentence.

Aminoguanidine (AG) is known to be an antiglycative agent and commonly used as standard (positive control).

Section 3.2.
Page 20 …The antiglycative activity is generally correlated to the polyphenolic composition of the extracts. In particular, previous structure-activity studies indicated that flavones are able to exhibit stronger inhibitory effects when compared to flavonols, flavanones, and isoflavones. Reference is needed for this paragraph.

We thank the Reviewer for the comment. We added references 72 (see page 28, lines 674-675).

…The composition of dittany infusion rich in apigenin, luteolin, kaempferol, and also hydroxycinnamic derivatives could justify the high antiglycative activity registered.
Correct to hydroxycinnamic acid derivatives

We thank the Reviewer for the comment. We corrected the text (line 456)

Authors stated the main objectives of the research were to study the chemical composition of the beverage and to evaluate its effect on the generation of early glycation products and AGEs. In my opinion it would be advisable to try to give some comments on the structure features of the polyphenols identified in the structure-activity (antiglycative) relationship sense.

As stated we tested the activity of the whole infusion and identified its chemical composition. At this stage it is hard to hypothesize any correlation between the structures of the identified compounds and the activity of the infusion because it would be necessary to test each single compound.

Literature
Correct the names in the reference No 41 …M. M. Natic´, D. C. Dabic´, M. M. Fotiric´ Akšic´, M. Ljubojevic´and Z. Tešic, to M. M. Natić, D. Č. Dabić, M. M. Fotirić Akšić, M. Ljubojević, Ž. Tešić

We thank the Reviewer for the comment. We corrected the Reference

Table 1 Please reorganize table by putting (µmol g-1) into the column.
Statistical test on the antioxidant activity is not relevant.

We thank the Reviewer for the comment. Table 1 was modified

Table 2 Correct the names of the compounds in the table… e.g. Kampferol

We thank the Reviewer for the comment. Table 2 was corrected

Dipartimento di Scienze del Farmaco
Università degli Studi di Pavia

January 9th , 2018

Dear Editor,

We revised our manuscript according to the suggestion and comments of the Reviewers'. Please, find the new version with the response to the Reviewers' comments.

I am looking forward to hearing from you soon.

Best regards,

Dr. Adele Papetti, PhD

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Cretan tea (*Origanum dictamnus* L.) as a functional beverage: investigation on antiglycative and carbonyl trapping activities

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Abstract

Accumulation of advanced glycation end products (AGEs) *in vivo* is associated with many chronic disorders such as diabetes, renal failure, aging, and Alzheimer's disease. The aim of this study was to expand the knowledge about the functional properties of *Origanum dictamnus* L. beverage (Cretan tea) by an investigation about the inhibitory effects on the formation of AGEs and the capacity to trap dicarbonyl compounds. Dittany infusion was characterized in its polyphenolic composition by RP-HPLC-DAD-ESI-MS^n and twenty compounds were detected. Antiglycative property was evaluated by *in vitro* BSA-sugars (glucose, fructose, and ribose) and BSA-methylglyoxal (MGO) assays, formation of Amadori products and dicarbonyl compounds tests, direct glyoxal (GO) and MGO trapping capacity. The infusion showed the highest inhibitory effect on the formation of dicarbonyl compounds and of AGEs (activity values range 72-100%) and only a weak effect on the formation of Amadori products, indicating that the antiglycative action occurred primarly during the last two phases of the non-enzymatic glycation reaction. These activities are partially correlated with the antioxidant/antiradical activity, as demonstrated by the scavenger capacity against ABTS cation and DPPH stable radicals, and the reducing power. The registered high anti-AGEs capacity could probably be ascribed to dittany polyphenolic composition particularly rich in flavone derivatives. These findings support further investigations to study the feasibility of dittany as antiglycative agents in food or cosmetic preparation.

Keywords: glycation inhibitors; carbonyl trapping agents; advanced glycation end products (AGEs); Cretan tea; *Origanum dictamnus* L.; RP-HPLC-DAD-ESI/MS^n

1. Introduction

Origanum dictamnus L. (known as dittany) is an endemic plant of the island of Crete (Greece) belonging to the family of *Lamiaceae*, genus *origanum*.[1] Dittany was considered as "panacea" by the ancient Greeks and it was used in folk medicine for its healing effects.[2-4] Nowadays, it is widely cultivated on the Crete island as its uses as a herbal tea plant, a spice in distilleries, and a condiment contribute to sustain the local economy.[5] In the last two decades, a certain number of studies describe the biological effects of *O. dictamnus* decoctions, hydro-alcoholic extracts, and essential oils, such as antimicrobial activities against Gram-negative clinical strains, phytopathogenic bacteria, and pathogenic fungi.[6-12] Antioxidant capacity, cytotoxic activity against human bronchial epidermoid cancer NSCLC-N6 (non-small cell lung cancer) and P388 (murine leukemia) cell lines, and inhibitory effects on HT29 colon and PC3 prostate cancer cells growth have been also ascribed to dittany components.[9, 13-19]

Because of the biological effects, research has also been focused on the characterization of volatile and not volatile dittany components. More than 80 volatile compounds were identified in the essential oils[20-24] and several polyphenols were characterized in the aqueous extract (hydroxycinnamic acids[17, 25-28] and catechins[17]) and in different polar solvent extracts (phenolic acids [11,14,16,18,28,29] and flavonoids[11,14,18, 28]).

In the last decade, beverages rich in polyphenols and phenolic acids have been indicated as sources of Advanced End Glycation products (AGEs) inhibitors.[30,31] AGEs are the final products deriving from the Maillard reaction or non-enzymatic glycation process that starts with the interaction between the carbonyl group of reducing sugars and the free amino group of proteins, lipids, and nucleic acids, when Amadori rearrangement compounds react with amino groups by either oxidative or non-oxidative pathways.[32] Maillard reaction takes place not only in food during processing and storage, but also in living bodies, giving origin to AGEs accumulation *in vivo* and by consequence to the development of age-related disorders.[33,34] Therefore, the search for new

AGE-inhibitors, especially from natural rather than synthetic sources, is considered of particular interest.

The aim of the present research is to extend the knowledge of the functional properties of dittany infusion. The main objectives were to study the chemical composition of the beverage by RP-HPLC-DAD-ESI-MS^n and to evaluate its anti-glycation effect monitoring different reaction stages and products. For this purpose, different *in vitro* models were studied: (i) inhibition of fructosamine formation (early stage of non-enzymatic protein glycation); (ii) inhibition of the α-dicarbonyl compounds formation (generated after oxidation and dehydration steps in the Maillard reaction); (iii) inhibition of BSA-MGO system (middle stage of non-enzymatic protein glycation) and of BSA-sugar systems (last phase of non-enzymatic protein glycation). Furthermore, the ability of infusion to directly trap MGO and glyoxal (GO) (important intermediates of Maillard reaction) was assessed. A potential relationship of antioxidant activity with AGE-inhibitory effect was also evaluated.

2. Materials and methods

2.1. Chemicals

Ethanol, methanol, D(+) glucose (GLC), D(-) fructose (FRU), disodium hydrogen phosphate dodecahydrate, sodium carbonate decahydrate, sodium bicarbonate were purchased from Carlo Erba (Milano, Italy). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C), HPLC-grade and HPLC-MS-grade formic acid, methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), nitrotetrazolium blue chloride (NBT), methylglyoxal (MGO, 40% aqueous solution), glyoxal (GO, 40% aqueous solution), 5-methylquinoxaline (5-MQ), *o*-phenylendiamine (grade purity ≥ 98%, OPD), bovine serum albumin (grade purity ≥ 98%, BSA), D(-)- ribose (grade purity ≥ 98%, RIB), aminoguanidine hydrochloride (grade purity ≥ 98%, AG), sodium dihydrogen phosphate monohydrate, potassium dihydrogen phosphate, sodium hydroxide pellets, sodium azide (grade purity 99.5%), (-)-

gallocatechin (grade purity ≥ 98%), rosmarinic acid (grade purity ≥ 98%), apigenin-7-*O*-glucuronide (grade purity ≥ 95%), and luteolin-7-*O*-rutinoside (grade purity ≥ 95%) were provided by Sigma-Aldrich (St. Louis, Mo, USA). Water was obtained from a Millipore Direct-QTM system (Merck-Millipore, Milan, Italy).

2.2. Equipment

Vacuum freeze-drier Modulyo System (5Pascal, Italy) was used for freeze-drying process. HPLC-DAD-ESI/MS^n analyses were performed using a Thermo Finningan Surveyor Plus HPLC apparatus (Thermo Fischer Scientific, Waltham, MA, USA) equipped with a quaternary pump, a Surveyor UV-Vis photodiode-array detector, a Surveyor Plus autosampler, and a vacuum degasser connected to a LCQ Advantage Max ion trap spectrometer through an ESI source. Spectrophotometer Perkin-Elmer Lambda25 was used for antioxidant assays. Spectrofluorometer Perkin Elmer L550B was used for antiglycative assays.

2.3. Preparation of Cretan tea infusion

Cretan tea (*Origanum dictamnus* L., dittany) dried herb was directly purchased from Anna Herbs, (Heraklion, Crete, Greece). Preparation of Cretan tea infusion was alike in local traditional preparation. Five grams of herb were infused into 200 mL of boiling water (equivalent to a teacup) and the infusion was left to room temperature to cool for 30 min before filtration through filter paper. The beverage was freeze-dried and stored at – 20 °C for further assays.

2.4. *In vitro* glycation of bovine serum albumin (BSA) induced by monosaccharides (glucose, ribose, fructose) and methylglyoxal and determination of fluorescent Advanced Glycation End-Products (AGEs)

The BSA-GLC, BSA-RIB, BSA-FRU, and BSA-MGO assays were performed according to the methods proposed by Mesías *et al.*[35] with slight modifications. Briefly, 35 mg mL^{-1} BSA was incubated with sugars or MGO (175 mg mL^{-1} GLC, 150 mg mL^{-1} RIB, 175 mg mL^{-1} FRU, and 0.4 mg mL^{-1} MGO) in 100 mM phosphate buffer (pH 7.4) containing 0.02% (w/v) of sodium azide (to ensure aseptic conditions) at 37 °C in absence or presence of dittany solution (or positive control AG) or phosphate buffer (blank). The final reaction mixture contained 500 μL of BSA solution, 1 mL of sugar solution, and 375 μL of dittany (5 mg dry matter mL^{-1} distilled water) or AG (0.5 mg mL^{-1} 100 mM phosphate buffer, pH 7.4). The systems containing GLC and FRU were incubated for 7 and 14 days, respectively, with four (at 1, 3, 5, and 7 days) and five analytical determinations (at 1, 3, 5, 7, and 14 days), respectively; the system containing RIB was incubated for 1 day monitoring the AGE formation after 1, 3, 6, and 24 h; the system containing BSA-MGO was incubated for 7 days, with analytical determination at 1, 3, and 6 h, and at 1, 5, 7 days. Different incubation and monitoring times are consequence of different sugar and MGO reactivities.[36]

Vesperlysine-like (λ_{exc} 370 nm; λ_{em} 440 nm) AGE fluorescence was measured. Pentosidine-like (λ_{exc} 335 nm; λ_{em} 420 nm) and argpyrimidine-like (λ_{exc} 335 nm; λ_{em} 440 nm) AGE fluorescence were also monitored for the systems containing RIB and MGO, respectively.

The percentage inhibition of AGE formation was calculated for each incubation time as follows:

Inhibition % = {1 – [(Fluorescence of incubation mixture containing sample – Intrinsic fluorescence of sample) / Fluorescence of incubation mixture without sample]} x 100

The reported values are the means of three independent experiments, each performed in duplicate.

2.5. Evaluation of fructosamine (Amadori products) formation

The Amadori products were measured as fructosamine by nitroblue-tetrazolium (NBT) salt according to the assay described by Zhang *et al.*[37] with slight modifications. Glycated material was prepared incubating BSA-GLC, BSA-FRU, and BSA-RIB in presence or absence of dittany solution for 14 days. Glycated BSA (0.5 mL) was mixed with 0.3 mM NBT reagent (2.0 mL) in

100 mM bicarbonate-carbonate buffer solutions (pH 10.35) at room temperature for 20 min. Fructosamine present in the systems has the ability to reduce NBT to tetrazinolyl radical forming monoformazano (MF^+), a coloured compound whose presence can be measured spectrophotometrically at 530 nm.

The ability of samples to inhibit the fructosamine formation was calculated as follows:

Inhibition % = {1 – [(Absorbance of glycated system containing BSA, sugar, and inhibitor – Intrinsic absorbance of system containing BSA and inhibitor) / (Absorbance of glycated system without inhibitor – Absorbance of BSA)]} x 100

The reported values are the means of three independent experiments, each performed in duplicate.

2.6. Evaluation of direct MGO and GO-trapping capacity by RP-HPLC-DAD method

Following the method proposed by Mesías *et al.*[35] with slight modifications, 100 μL aliquot of 5.2 mM GO (in 100 mM phosphate buffer, pH 7.4) or 5.2 mM MGO (in 100 mM phosphate buffer, pH 7.4) solutions were added to 50 μL of 5-MQ (internal standard, 6.1 mmol L^{-1} in water/methanol, 50:50, v/v), and to 100 μL of dittany aqueous solution (10 mg dry matter mL^{-1}) or phosphate buffer (control). After the dilution with phosphate buffer to a final volume of 1 mL, the reaction mixture was incubated at 37 °C for 1, 3, 6, 24, 72, and 168 h. After the addition of 200 μL of OPD (10.8 mg mL^{-1} in phosphate buffer), the mixtures were submitted to a derivatization step at 37 °C for 30 min and the residual GO and MGO were quantified as quinoxaline derivatives by HPLC-DAD. The separations were carried out on a Gemini® C18 analytical column (150 × 2.0 mm i.d., 5 μm, Phenomenex, Torrance, CA) with a Hypersil Gold C18 guard column (10 × 2.1 mm i.d., 5 μm, Phenomenex, Torrance, CA) at a constant flow rate of 0.3 mL/min (injection volume 5 μL). The elution was carried out in isocratic mode using as mobile phase a solvent mixture consisting of 0.5% acetic acid aqueous solution and methanol, 50/50, v/v. Chromatograms were registered at 315 nm. The amount of unreacted GO or MGO was calculated from the ratio of quinoxaline or 2-

182 methylquinoxaline (2-MQ) obtained by the conversion of GO and MGO, respectively, and 5-MQ
183 used as internal standard.
184 The percentage of GO and MGO trapped was calculated as follows:
185 GO or MGO decrease (%) = [(Amount of GO or MGO in control sample – Amount of GO or MGO
186 in sample containing extract) / Amount of GO or MGO in control sample] x 100
187 The reported values are the means of three independent experiments, each performed in duplicate.
188

189 **2.7. Spectrophotometric analysis of dicarbonyl compounds**

190 Alpha dicarbonyl compounds were measured spectrophotometrically by Girart-T assay according to
191 the method described by Xu *et al.*.[38] Briefly, 0.4 mL glycated material was incubated with 0.2 mL
192 Girart-T solution (500 mM) and 3.4 mL sodium formate (500 mM, pH 2.9) at room temperature for
193 1 h. The absorbance was registered at 294 nm against blank containing all reagents except Gerart-T
194 solution. The content of dicarbonyl compounds was calculated as glyoxal equivalent after the
195 construction of a calibration curve in the concentration range of 0.5 - 40 μM for the system
196 containing GLC and of 40 – 250 μM for the systems containing FRU and RIB.
197

198 **2.8. DPPH assay**

199 Antiradical activity was evaluated as scavenging activity against the stable coloured DPPH free
200 radical.[39] Briefly, a 100 μL aliquot of sample solutions (40 mg dry matter mL^{-1}) or KH_2PO_4/NaOH
201 buffer, pH 7.4, (control) was added to 3.9 mL of a 6 $x10^5$ mol L^{-1} methanol/KH_2PO_4/NaOH buffer
202 (50:50 v/v) DPPH radical solution. The mixture discoloration was monitored at 515 nm after 20
203 min of reaction. The antiradical activity was calculated as follows:
204 Anti-DPPH activity (%) = [(Absorbance of control – Absorbance of sample)/Absorbance of
205 control] x 100

Methanolic solutions of Trolox C in the concentration range of 15-300 μmol L^{-1} were used for calibration. Results were expressed as μmol Trolox equivalent antioxidant capacity (TEAC) g^{-1} dry sample. LOQ was 2.4 μmol TEAC g^{-1} dry sample.

The reported values are the means of three independent experiments, each performed in duplicate.

2.9. ABTS assay

Scavenging activity against ABTS cation ($ABTS^{\bullet+}$) was evaluated as described by Delgado-Andrade *et al.*[40] with slight modifications. ABTS stock solution was prepared by reacting 7 mM cation radical with 2.45 mM potassium persulfate and allowing the reaction mixture to stand in the dark at room temperature for 16 h before use. Then the mixture was diluted with distilled water (absorbance value of 0.70 ± 0.02 at 734 nm). A 40 μL aliquot of dittany aqueous solution (50 mg dry matter mL^{-1}) or water (control) was added to 500 μL of diluted $ABTS^{\bullet+}$ solution and to 1460 μL of water. The absorbance of the reaction mixture was taken at 734 nm after 10 min. The ability to scavenge $ABTS^{\bullet+}$ was calculated as follows:

$ABTS^{\bullet+}$ scavenging activity (%) = [(Absorbance of control – Absorbance of sample) / Absorbance of control] × 100

Methanolic solutions of Trolox C in the concentration range of 0.05-50 μmol L^{-1} were used for calibration. Results were expressed as μmol Trolox equivalent antioxidant capacity (TEAC) g^{-1} dry sample. LOQ was 0.03 μmol TEAC g^{-1} dry sample.

The reported values are the means of three independent experiments, each performed in duplicate.

2.10. Reducing power assay

The reducing power was determined as previously described by Natic *et al.*[41] with slight modifications. A 1 mL aliquot of infusion (2.55 mg dry material mL^{-1}) was mixed in a test tube with 0.4 mL of potassium hexacyanoferrate (III) (0.02 M), 0.05 mL of chloridric acid (0.01 M), 0.4

mL of iron(III) chloride (0.02 M), and 0.7 mL of distilled water. The absorbance of the reaction mixture was taken at 720 nm after 15 min of incubation in the dark. Methanolic solutions of Trolox C in the concentration range of 10-200 μmol L^{-1} were used for calibration. Reducing substances were expressed as μmol Trolox equivalent antioxidant capacity (TEAC) g^{-1} dry sample.

The reported values are the means of three independent experiments, each performed in duplicate.

2.11. RP-HPLC-DAD-ESI/MS^n analysis

HPLC-DAD-ESI/MS^n analysis were carried out on an Ascentis® Express C18 analytical column (150 x 2.0 mm i.d., 2.7 μm, Supelco®, Bellofonte, PA) at a constant flow rate of 0.3 mL min^{-1} (injection volume 20 μL). The mobile phase consisted of A (0.1% formic acid in water), and B (methanol) with the following gradient table: 0-10 min, 5-8% B; 10-60 min, 8-40% B; 60-70 min, 40-60% B; 70-80 min, 60-100% B; 80-90 min, 100% B, followed by column reconditioning in 10 min. Chromatograms were recorded at 254 nm (flavones), 280 nm (phenolic acids), 314 nm (hydorxycinnamic acids), and 370 nm (flavonols). The ion trap operated in data-dependent, full scan (60-1500 *m/z*), and MS^n mode to obtain fragment ion *m/z* with a 35% and an isolation width of 3 *m/z*. The mass spectrometric parameters were set by flow injection analysis of gallocatechin and apigenin-7-*O*-glucuronide (10ppm in 0.1% formic acid –ACN solution, 50:50, v/v) as below: ionization polarity, negative and positive; sheat gas glow rate, 50 arbitrary units (AU); auxiliary gas flow rate, 20 AU; ionization voltage, 5 kV; capillary temperature, 400 °C. The Thermo Fischer Scientific Excalibur 2.1 software was used for data acquisition and processing.

2.12. Statistical Analysis

Data from at least three replicated experiments performed in duplicate were expressed as mean ± standard deviations (SD) of measurements. Differences were considered to be significant at $P < 0.05$ and $P < 0.01$. All statistical analysis was carried out using Microsoft Excel 2010.

3. Results and Discussion

3.1 Chemical characterization of infusion by RP-HPLC-DAD-ESI-MS^n

The qualitative profile of the infusion was performed using RP-HPLC-DAD-ESI-MS^n using data-dependent acquisition that combines the putative molecular mass (MM) obtained by single stage MS with UV detection and tandem MS analysis acquired by applying user-specified criteria to select the ion of interest for subsequent fragmentation. Additional targeted MS^n experiments have been performed on selected pseudomolecular ions for structure confirmation, if necessary.

Among the eight flavones detected in the infusion, compound 3 was a *C*-glycoside (Figure 1). This was putatively identified as 6,8-di-*C*-hexosyl-apigenin, a di-*C*-glycosylated flavone, by comparing its fragmentation pattern with that reported in literature.[42,43] It showed a molecular ion at *m/z* 593 in negative ionization mode. The fragmentation pattern consisted in the subsequent losses of 120 Da giving the peak base at *m/*z 473 and the secondary ion at *m/z* 353, respectively. The loss of 120 Da is characteristic of the presence of a hexosidic residue bound to aglycone through a C-C bond. The fragment at 353 *m/z* corresponds to the aglycone apigenin +113 Da and it is an ion that could be easily observed for C-glycosil flavones. Glucuronide derivatives of apigenin were also detected. In particular, apigenin-7-*O*-glucuronide was identified (compound 13); its structure was also confirmed by comparing its selectivity and fragmentation pattern with those obtained for the standard compound analysed in the same conditions. The molecular ion at *m/z* 445 produced an ion at *m/z* 269 corresponding to apigenin by the neutral loss of glucuronic acid (176 Da). Compound 9 showed a molecular ion at *m/z* 621 which fragmented producing a base peak at *m/z* 351 due to the loss of the aglycon and a secondary peak at *m/z* 269 corresponding to apigenin aglycon and due to the loss of two glucuronide residues [M-H-(176 x 2)]$^-$. The molecular structure hypothesized was apigenin-7-*O*-diglucuronide. Two isobaric derivatives of apigenin with molecular ion at *m/z* 797 (compounds 5 and 8) were putatively identified as apigenin-*O*-triglucuronide. In both cases a base peak at *m/z* 527 [M-H-aglycon]$^-$ and a low abundance signal corresponding to aglycone ion at *m/z* 269 [M-527]$^-$ were registered in MS/MS spectra. Furthermore an ion at *m/z* 351 due to the loss of

283 apigenin-monoglucuronide residue was also detected.[44-46] Glucuronide derivatives of apigenin were
284 previously identified in aromatic species of *Origanum majorana*[46], but never before in *O.*
285 *dictamnus*.
286 Two luteolin derivatives (compounds 6 and 14) were also found in dittany infusion. The first
287 compound was putatively identified as luteolin-7-*O*-diglucuronide (6). MS^2 experiments produced
288 ions at *m/z* 351 (base peak) and *m/z* 285 from the fragmentation of the molecular ion at *m/z* 637 [M-
289 H]$^-$.[47,48] The second luteolin derivative had the same molecular mass of 6,8-di-*C*-hexosyl-apigenin
290 (594 Da), but the fragmentation pattern was different. In fact, a base peak at *m/z* 285 corresponding
291 to luteolin aglycone, which is generated by the loss of a rutinose residue [M-H-308]$^-$ and a radical
292 ion at *m/z* 286 with low abundance were registered. Considering that the ratio between the
293 intensities of aglycone and radical aglycone ions are closely correlated to the glycosylation position,
294 the compound was identified as luteolin-7-*O*-rutinoside (14).[46] The structure was also confirmed by
295 the analysis of pure standard. The last flavone derivative detected was 5,4'-dihydroxy-6,7,8-
296 trimetoxyflavone (xantomicrol) (16). Its MS and MS/MS spectra registered in positive ionization
297 mode showed a molecular ion at *m/z* 345 which fragmented giving a base peak at *m/z* 330 (loss of
298 15 Da [M+H-15]$^+$) and ions at *m/z* 315 (50%) and 313 (45%). Its molecular structure was
299 tentatively identified by comparing the obtained fragmentation with that reported in literature for
300 the same compound identified in different *Origanum* species.[49]
301 Considering flavonols, two keampferol derivatives were detected in dittany infusion (compounds 7
302 and 15). They showed a molecular ion at *m/z* 813 [M-H]$^-$ which fragmented in three glucuronic acid
303 residues [176 Da x 3]$^-$ generating a base peak at *m/z* 527 by the loss of kaempferol, and secondary
304 ions at *m/z* 351 and 285 following the loss of kaempferol monoglucuronide and three glucuronic
305 acid residues, respectively. The isobaric compounds were tentatively identified as kaempferol-7-*O*-
306 triglucuronide and kaempferol-3-*O*-triglucuronide.[44]
307 Compound 4 was identified as gallocatechin due to its molecular mass of 306 Da (*m/z* 305, [M-H]$^-$)
308 and its fragmentation pattern which showed a base peak at *m/z* 225. This structure was also

confirmed by the analysis of standard compound. This compound was previously identified in other Lamiaceae species and in particular in *O. majorana*.[46,50]

The analysis of the MS spectrum of *O. dictamnus* infusion revealed the presence of six hydroxycinnamic acid derivatives, among which five different isoforms of salvianolic acid. Compound 10 at *m/z* 359 was characterized as rosmarinic acid. The MS/MS spectrum of this compound provided fragment ions at *m/z* 161 (base peak) [caffeic acid-H- $2H_2O$]$^-$ by losing two molecules of water, and *m/z* 197 and 179 corresponding to 2-hydroxy derivative of hydrocaffeic acid and caffeic acid, respectively[46]. The presence of this acid in dittany and other vegetables belonging to Lamiaceae family was already reported in literature.[51-53] Regarding salvianolic acid derivatives, compound 12 was putatively identified as salvianolic acid K by comparing the fragmentation pattern with literature data.[43,54] It showed [M-H]$^-$ ion at *m/z* 555 which was subjected to a decarboxylation and a dehydratation, giving a base peak at *m/z* 493 and a secondary peak at *m/z* 359, which has a relative abundance of about 20% corresponding to rosmarinic acid after the loss of 3,4-dihydroxyvinilbenzenic mojety.[43] This compound was previously described in a methanolic extract of *O. dictamnus*[55] and also in *Thymus longicaulis*. [43] Compound 17 was putatively identified as salvianolic acid B (molecular ion at *m/z* 717), known to be present in *O. majorana*[46]; its fragmentation pattern consisted of a base peak at *m/z* 519 following the loss of 3-(3,4-hydroxyphenyl)lactic acid (danshensu) mojety [M-H-198]$^-$, an ion with very low intensity at *m/z* 475 [M-danshensu-CO_2-H]$^-$, an ion deriving from the loss of the second danshensu mojety at *m/z* 321 [M-H-198-198], and an ion at *m/z* 339 [M-danshensu-caffeic acid-H]$^-$.[43, 56,57]An isobaric compound was detected in our infusion and the hypothetic structure could be isosalvianolic acid B (20) due to the lack in its MS/MS spectrum of the ion at *m/z* 321, as previously reported by Taamalli. [46] Compound 18 was putatively identified as salvianolic acid I through MS^2 and MS^3 experiments. In fact, it showed a molecular ion at *m/z* 537 which fragmented to *m/z* 493 [M-H-CO_2]$^-$, to *m/z* 339 [M-H-198]$^-$ corresponding to danshensu mojety, and to *m/*z 313 [M-H-CO_2-180]$^-$. The molecular structure was confirmed by MS^3 experiments which led to the generation of an ion at

335 *m/z* 179 corresponding to caffeic acid. The presence of salvianolic acid I was not surprising because
336 several researches underlined the presence of salvianolic acids in different types of sage, which
337 belongs to the same family of dittany. [46] Another compound with MM 538 Da was detected
338 (compound 11). It was putatively identified as lithospermic acid by its fragmentation pattern very
339 similar to that of salvianolic acid I, but lacking of the fragment at *m/z* 339. The presence of this acid
340 was previously reported in *O. vulgare* by Martins *et al.*[58] and in *O. majorana* by Taamalli *et al.*.[46]
341 The last salvianolic acid derivative detected was a compound with a molecular mass of 494 Da (19)
342 putatively identified as salvianolic acid A.[59,60] In negative ionization mode its fragmentation
343 produced a base peak at *m/z* 295 by the loss of danshesu mojety, an intense secondary peak at *m/z*
344 185 $[M\text{-}H\text{-}C_6H_6O_2]^-$ and a low abundant ion at *m/z* 109 $[C_6H_6O_2\text{-}H]^-$. This compound here
345 tentatively identified was never before detected in *Origanum* species.
346 A *p*-hydroxyphenylpropionic acid-O-hexoside (compound 1) was tentatively identified by its MS
347 and MS/MS spectra. The molecular ion registered in negative ionization mode was at *m/z* 327, and
348 it is also present in the spectrum as formiate derivative. It fragmented to *m/z* 165 following the loss
349 of an hexose mojety $[M\text{-}H\text{-}162]^-$ and to a low abundant ion at *m/z* 121 due to the loss of carboxylic
350 group from *m/z* 165. Compound 2 showed a molecular mass of 388 Da. Its fragmentation pattern
351 consisted of a base peak at *m/z* 207 and secondary peaks at *m/z* 163 (due to the decarboxylation of
352 the base peak), and at *m/z* 369. The further fragmentation of the ion at *m/z* 207 gave an ion at *m/z*
353 163. Comparing these data with the literature ones, the compound could be 12-hydroxyjasmonic-*O*-
354 hexoside acid, before detected in the aerial part of a Greek *O. vulgare*. [61,62]
355 In conclusion, we identified twenty compounds in dittany infusion, among which nine flavones, two
356 flavonols, one catechin, and seven phenolic acids (Table 2). Only rosmarinic acid and salvianolic
357 acid B were previously identified in Cretan dittany.
358
359 **3.2. Antiglycative capacity of the infusion**

An herbal tea daily consumed by Greeks was investigated as a promising functional beverage useful in the reduction of non-enzymatic protein glycation and in the scavenging free radicals.

Firstly, the effect on the early stage of non-enzymatic glycation of protein was monitored by the inhibition of fructosamine formation. Fructosamine is an Amadori product generated by the rearrangement of Schiff's bases that originates from the reaction between the carbonyl groups of monosaccharides with the amino groups of proteins after condensations, rearrangements, and oxidative modifications. [63] Considering that different rates of protein glycation could be registered depending on the sugar, the formation of fructosamine was investigated under mild reaction conditions applied in *in vitro* system containing three different dietary monosaccharides (GLC, FRU, and RIB) by NBT assay. Amadori products formed after 14 days of incubation were spectrophotometrically monitored by the reduction of NBT yielding to a coloured compound. The reduction of NBT was inhibited by incubating dittany infusion with all the sugars-BSA systems used. The inhibitory percentage were 14.38 ± 0.05, 41.96 ± 1.77, and 9.17 ± 0.25 for GLC, FRU, and RIB, respectively. The values registered for FRU-glycation system were significantly higher than those registered for the other two monosaccharides ($P < 0.01$).

After oxidation and dehydration steps in the Maillard reaction, dicarbonyl compounds, considered as AGEs precursors, were generated. The inhibitory effect of the infusion on the α-dicarbonyl compounds formation was calculated using a standard curve for GO. As shown in Figure 2, both dittany infusion and AG, used as positive control, inhibited the formation of dicarbonyl compounds generated at different concentration levels depending on the sugar present in the glycated material. In fact, dittany showed a significantly stronger inhibitory effect than AG in the systems containing GLC and FRU starting from 2 and 1 day, respectively. Conversely, when RIB is present in the system dittany showed higher efficacy than AG which had no activity for a period shorter than 4 days.

As MGO can react with serum albumin acting as intermediator for AGEs formation, the system BSA-MGO could be useful to evaluate the middle stage of protein glycation, while the systems

386 BSA-sugar could be useful to evaluate the last phase of non-enzymatic glycation process.
387 Therefore, the ability of dittany infusion to inhibit AGEs formation promoted by the three above
388 mentioned sugars and MGO were monitored. In particular, the formation of fluorescent AGEs were
389 monitored. Different time monitoring were selected for each system according to the different
390 reactivity of the sugar considered and of MGO; in fact, the fluorescence intensity of the glycated
391 material significantly increased throughout the incubation period, but dramatically decreased after
392 14 days when GLC is present in the system, after 7 days in presence of FRU or MGO, and after 1
393 day with RIB. These kinetics are in accordance with those obtained by Sadowska-Bartosz *et al.*[64]
394 indicating RIB as the most reactive sugar, followed by FRU and GLC. In previous investigations,
395 the highest RIB reactivity was explained by its planar structure eliciting the unstable aldofuranose
396 ring to easily react with the amino groups.[43] Preliminary studies on the relationship between
397 infusion concentration and activity were performed using final concentrations in reaction medium
398 ranging from 20 μg to 1 mg mL^{-1}. One mg mL^{-1} was the concentration able to generate the highest
399 inhibitory activity in all the systems (data not shown) and therefore all tests were performed
400 incubating this concentration. AG was always used as positive control due to its well-known action
401 in the glycation process. Figure 3 shows the effect of the infusion on fluorescent AGEs formation in
402 BSA-GLC system. The inhibitory capacity at the beginning of the monitoring period was about 72
403 % and it was significantly lower than the activity of the positive control AG. Then, the effect of the
404 infusion increased with the reaction time, differently from that of AG showing a particular kinetic in
405 the inhibition of AGE formation, as evident in the decreased inhibition value registered for a longer
406 time; in fact, after 14 days of incubation dittany was able to inhibit almost completely vesperlysine-
407 like AGEs generated in the system. Differently, the high activity values registered after 1 day of
408 incubation in the system containing FRU were close to those registered for AG (> 90%) and then
409 increased till 5 days when dittany completely inhibited AGEs formation; at the end of the
410 monitoring period (7 days) the activity decreased reaching 90% of inhibitory capacity (Figure 4).

In the BSA-RIB system (Figure 5), the activity registered in the inhibition of vesperlysine-like and pentosidine-like AGEs was very similar during the entire monitoring time with values ranging from about 84 to 96%, and always significantly higher ($P<0.01$) than the activity registered for AG at the same times. After 24 h a slight but significant ($P<0.05$) reduction in the capacity of inhibiting the formation of both types of AGEs in respect to the values registered at 1h was recorded. The kinetic profiles registered for vesperlysine-like and pentosidine-like AGEs seemed to be less affected by the chemical structures of the compounds present in dittany infusion in comparison to other extracts, as previously reported in literature. In fact, extracts particularly rich in hydroxycinnamic acid derivatives, showed a different activity profile according to the different type of AGEs generated probably because of molecular structural changes under simil-physiological conditions as in the systems here studied.[65, 66]

As regards the action of the infusion in the system consisting of BSA and MGO, the capacity of the tested extract to inhibit vesperlysine-like and argpyrimidine-like AGEs was demonstrated (Figure 6). In particular, considering the inhibition of the formation of versperlysine-like AGEs, dittany showed activity values significantly higher ($P<0.01$) than those registered for AG at all the monitoring times; it was able to totally counteract AGEs formation after 1 h, decreasing its activity progressively till 1 day when a value of 82% was reached, and then increasing its activity till 7 days. Conversely, similar very high activity values close to 100% were registered both for dittany and AG when considering argpyrimidine-like AGEs formation after 1 and 3 h of incubation. Increasing the incubation time, only a slight decrease in the inhibitory capacity was registered for dittany, differently from AG which showed statistically significant ($P<0.01$) reduction.

Considering that GO and MGO are important precursors in the formation of AGEs due to their comparatively higher reactivity than reducing sugars to attack amino groups in proteins, trapping these carbonyl should be an effective strategy to alleviate the carbonyl stress. Therefore, the capacity to directly trap GO and MGO was also evaluated. In Figure 7 the ability of dittany infusion to act as trapping-agent is recorded at different times starting from 1 h up to 7 days (168 h). The

results showed that the trapping capacity of MGO is generally higher than that of GO in the first 24 h of monitoring; conversely, for longer time both of them were completely trapped.

Considering that the second period of the glycation reaction was characterized by the free-radical mediated conversion of the Amadori products to AGEs, in order to verify if the antiglycative effect registered for the infusion may be associated with its antioxidant/antiradical acitivity, three different assays, i.e. ABTS cation radical scavenging activity test, DPPH free radical scavenging capacity method, and reducing capacity assay were used. In Table 1 the obtained results are reported. Dittany showed a high capacity in reducing oxidative substances and in acting as anti-DPPH radical. Conversely, only a weak ABTS cation radical scavenging capacity was registered. Therefore, a correspondence between DPPH or reducing power and antiglycative capacity was found in all the methods used.

Several studies previously pointed out the antioxidant and antiglycation capacities of herbal infusions, such as black and green tea (*Camellia sinensis*), balm (*Melissa officinalis*), sage (*Salvia officinalis*), common verbena (*Verbena officinalis*), some Thai herbal teas, and mate tea (*Ilex paraguariensis*).[67,68] The inhibitory effects on AGEs registered for dittany infusion are similar with those reported in particular for black and green tea in BSA-GLC and BSA-MGO systems by Ho *et al.* [67] and for Guava tea in BSA-GLC[69], and the activity values are higher than those registered for sage, common verbena and lemon grass (*Cymbopogon citratus*) herbal tea.[67,68] Moreover, the antiglycative activity of dittany is higher than that of mate tea in BSA-MGO system[70] and than that of mate tea and coffee in BSA-FRU system.[71] The antiglycative activity is generally correlated to the polyphenolic composition of the extracts. In particular, previous structure-activity studies indicated that flavones are able to exhibit stronger inhibitory effects when compared to flavonols, flavanones, and isoflavones. [72] The composition of dittany infusion rich in apigenin, luteolin, kaempferol, and also hydroxycinnamic acid derivatives could justify the high antiglycative activity registered.

4. Conclusion

This study illustrated that dittany infusion was a complex mixture consisting of several flavones, flavonols, and hydroxycinnamic acid derivatives. Cretan tea was proved to have inhibitory effects on AGEs formation being very active as antiglycative agent in the incubation systems consisting of a model protein and different sugars or MGO. This effect may be partially related with its antioxidant/antiradical activity and could be ascribed to its composition in flavones, flavonols, and hydroxycinnamic acid derivatives.

As the capacity of inhibiting the formation of dicarbonyl compounds and AGEs was stronger than that of reducing Amadori products, we could indicated that the antiglycative action of the tested beverage occurred primarly during the last two phases of the non-enzymatic glycation reaction.

These findings are relevant for enhancing the functional properties of Cretan tea beverage even if more investigations are highly recommended to evaluate the potentially use of such beverage as food supplement useful as inhibitor of the non-enzymatic glycation process, involved in chronic and degenerative disorders. To this purpose, the protective effects of the infusion will be tested with novel *in vitro* approaches based on the use of dynamic bioreactors, which are more similar to physiological compartments *in vivo*.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This research was supported by Merck KGaA for consumable materials.

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686 **Table 1.** Antioxidant activity of dittany infusion. The results are expressed as mean ± DS for *n*=6.

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μmol g^{-1}	Dittany infusion
	689
ABTS TEAC	2.8 ± 0.3
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DPPH TEAC	177.1 ± 1.5
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Reducing power	308.6 ± 3.4
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Table 2. MS, MS/MS, and MS^3 data (negative and positive ionization modes) of the compounds identified in dittany infusion (Compounds are reported in order of elution; [a] positive ionization mode;*compared with standard compounds).

Compound	Precursor ion (*m/z*)	HPLC-ESI/MS^n *m/z* (% of base peak)	Compound identity
1	327	MS^2[327]: 165(100), 121(25)	*p*-hydroxyphenylpropionic acid hexoside
2	387	MS^2[387]: 369(30),207(100),163(90)	12-Hydroxyjasmonic acid *O*-hexoside
		MS^3[207]: 163(100)	
3	593	MS^2[593]: 473(100),383(35),353(70), 279(5)	6,8-di-*C*-hexosylapigenin
4	305	MS^2[305]: 225(100),97(50)	Gallocatechin*
5	797	MS^2[797]: 527(100),351(50), 269(12)	Apigenin-*O*-triglucuronide isomer 1
6	637	MS^2[637]: 351(100),285(40)	Luteolin-7-*O*-diglucuronide
7	813	MS^2[813]: 527(100),351(60),285(20)	Kaempferol-*O*-triglucuronide isomer 1
8	797	MS^2[797]: 527(100),351(60),269(15)	Apigenin-*O*-triglucuronide isomer 2
9	621	MS^2[621]: 351(100),269(10)	Apigenin-7-*O*-diglucuronide
10	359	MS^2[359]: 197(20),179(25),161(100)	Rosmarinic acid*
11	537	MS^2[537]: 493(100),295(25)	Lithospermic acid
12	555	MS^2[555]: 493(100),359(20)	Salvianolic acid K
13	445	MS^2[445]: 269(100)	Apigenin-7-*O*-glucuronide*
14	593	MS^2[593]: 286(60),285(100)	Luteolin-7-*O*-rutinoside*
15	813	MS^2[813]: 527(100),351(55),285(40)	Kaempferol-*O*-triglucuronide isomer 2
16	345[a]	MS^2[345]: 330(100),315(55),313(50)	5,4'-dihydroxy-6,7,8-trimethoxyflavone
17	717	MS^2[717]: 519(100),475(50),339(30),321(30)	Salvianolic acid B
18	537	MS^2[537]: 493(100),339(35),313(15),295(25)	Salvianolic acid I
		MS^3[439]: 179(100)	
19	493	MS^2[493]: 295(100),185(75),109(10)	Salvianolic acid A
20	717	MS^2[717]: 519(100),475(40),339(50)	Isosalvianolic acid B

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719 **Figure captions**

720 **Figure 1.** HPLC-DAD chromatogram of dittany infusion registered at 320 nm.

721 **Figure 2.** Inhibitory effect of dittany infusion on the formation of dicarbonyl compounds in BSA-
722 GLC (A), BSA-FRU (B), and BSA-RIB (C) systems.

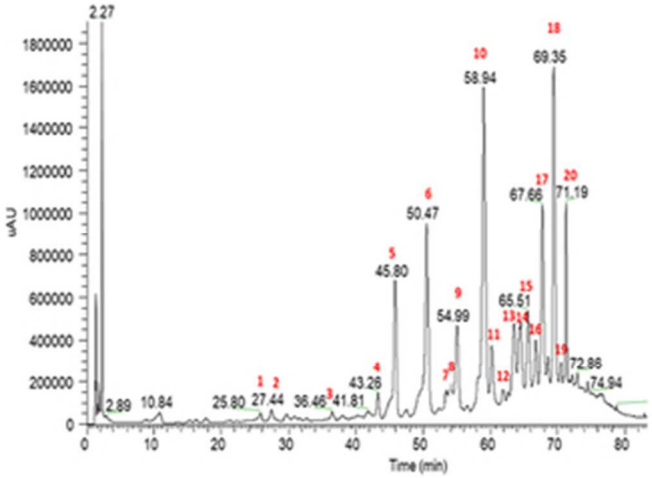
723 **Figure 3.** Antiglycative activity of dittany infusion and aminoguanidine (AG) on the formation of
724 vesperlysine-like AGEs in BSA-GLC assay. Different superscript letters within each monitoring
725 time indicate significant differences ($P < 0.05$) among AG and infusion.

726 **Figure 4.** Antiglycative activity of dittany infusion and aminoguanidine (AG) on the formation of
727 vesperlysine-like AGEs in BSA-FRU assay. Different superscript letters within each monitoring
728 time indicate significant differences ($P < 0.05$) among AG and infusion.

729 **Figure 5.** Antiglycative activity of dittany infusion and aminoguanidine (AG) on the formation of
730 (A) vesperlysine-like AGEs and (B) pentosidine-like AGEs in BSA-RIB assay. Different
731 superscript letters within each monitoring time indicate significant differences ($P < 0.05$) among
732 AG and infusion.

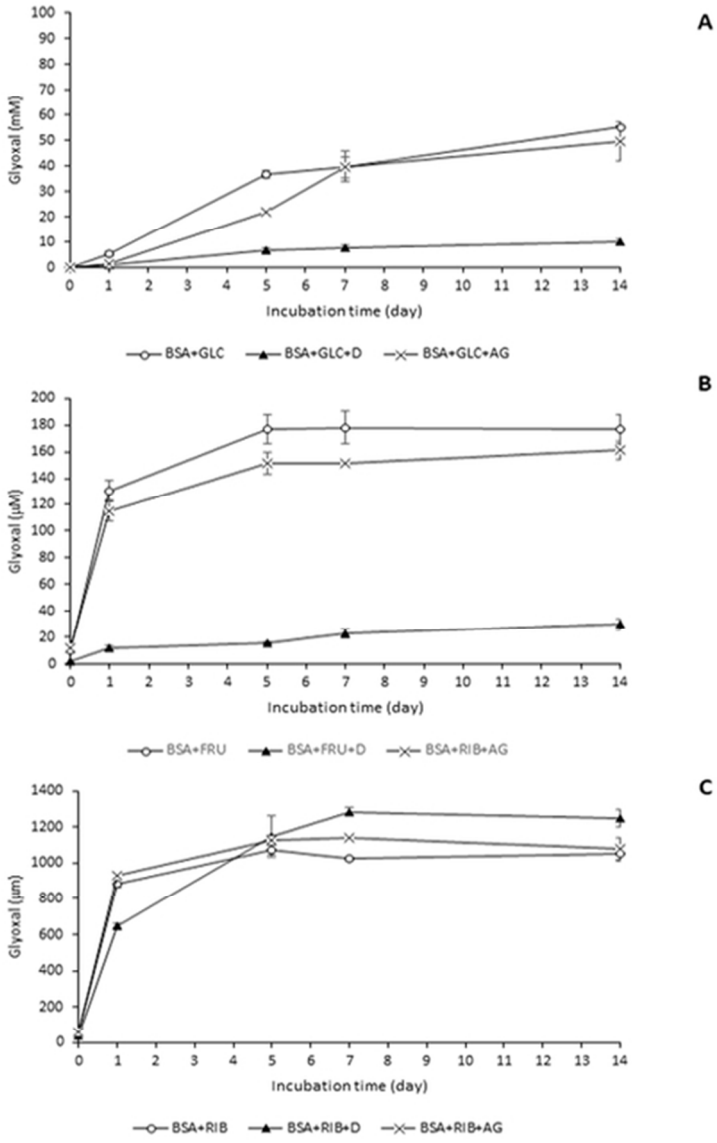
733 **Figure 6.** Antiglycative activity of dittany infusion and aminoguanidine (AG) on the formation of
734 (A) vesperlysine-like AGEs and (B) argpyrimidine-like AGEs in BSA-MGO assay. Different
735 superscript letters within each monitoring time indicate significant differences ($P < 0.01$) among
736 AG and infusion.

737 **Figure 7.** Kinetic study of direct MGO (A) and GO (B) trapping capacity of dittany infusion after
738 incubation (0-168 h).



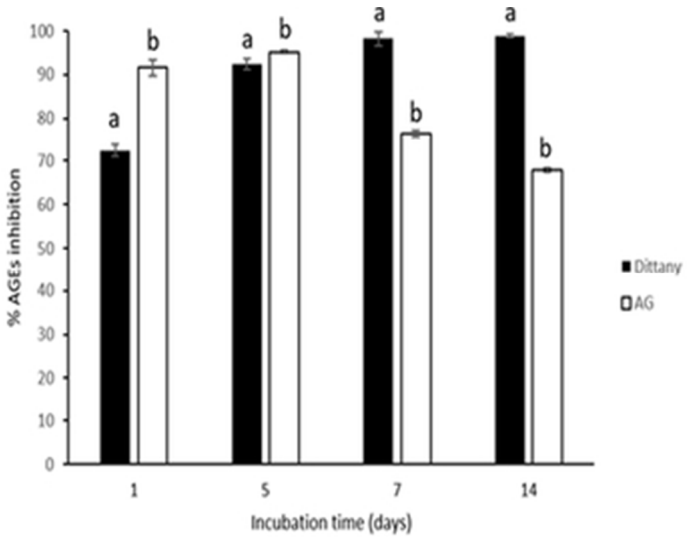
HPLC-DAD chromatogram of dittany infusion registered at 320 nm.

14x11mm (600 x 600 DPI)



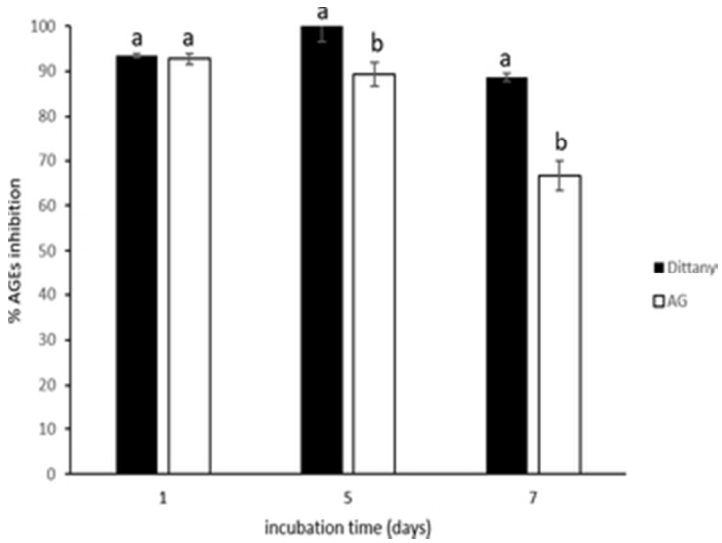
Inhibitory effect of dittany infusion on the formation of dicarbonyl compounds in BSA-GLC (A), BSA-FRU (B), and BSA-RIB (C) systems.

22x34mm (600 x 600 DPI)



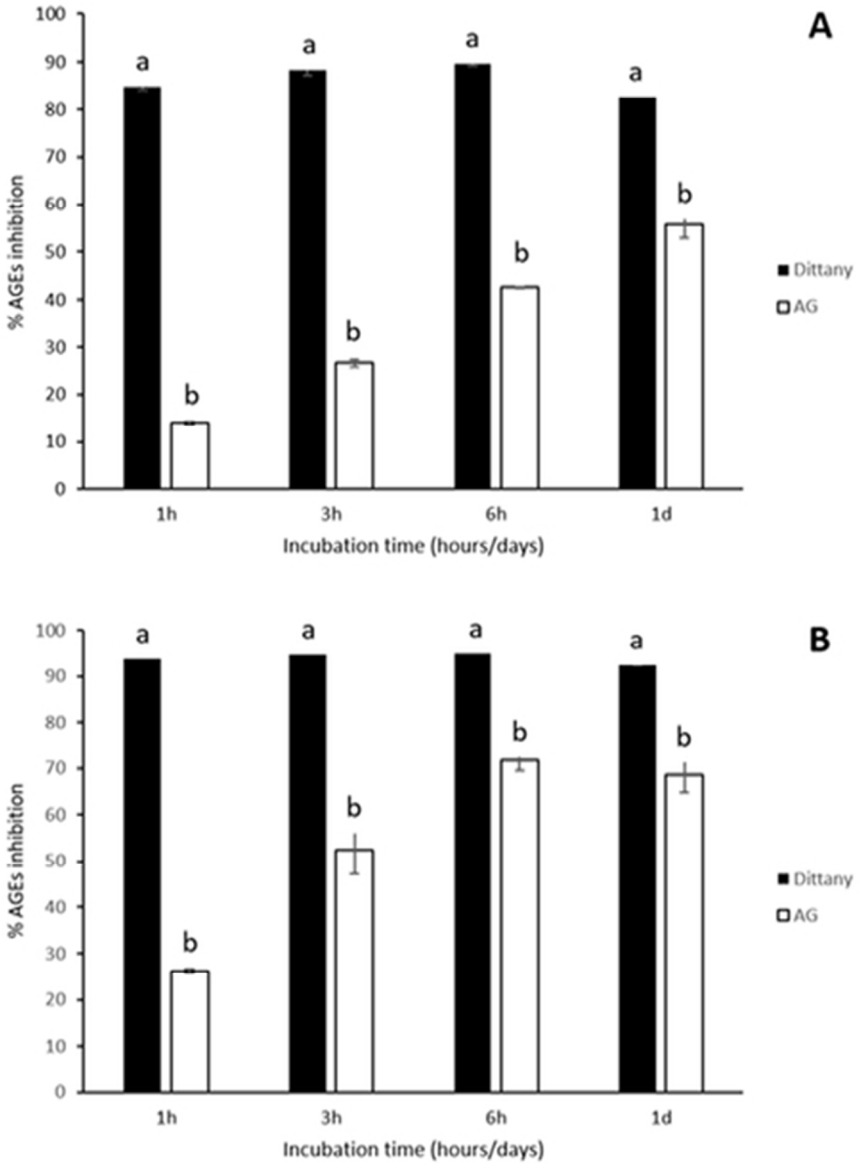
Antiglycative activity of dittany infusion and aminoguanidine (AG) on the formation of vesperlysine-like AGEs in BSA-GLC assay. Different superscript letters within each monitoring time indicate significant differences ($P < 0.05$) among AG and infusion.

14x11mm (600 x 600 DPI)



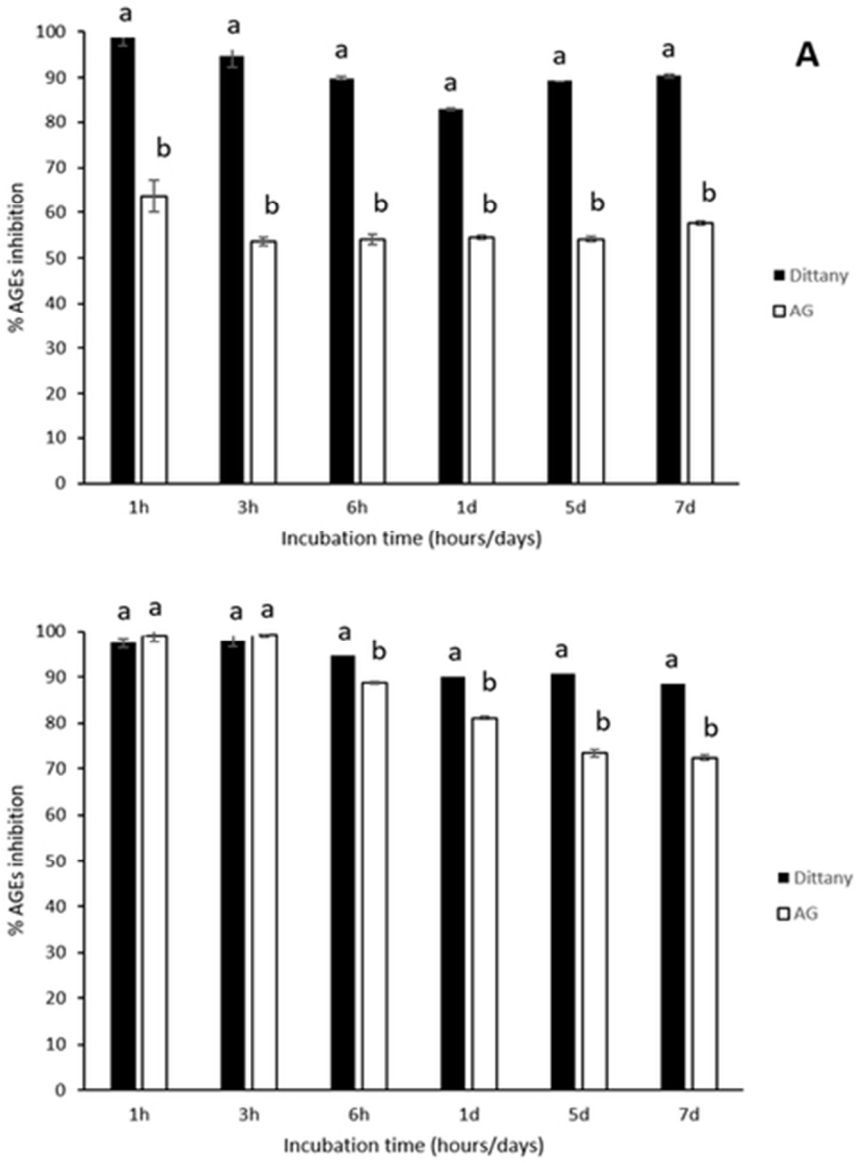
Antiglycative activity of dittany infusion and aminoguanidine (AG) on the formation of vesperlysine-like AGEs in BSA-FRU assay. Different superscript letters within each monitoring time indicate significant differences ($P < 0.05$) among AG and infusion.

14x11mm (600 x 600 DPI)



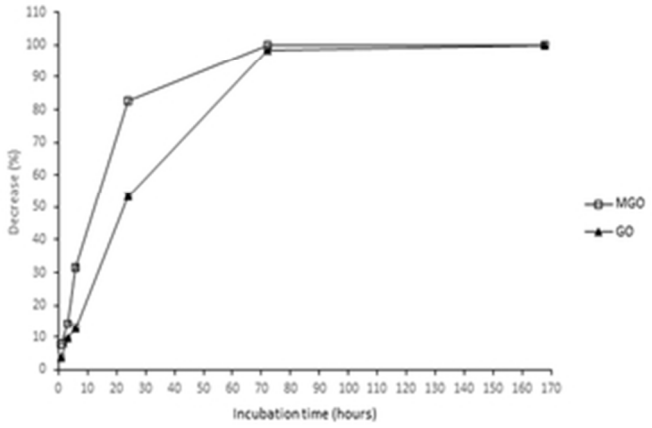
Antiglycative activity of dittany infusion and aminoguanidine (AG) on the formation of (A) vesperlysine-like AGEs and (B) pentosidine-like AGEs in BSA-RIB assay. Different superscript letters within each monitoring time indicate significant differences ($P < 0.05$) among AG and infusion.

19x26mm (600 x 600 DPI)



Antiglycative activity of dittany infusion and aminoguanidine (AG) on the formation of (A) vesperlysine-like AGEs and (B) argpyrimidine-like AGEs in BSA-MGO assay. Different superscript letters within each monitoring time indicate significant differences ($P < 0.01$) among AG and infusion.

19x26mm (600 x 600 DPI)



Kinetic study of direct MGO (A) and GO (B) trapping capacity of dittany infusion after incubation (0-168 h).

14x11mm (600 x 600 DPI)