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# Impact of "Golden" tomato juice on cognitive alterations in metabolic syndrome: Insights into behavioural and biochemical changes in a high-fat diet rat model

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ABSTRACT

"Golden" tomato (GT) plays a protective role in metabolic dysfunction induced by High-Fat Diet (HFD). Our aim is to characterize the phytonutrient composition of the juice and explore the influence of GT, orally administered for one month, on cognitive impairment associated with Metabolic Syndrome (MetS) in male rats. We investigated reactivity, stress response and memory, together with brain neurotrophic/inflammatory signaling. Our data showed that HFD-induced functional modifications were ameliorated by GT nutritional supplementation. In particular, the behavioural reactivity improved in HFD/GT rats, that also showed a better performance in tests measuring anxiety and anhedonia. Furthermore, GT consumption rescued the declarative memory impairment. Lastly, GT supplementation counteracted HFD-induced brain alterations in PI3K\Akt and MAPK/ERK signalling pathways. In conclusion, this study provides evidence of the importance of food supplementation with GT in the protection from neuroinflammation and cognitive alterations associated with MetS.

## 1. Introduction

The intriguing *liason* between central oxy-inflamation and cognitive decline in metabolic syndrome (MetS), as well as their underlying neural mechanisms, has awaken avid interest (Higgs, 2023). Very recently, it was postulated that MetS could influence functional connectivity in cortical default mode network and that these alterations impact on superior functions and cognitive alterations (Zheng et al., 2023). The encountered alteration of brain homeostasis in MetS, due to hypertension and chronic inflammation, was found to closely correlate with compromised executive, attentive and global functions that lead to cognitive decline and dementia in humans (Zheng et al., 2023). Strikingly important is that MetS-induced alterations at the brain level have been reported to cause damage in discrete neuronal structure and function in crucial brain areas (Cope et al., 2018), but also to boost inflammatory state that impaired learning and memory, the response to stress and to anxiety that could be investigated via specific behavioural paradigms (Fuentes et al., 2023; Lakhan & Kirchgessner, 2013; Pinto et al., 2022). It has been hypothesized that the MetS-mediated molecular mechanisms that trigger neurodegeneration may have an impact on neurodegenerative diseases such as Alzheimer's disease, and depression (Blázquez et al., 2014; Wahl et al., 2018). This could be due to the overgeneration of reactive oxygen species (ROS), to the alteration of endogenous antioxidant systems and to neuroinflammation determining reactive astrogliosis, or even to the alteration of microtubular cellular transport system for nutrients that is supported by the protein tau (Blancas-Velazquez et al., 2018; de Aquino et al., 2018; Gambino et al., 2022c; Gauthier et al., 2022; Treviño et al., 2015). This is particularly true in brain regions such as the hippocampus, the hypothalamus and cerebral cortex where the neuroinflammatory and the neurodegenerative condition associated with MetS leads to modifications in the activation of several intracellular signaling cascades, including

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Mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated kinase (Erk) and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) (Huang et al., 2018; Milstein & Ferris, 2021).

Considering this, MetS induced by High-Fat Diet (HFD) in rats represents an invaluable model for exploring the eventual neuroprotective role of novel health-enhancing foods (Q. Wang et al., 2018). Among these, "neuro-nutraceuticals" have catched attention for their modulatory activity in brain pathophysiological processes, at nutritionally-relevant amount, with a view to enhance preventive and protective strategies in clinical practice that could benefit from nutritional supplementation(Naoi et al., 2019; Wal et al., 2023). For instance, a huge amount of studies have focused so far on beneficial properties of anti-oxidant polyphenolic compounds that can elicit synaptic plasticity by activating specific signalling pathways, can promote vascular effects proved to be functional in hippocampal nerve cells growth and ultimately affect cognition(Caruso et al., 2022; Godos et al., 2023; Spencer, 2009). Polyphenols, curcumin, berberine, rutin and indicaxanthin can be cited among some of the neuro-nutraceuticals able to cross the blood brain barrier (BBB), maintain their molecular integrity, accumulate in some regions of interest, and exert a protective activity on the Central Nervous System (CNS)(Figueira et al., 2021; Gambino et al., 2018; Meng et al., 2015). Importantly, nutritional supplementation with neuro-active substances has been found to powerfully influence neuronal excitability(Gambino et al., 2022a; Williams et al., 2004), which impacts on cognitive and behavioural function(Abu-Elfotuh et al., 2022; Wal et al., 2023).

"Golden" tomato (GT) is a food product, belonging to the cultivar *"Brigade"*, harvested before full maturation with respect to red tomato (RT), comprising a plethora of bioactive phytonutrients (Gambino et al., 2023). GT extracts from fresh fruits have been recently characterized in terms of antioxidant composition and protective effects on the metabolic dysfunction determined in a HFD rat model of MetS (Gambino et al., 2023, Pipitone et al., 2023). In detail, GT extract has already proven to be effective on altered biometric parameters, glucose tolerance and lipid dysmetabolism when chronically administered in MetS rats(Gambino et al., 2023). Even more intriguing, it ameliorated robust systemic and hepatic biomarkers of oxidative unbalance in MetS, such as disulfide coumpounds, boosting endogenous protection from plasmatic and hepatic lipid peroxidation products and increasing the glutathione ratio in the liver(Gambino et al., 2023). Furthermore, we have previously uncovered that GT extracts with respect to red tomatoes at full maturation significantly differs in terms of phytonutrients, anti-oxidant and anti-dysmetabolic effects exerted in non-alcoholic fatty disease (Pipitone et al., 2023). The GT powerful antioxidant activities could play a crucial role on oxidant-driven cognitive impairment typical of MetS model, both at behavioural and molecular level.

With this in mind, we aimed at assessing whether the juice of GT could represent a functional food exerting neuroprotective effects on HFD-induced rat model of MetS. To this purpose, we first characterized the phytonutrient composition of this food matrix, i.e. GT juice, and then designed a nutritional supplementation approach for one month with GT juice to rats that were fed with HFD for 8 weeks to induce MetS. At the end of the experimental protocol, we evaluated if GT supplementation influenced exploratory behaviour, reactivity, the response to stress and anxiety, depressive-like behaviour and memory, by means of an ample set of widely used behavioural tests. Furthermore, brain neurotrophic/inflammatory signaling was assessed to research targets that could be implicated in eventual GT-mediated neuroprotection in specific brain areas. In detail, we investigated the modulation of MAPK/ERK and PI3K/Akt pathways in rat hippocampus, prefrontal cortex and hypothalamus, three crucial regions involved in energetic homeostasis and behaviour. The current investigation on GT aligns with the theme of food supplementation with neuro-nutraceuticals in the protection from neuroinflammation and cognitive alterations associated with MetS.

## 2. Methods

### 2.1. Determination of nutraceutical properties in golden tomato juice

As reported in our previous paper, the food matrix named "Golden Tomato" refers to a tomato harvested at the veraison stage and defined as "golden" on the basis of the degree of coloring it possesses (Gambino et al., 2023). It is a tomato from the cultivar Brigade, grown in a sandy soil type with a northwest exposure and 650 m of altitude. The golden tomato juice was obtained at the farm through the following steps: cold cutting of selected samples of golden tomatoes, treatment at 95 °C to inactivate the pectinases, concentration and pasteurisation. The juice, that has a water content of 84%, has been characterised according to phytonutrients and antioxidant properties.

#### 2.1.1. HPLC-mass analysis for the determination of phytonutrients

*2.1.1.1. Phytonutrient extraction stage from golden tomato juice.* The extraction of phytonutrients has required a series of preliminary tests carried out in both tetrahydrofuran (THF) and dichloromethane/methanol at a 1/1 ratio, purchased from the company Merck-life Science. The solvents tested for the extraction, dichloromethane/methanol at a 1/1 ratio and tetrahydrofuran, were chosen the first for its medium polar characteristics, while the second was used because it allows the immediate and complete dissolution of the components involved. Based on the recoveries of the substances, tetrahydrofuran was preferred.

*2.1.1.2. Chromatographic analysis of phytonutrients in golden tomato juice.* Chromatographic separation was achieved on Luna PFP(2) (150 × 2.0 mm, 3 μm) column equipped with precolumn, purchased from the Phenomenex company, with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B).

A gradient method at 400 μL/min flow rate was applied as follows: start at 60% B, stay for 2 min; increase to 100% B over 8 min, held for 7 min; then decrease to 60% B over 2 min; maintained constant for 3 min. for a total run time of 20 min. Injection volume was 1 μL. A full mass/targeted SIM (t-SIM) scan methods were applied. Phytonutrients were analyzed by LC-MS/MS using instrumentation: Q-Exactive LCq/Orbitrap MS (ThermoFisher Scientific), interfaced with UHPLC Ultimate 3000 RS (Dionex) in ESI (Electrospray Ionization), in T-SIM positive ion/negative ion mode. The Orbitrap parameters were set as follows: alternate switching (-)/(+) ESI full scan mode and t-SIM, sheath gas flow rate 30 AU, discharge voltage 3.5 kV, capillary temperature 300 °C, resolution 35,000 FWHM, AGC target $5x10^6$, maximum injection time 200 ms and scan range 100–1000 *m/z*.

### 2.2. Animals and diet composition

Male Wistar rats were provided by Envigo S.r.l (Indianapolis, Indiana). They were housed two per cage and maintained on a 12 h on/off cycle (8:00–20:00 h) at a constant temperature (22–24 ∘C) and humidity (50 ± 10%). During the acclimation period of seven days, animals were first fed with a standard chow diet providing 3.94 kcal/g and then divided into two homogenous groups with balanced weight (7 week-old, n = 20, weighing 240 ± 13.04 g). These groups were fed with standard laboratory food (code PF1609, certificate EN 4RF25, Mucedola, Milan, Italy), or fed with HFD food with 60% of energy coming from fats (code PF4215-PELLET, Mucedola, Milan, Italy), whose detailed characteristics are described in our previous papers(Di Majo et al., 2022; Gambino et al., 2023). The control group (NPD: normal pelletized diet) was fed with a low-fat diet represented by a standard rat chow (3.94 kcal/g, 55.50% carbohydrate, 22% protein, 3.50% lipid, 4.50% fiber). The second experimental group (HFD: high-fat diet) was fed with a hypercaloric pelletized diet (5.5 Kcal/g) consisting of 34% fat, 23% protein, 38% carbohydrates and 5% fiber, until MetS was induced and assessed

following criteria already established by previous literature (Gambino et al., 2023; Giammanco et al. 2016; Rodríguez-Correa et al., 2020). All rats had free access to food and water. Animal care and handling throughout the experimental procedures were in accordance with the ARRIVE guidelines and the European Directive (2010/63/EU). The experimental protocols were approved by the animal welfare committee of the University of Palermo and authorised by the Ministry of Health (Rome, Italy; Authorization Number 14/2022-PR).

#### 2.2.1. Induction of MetS and experimental groups

The experimental timing of the study has been subdivided into three points: T0 (starting point), T1(induction of MetS) and T2 (end of the experiment).

1) At the starting point (T0), after the acclimatation period, animals were subdivided in NPD or HFD on the basis of the diet administered for 8 weeks until induction of MetS.
2) At T1, MetS induction was verified according to criteria widely employed in literature(Di Majo et al., 2022; Rodríguez-Correa et al., 2020), considering increased triglyceride levels, body weight gain and glucose tolerance with respect to normally-fed rats. Then, animals were divided into 3 groups based on the type of diet (NPD and HFD) and treatment administered (golden tomato juice or vehicle) from T1 until T2, which was reached 4 weeks after T1. In particular, the normal control was fed with a normal diet (NPD) until T2 and the second one (HFD group), representing the MetS control, was fed with the HFD throughout the study (from T0 to T2). The control NPD (n = 6) and HFD (n = 6) groups were subjected to the same stress conditions as the treated group, since they received during the last month of the experiment, from T1 to T2, a volume of vehicle (water) by oral gavage equal to the tomato solution administered to the treated groups. Lastly, one group (HFD/GT, n = 8) was treated with GT juice in the last month of the trial (T1-T2).
3) T2 is the end of the experimental study that occurs 12 weeks after the starting point (T0).

#### 2.2.2. Nutritional supplementation with GT

GT juice was administered by oral gavage for one month at a daily dose of 2 mL/kg body weight during the last month of the study (T1-T2).

### 2.3. Behavioural paradigms

All the paradigms, except for burrowing and saccharin preference, were assessed using a behavioral tracking software (AnyMaze, Version 7.20) and evaluated by an expert scientist blind to the experimental groups.

#### 2.3.1. Exploratory behaviour and reactivity

*2.3.1.1. Open field test.* The open field test (OFT) was used to determine locomotor and exploratory activity of the animals. The animals were placed in a square open field (70x70cm) facing the wall from a distance of 10 cm and were then recorded for 10 min. Activity parameters recorded were: total distance travelled, immobility time, time spent in the centre zone and number of entries in the zone.

*2.3.1.2. Burrowing test.* The burrowing test was carried out as previously described(Seiffert et al., 2019). Plastic burrowing tubes (32 cm long × 10 cm Ø, elevated on one site by 6 cm) were filled with 2.5 kg gravel (quartz-light, grain size 2–4 mm) and placed in the cage. Every rat was tested separately. All rats received a training prior to the assessment of baseline performance (T0). On day 1 of the training phase, rats received an empty burrowing tube for 60 min; on day 2–4, rats received a gravel-filled tube for 60 min after a habituation time of 30 min in the empty cage. Burrowing behavior was tested on day 5 (baseline, T0), at T1 and T2. As soon as the burrowing tube was placed in the cage the latency time to start burrowing was measured. The remaining gravel in the tubes was weighed and the difference to the initial value was calculated to determine the displaced gravel (amount burrowed).

#### 2.3.2. Anxiety-associated and anhedonia-associated behaviour

*2.3.2.1. Novelty-suppressed feeding test.* In the novelty-suppressed feeding test (NSFT), after a 24-hour food deprivation, rats were exposed to an open-field maze where a food pellet (2 g of HFD or NPD chow) was positioned in a white rounded disc at the center of the arena. Animals had a 10-minute cut-off to reach food and eat it, only rats that completed the test were taken into consideration(Blasco-Serra et al., 2017). The feeding time was monitored so that, once rats completed the test, they were removed from the arena and brought back to their home cage. In the home-cage the amount of food consumed by animals in the following 10 min was weighed in order to verify if all the rats completed the test in the deprived conditions.

*2.3.2.2. Black-white box test.* A plastic box (60 cm length × 30 cm height × 30 cm width) divided into two equal-size compartments connected through a poorly illuminated tunnel was used for the black-white box test (BWB). One of the compartments was painted black and was covered with a lid and the other compartment was painted white and illuminated with a 45 W light bulb positioned 40 cm above the box (Frinchi et al., 2020). The animals were placed in the white compartment facing the tunnel. The parameters evaluated for five minutes were number of entries and the time spent in the light zone.

*2.3.2.3. Saccharin preference test.* The saccharin preference test was performed in accordance with a protocol described in(Di Liberto et al., 2018). Two water bottles were filled with 500 g of the solutions Initially, we evaluated water intake from both bottles over 24 h in order to assess a putative side preference. Next, one of the two bottles provided was filled with 0.1% saccharin solution (Saccharin ≥ 98%, Sigma-Aldrich) and the intake was determined for another 24 h. At the third day both bottles were filled again with regular water and at the fourth day the saccharin-containing solution was provided in the bottle on the other side.

#### 2.3.3. Learning and Memory: Object recognition test

This Object Recognition test (ORT) is considered a reliable and widely used paradigm of learning and memory by evaluating the declarative memory system in experimental models(Plescia et al., 2014; Squire & Zola, 1996). Declarative memory system is implicated in the recognition of novel stimuli in a non-familiar environment. To this purpose, rats were first habituated to the arena (open field maze with the same characteristics as in 2.3.1.1). On the test day, animals were exposed to two identical objects at a fixed distance from each other. After 1 h retention, one of the two objects was substituted with a novel one, while the other remained the same ("familiar object"). The objects were chosen and 3D printed according to specific literature on this paradigm (Inayat et al. 2021). For the evaluation of ORT we took into consideration the retention index (RI%), i.e. the time spent exploring the novel object with respect to the total time spent to investigate both the familiar and novel one. This parameter allows to assess the animal's ability to discriminate between a novel and a familiar stimulus after one hour from the exposure to the familiar ones, as main index of retention (Botton et al., 2010).

### 2.4. Analyses of biochemical parameters

#### 2.4.1. Tissue preparation and protein extraction

At the end of the behavioral testing, rats were sacrificed by decapitation to collect blood and to quickly remove the brain. The left

hemisphere was immediately frozen in cold isopentane for histological analysis, while the right hemisphere was dissected under stereomicroscopy with refrigerate support in order to sample the hippocampus, the prefrontal cortex and hypothalamus. Sampled tissues were processed for protein extraction, as previously described(Di Liberto et al., 2017). Briefly, tissues were homogenized at 4 °C in cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton, SDS 0.1%), supplemented with protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich P5726). Samples were sonicated (30 pulsations/min), quantified by the Lowry method (Lowry et al. 1951), and stored at −80 °C for western blotting analyses.

#### 2.4.2. Western blotting

Western blotting was performed as previously described(Di Liberto et al., 2011). Protein samples (50 μg) and molecular weight marker (PageRulerTM Plus Prestained Protein Ladder, 26619 ThermoFisher Scientific), were run on 10% polyacrylamide gel and electrophoretically transferred onto nitrocellulose membrane (RPN303E, Hybond-C-extra, GE Healthcare Europe GmbH). The membranes were incubated for 1 h in blocking buffer (1 × TBS, 0.1% Tween–20, 5% w/v nonfat dry milk) and incubated with gentle shaking overnight at −4 °C with specific antibodies in blocking buffer. The following antibodies were used: anti-phosphorylated (p) Erk1/2 antibody (Rabbit phospho-p44/42 MAPK, Thr202/Tyr204 antibody 1:1000; 9101 Cell Signaling), or anti-pAkt (rabbit phospho-Akt (Ser473) antibody, 1:1000; 9271 Cell Signaling). The day after, membranes were washed for three times for 5 min with TBS/T, and incubated for 1 h at room temperature with goat anti-rabbit IgG (sc-2357 Santa Cruz Biotechnology) horseradish peroxidase-conjugated diluted 1:10,000. For normalization of the densitometric signal, membranes were exposed to horseradish peroxidase-conjugated β-Actin primary antibody (sc-2005 Santa Cruz Biotechnology), diluted 1:10,000, for 1 h. After three washings with TBS-T, immunocomplexes were visualized with chemiluminescence reagent (SuperSignalTM West Pico PLUS, ThermoFisher Scientific) by using iBright FL1000 Imaging System (ThermoFisher Scientific). The densitometric evaluation of bands was performed by measuring the optical density (O.D.) using NIH ImageJ software.

### 2.5. Statistical analyses

Statistical analysis was performed by using GraphPad Prism 9.02 (San Diego, CA, USA). Behavioural and biochemical parameters were compared by a one-way ANOVA test followed by Bonferroni post hoc evaluations for differences between means and represented by scattered bar graphs. Differences were considered significant when $p < 0.05$. The statistical power (g-power) was considered only if $> 0.75$ and the effect size if $> 0.40$. The results are presented as the mean ± standard error of the mean (S.E.M.).

## 3. Results

### 3.1. Phytonutrient composition of golden tomato juice

Analyses of the GT juice reveal different amounts of phytonutrients compared with the fresh fruits of golden and red tomatoes. Statistical values obtained by one-way ANOVA comparing data from GT juice versus GT fresh fruits and vs RT fresh fruits are provided in Table 1. The phytonutrient values shown in the table are expressed as the mean and standard deviation of three repetitions performed on three samples of the same tomato (red and golden), cultivar Brigade, from the same farm and harvested at the same time. In the case of GT juice, these are analyses performed in triplicate on three different bottles, prepared under the same conditions and on the same day. Of interest is the finding regarding rutin (quercetin-3-O-rutinoside), which, compared to the other phytonutrients, is present in significantly higher concentrations in the GT juice than in both fresh golden and red tomatoes. As can be seen in the table, 100 g of juice possesses 88% more rutin than 100 g of fresh golden tomato and 58% more than 100 g of red tomato, respectively. It can be hypothesized that during the process of tomato juice production the formation of rutin is activated from its precursors as it occurs during the ripening stage of tomato(Capanoglu et al., 2012). Furthermore, significant differences emerged in the lycopene and β-carotene concentrations comparing GT juice with GT and RT fresh tomatoes. The concentration of naringenin, lutein and phytoene resulted non-significant (n.s.) between groups.

**Table 1**
Phytonutrients present in 100 g of golden tomato juice and in 100 g of the fresh fruits (red and golden tomato).

| Phytonutrients | mg/100 g of gold tomato juice | mg/100 g of fresh golden tomato | mg/100 g of fresh red tomato | Statistical Values |
|---|---|---|---|---|
| Lycopene | 1.24 ± 0.52 | 16.65 ± 2.78 | 98.55 ± 3.56 | $F_{(2,8)} = 1191$; $p < 0.0001$ |
| β-carotene | 5.97 ± 0.76 | 1.95 ± 0.97 | 6.31 ± 1.25 | $F_{(2,8)} = 17.18$; $p = 0.0033$ |
| Rutin | 1.97 ± 0.04 | 0.234 ± 0.05 | 0.35 ± 0.01 | $F_{(2,8)} = 2018$; $p < 0.0001$ |
| Naringenin | 2.29 ± 0.84 | 1.92 ± 1.02 | 0.83 ± 0.05 | n.s. |
| Lutein | 0.42 ± 0.03 | 0.39 ± 0.07 | 0.32 ± 0.02 | n.s. |
| Phytoene | 0.78 ± 0.01 | 0.32 ± 0.01 | 0.84 ± 0.03 | n.s. |

### 3.2. Exploratory behaviour and reactivity

#### 3.2.1. Open field test

Animals were tested in an open field arena in order to assess their behavioural reactivity and exploratory behaviour. As for total distance travelled, one-way ANOVA followed by Bonferroni post-hoc test highlighted a significant increase in HFD/GT group vs HFD, and not different versus NPD ($F_{(2,17)} = 11.65$, $p = 0.0007$, Fig. 1A). The evaluation of immobility time outlined that HFD rats spend more time motionless than NPD, but this is rescued in HFD/GT group that returns to basal values of NPD ($F_{(2,17)} = 12.96$, $p = 0.0004$, Fig. 1B). Lastly, time spent at the center of the maze was higher in HFD/GT group versus HFD and not different versus NPD ($F_{(2,17)} = 11.61$, $p = 0.0007$, Fig. 1C) and also the number of entries at the center ($F_{(2,17)} = 6.84$, $p = 0.0066$, Fig. 1D).

#### 3.2.2. Burrowing test

Natural rodent burrowing behavior was significantly affected by GT supplementation in MetS. Indeed, HFD/GT rats burrowed significantly more gravel compared to HFD and resulted not-significantly different from NPD group ($F_{(2,17)} = 4.482$, $p = 0.0273$, Fig. 2A). In addition, the time until onset of first burrowing proved to be prolonged in HFD animals but this latency was reduced by GT supplementation ($F_{(2,17)} = 11.96$, $p = 0.0006$, Fig. 2B).

### 3.3. Anxiety-associated and anhedonia-associated behaviour

#### 3.3.1. Novelty-suppressed feeding test

After an overnight food deprivation, rats underwent the novelty-suppressed feeding test in an open-field maze for evaluation of depression and anxiety. Only 4 out of 6 HFD rats managed to complete the NSFT. The evaluation of the latency to eat revealed that GT consumption ameliorated the feeding time versus HFD and was not different than NPD ($F_{(2,15)} = 7.38$, $p = 0.005$, Fig. 3A). Lastly, the evaluation of in-cage parameter, i.e. the amount of food eaten in the following 10 min, showed that no differences were encountered between HFD and NPD. However, only HFD/GT group consumed more food once back in the cage with respect to NPD ($F_{(2,15)} = 6.46$, $p = 0.0095$, Fig. 3B).

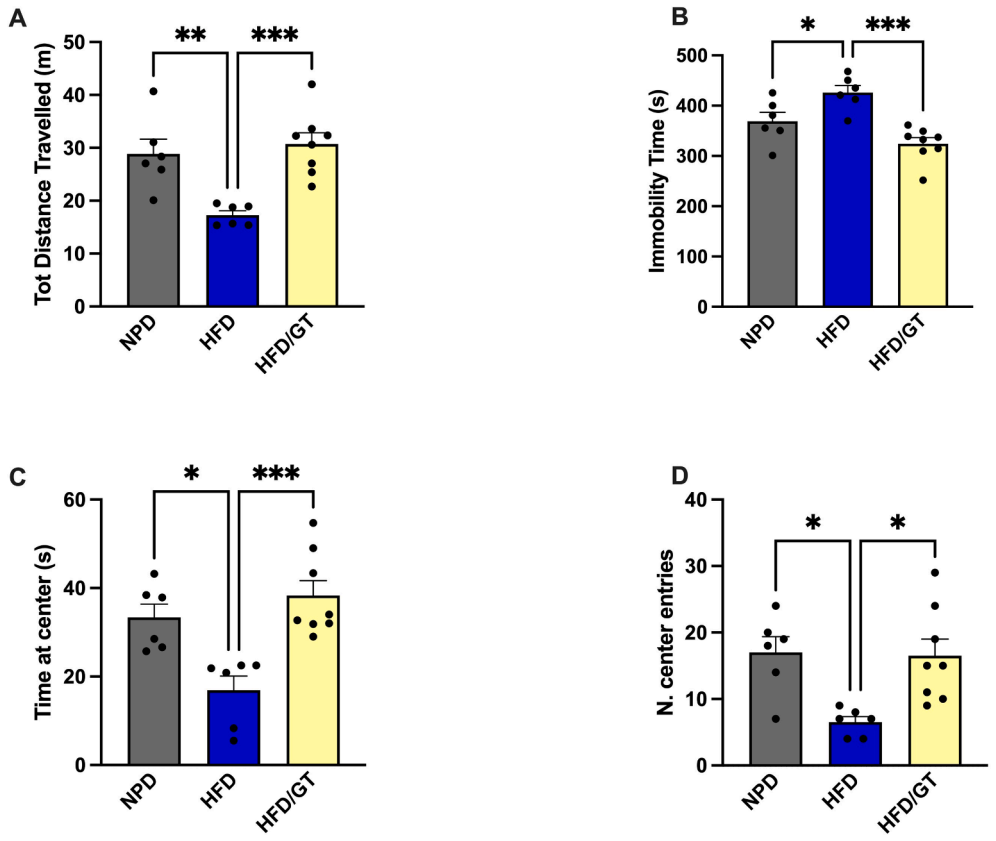


**Fig. 1.** Behavioural parameters assessed in the Open Field Test in NPD (n = 6), HFD (n = 6) and HFD/GT (n = 8) groups. **A.** Total Distance Travelled (m). **B.** Immobility time (s). **C.** Time spent in the center zone (s). **D.** Number of entries in the center zone.*p < 0.05 **p < 0.001, ***p < 0.0001.

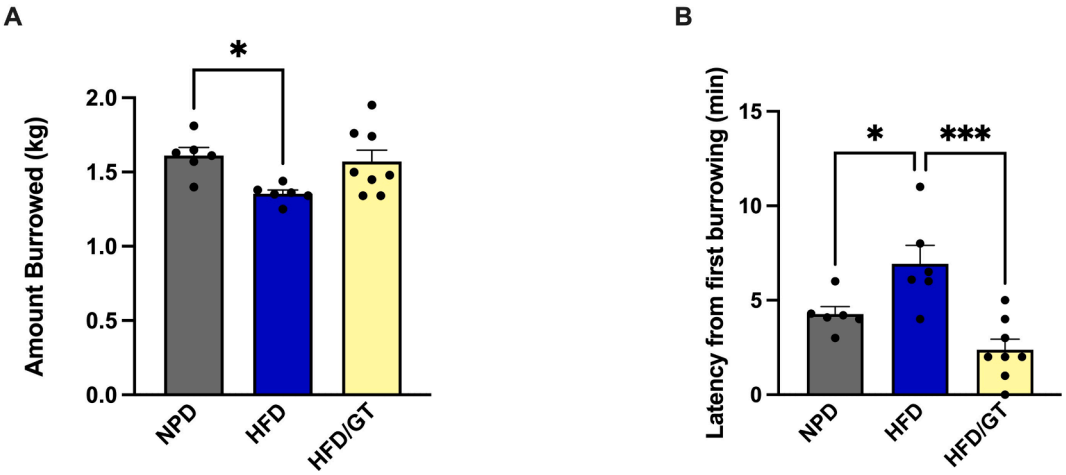


**Fig. 2.** Burrowing behaviour assessed in the NPD (n = 6), HFD (n = 6) and HFD/GT (n = 8) rats. **A.** Amount of gravel burrowed (Kg) **B.** Latency from first burrowing (min). *p < 0.05 and ***p < 0.0001.

### 3.3.2. *Black-white box*

The behavioural parameters evaluated in this paradigm revealed that GT nutritional supplementation improved the anxiety-associated behaviour of HFD rats. Indeed, even though the number of entries in the light zone was significantly reduced in HFD vs NPD ($F_{(2,17)} = 4.05$, $p = 0.036$, Fig. 3C) but was not rescued in HFD/GT, the time spent by HFD/GT animals in the light zone was markedly higher that HFD and not different versus NPD ($F_{(2,17)} = 8.12$, $p = 0.0033$, Fig. 3D).

### 3.3.3. *Saccharin preference*

No side preference emerged from the evaluation of water intake. Statistical differences in the consumption of saccharin were revealed in the experimental groups, supporting the idea of anhedonia-associated behaviour induced by HFD. In detail, both HFD and HFD/GT assumed less saccharin with respect to NPD, suggesting that GT does not manage to rescue ($F_{(2,17)} = 6.79$, $p = 0.0068$, Supplementary Figure).

## 3.4. *Learning and Memory: Object recognition test*

The Object recognition Test was employed to assess declarative memory by means of recognition of novel objects from familiar ones. Rats were first exposed to training with two identical objects and

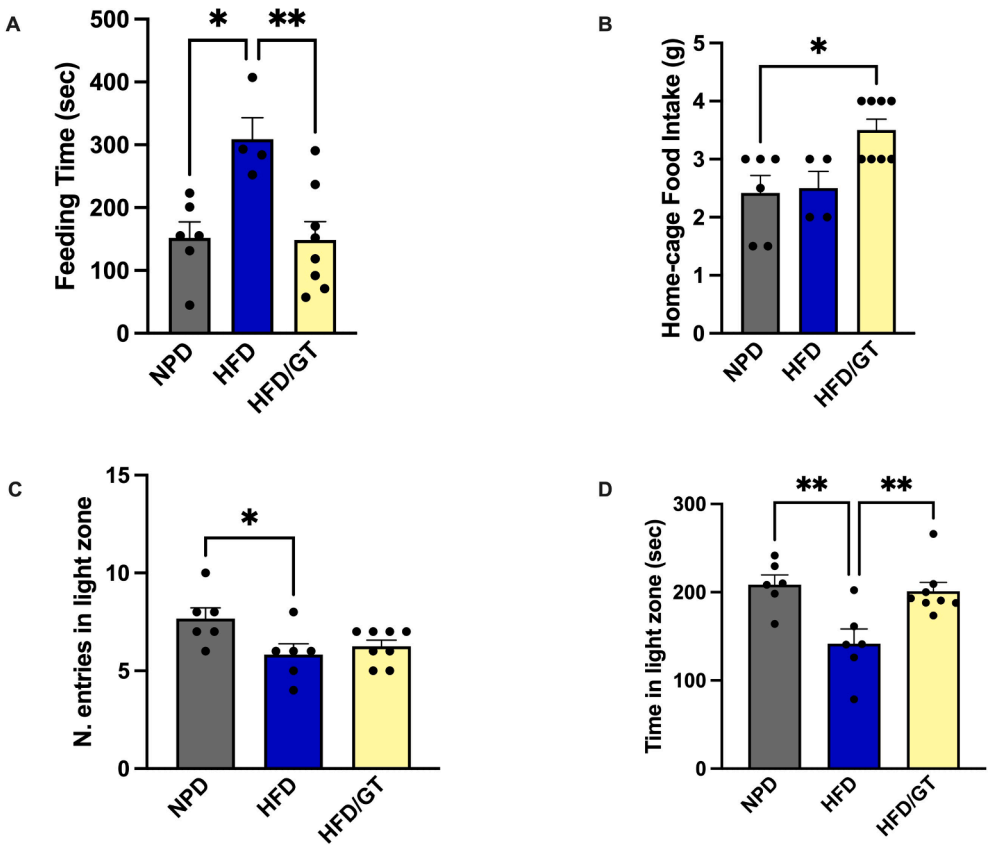


**Fig. 3.** Parameters of the novelty-suppressed feeding test (A,B) and dark-white box (C,D) in the NPD (n = 6), HFD (n = 6) and HFD/GT (n = 8) rats. **A.** Latency from feeding (feeding time, sec) **B.** Amount of food consumed back in the home-cage (food-intake, g). **C.** Time spent in the light zone (sec) **D.** Number of entries in the light zone. *p < 0.05 and **p < 0.001.

statistical analyses test did not show any significant variation in the recognition index, which was approximately 50% for all experimental groups as expected (Fig. 4A). After 1-hour retention, animals were exposed to a novel object and a familiar one. One-way ANOVA followed by Bonferroni post-hoc test unveiled that RI% of HFD/GT rats was higher than HFD alone and not significantly different from NPD ($F_{(2,17)}$ = 9.71, p = 0.0015, Fig. 4B). Indeed, HFD rats did not manage to recognize the novel stimulus and spent more time in the familiar one (RI %<50%) with respect to NPD (p < 0.05).

### 3.5. Modulation of PI3K/Akt and MAPK/ERK signaling

#### 3.5.1. Modulation of PI3K/Akt and MAPK/ERK signaling in the hippocampus

In the hippocampal tissue, we observed a significant modulation of the PI3K/Akt signaling. HFD was associated with a significant reduction in p-Akt levels, counteracted by GT consumption ($F_{(2,17)}$ = 22.46, p < 0.0001, Fig. 5A). On the contrary, no significant changes in p-ERK1/2 levels between the different experimental groups were detected ($F_{(2,17)}$ = 0.09117, p = 0.9133, Fig. 5B).

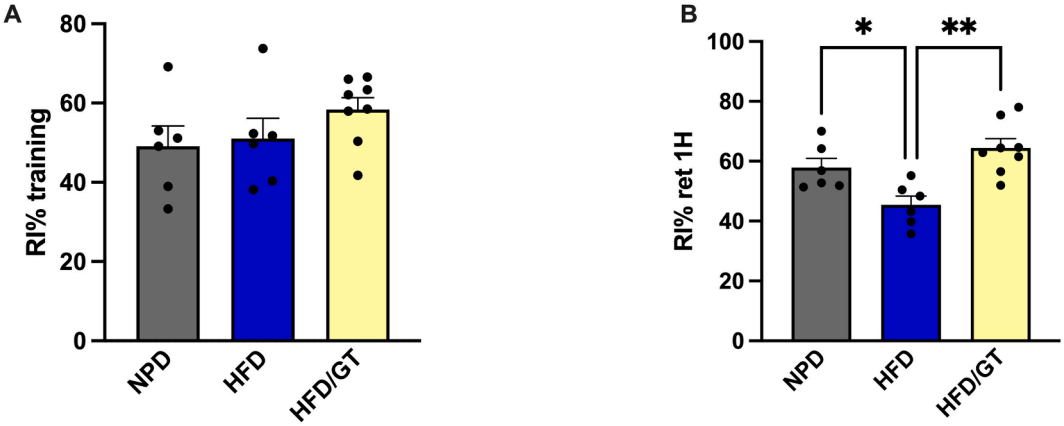


**Fig. 4.** Object recognition test evaluated in the NPD (n = 6), HFD (n = 6) and HFD/GT (n = 8) groups. **A.** Recognition index (RI%) during training phase **B.** RI% after 1-hour retention (ret 1 h). *p < 0.05 and **p < 0.001.

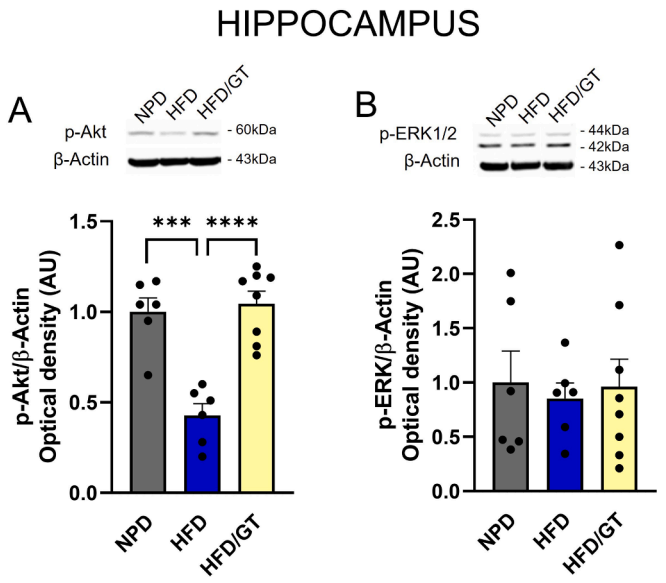


**Fig. 5.** Modulation of Akt and ERK1/2 activation by GT supplementation in the hippocampus. **A.** Representative images of phosphorylated (p)-Akt and β-Actin Western blotting bands and histogram of p-Akt normalized to β-Actin Optical density in NPD, HFD and HFD/GT hippocampus. **B.** Representative images of phosphorylated (p)-ERK1/2 and β-Actin Western blotting bands and histogram of p-ERK1/2 normalized to β-Actin Optical density in NPD, HFD and HFD/GT hippocampus. ***$p < 0.001$, ***$p < 0.0001$.

### 3.5.2. *Modulation of PI3K/Akt and MAPK/ERK signaling in the prefrontal cortex*

In the prefrontal cortex, neither HFD nor GT consumption significantly modified p-Akt levels with respect to NPD group $F_{(2,17)} = 0.4513$, $p = 0.6442$, Fig. 6A). On the contrary, MAPK/ERK signaling was significantly affected by GT consumption in MetS $F_{(2,17)} = 6.051$, $p = 0.0104$, Fig. 6B). Indeed, GT consumption reversed HFD-induced increase in the levels of phosphorylated ERK1/2.

### 3.5.3. *Modulation of PI3K/Akt and MAPK/ERK signaling in the hypothalamus*

Similarly to the prefrontal cortex, neither HFD nor GT consumption significantly modified p-Akt levels with respect to NPD group $F_{(2,17)} = 0.3722$, $p = 0.6947$, Fig. 7A) in the hypothalamus. Conversely, MAPK/ERK signaling was significantly modulated by GT consumption in the hypothalamus $F_{(2,17)} = 9.974$, $p = 0.0014$, Fig. 7B). Indeed, GT consumption counteracted HFD-induced increase in the levels of phosphorylated ERK1/2.

## 4. Discussion

Neuroprotective effects of nutritional supplementation with GT juice emerged in our model of MetS induced after 8 weeks of HFD.

To begin with, we presented the quali-quantitative composition in terms of phytonutrients encompassed in GT juice, as a food matrix with specific characteristics that differ from GT and from RT fresh extracts, as recently characterized (Gambino et al., 2023). As shown by HPLC-mass, only one phytonutrient is present in increased quantity in GT juice with respect to fresh fruits, therefore we assume that it could be responsible for GT juice specific effects, i.e. rutin. Indeed, rutin and its metabolites are able to cross the blood–brain barrier affecting behavioral and cognitive aspects in neurodegenerative disorders("The Beneficial Role of Rutin, A Naturally Occurring Flavonoid in Health Promotion and Disease Prevention: A Systematic Review and Update," 2019). However, it is undeniable that the high levels reported of β-carotene, naringenin and lycopene could be implicated as well in the putative neuro-active effects of GT juice.

As for behavioural investigation, rats were first exposed to OFT to evaluate their general locomotor activity and behavioural reactivity. As expected, HFD rats moved less than normally-fed ones and spent much less time in the center zone of the maze, which confirms that their exploratory behaviour was severely impaired by the increased body weight and dysmetabolism (Nesci et al., 2020; Vieira et al., 2023). Whereas, HFD rats chronically supplemented with GT juice enhanced their ability to explore the open field arena by increasing the amount of distance travelled, staying less time motionless and spending more time in the center zone, similarly to control rats.

The evaluation of burrowing behaviour also revealed that GT juice

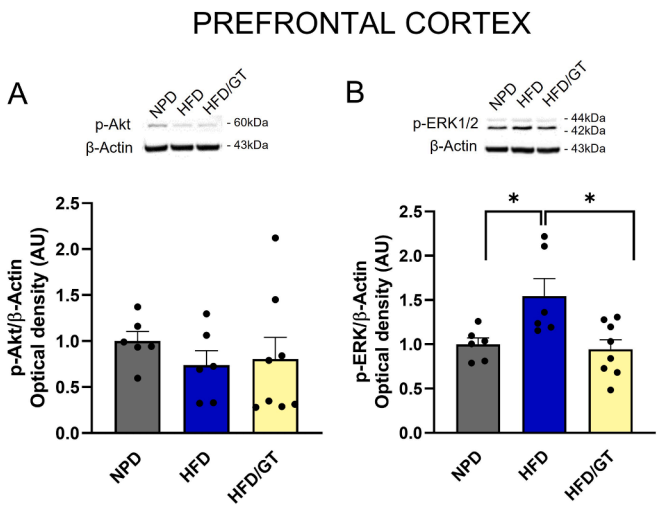


**Fig. 6.** Modulation of Akt and ERK1/2 activation by GT supplementation in the prefrontal cortex. **A.** Representative images of phosphorylated (p)-A and β-Actin Western blotting bands and histogram of p-Akt normalized to β-Actin Optical density in NPD, HFD and HFD/GT prefrontal cortex. **B.** Representative images of phosphorylated (p)-ERK1/2 and β-Actin Western blotting bands and histogram of p-ERK1/2 normalized to β-Actin Optical density in NPD, HFD and HFD/GT prefrontal cortex. *$p < 0.05$.

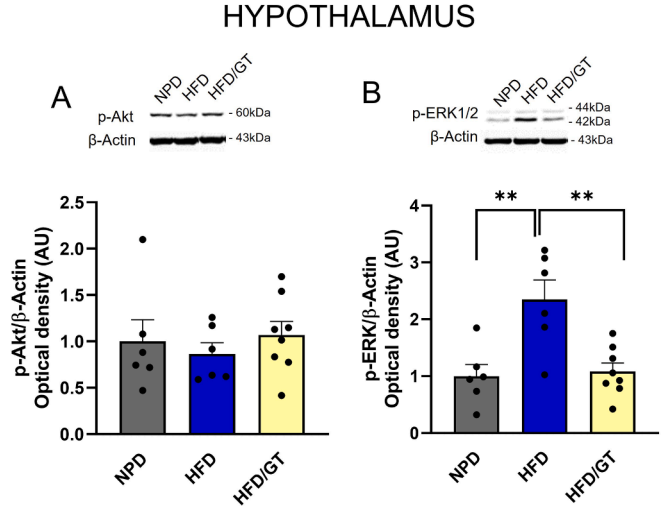


**Fig. 7.** Modulation of Akt and ERK1/2 activation by GT supplementation in the hypothalamus. **A.** Representative images of phosphorylated (p)-Akt and β-Actin Western blotting bands and histogram of p-Akt normalized to β-Actin Optical density in NPD, HFD and HFD/GT hypothalamus. **B.** Representative images of phosphorylated (p)-ERK1/2 and β-Actin Western blotting bands and histogram of p-ERK1/2 normalized to β-Actin Optical density in NPD, HFD and HFD/GT hypothalamus. **$p < 0.01$.

was able to restore a physiological behaviour like NPD group, as in the OFT, since HFD/GT rats showed a lower latency to begin the test versus HFD rats (Fisher et al., 2023; Lippi et al., 2023). Several studies already reported that after obesogenic procedures animals have a lower innate activity that could account for a reduced overall energy expenditure (Mort et al., 2023). The results obtained in enhancing locomotion in HFD/GT rats could be ascribed to an improved body mass, but also to a higher motivation to explore the surrounding novel environment.

Among MetS-induced brain impairment, it has been reported an alteration of anxiety-related and depressive-like behaviours(Gancheva et al., 2017; Rebolledo-Solleiro et al., 2017). Data from NSFT evidenced that GT supplementation in HFD rats allowed to restore a normal latency to feed after 24-hour deprivation in a novel environment, thus reverting the ability of the HFD to determine deleterious effects in this paradigm. A complete recovery of performance was detected also in the BWB test, showing that HFD/GT rats spent markedly more time in the light zone that represents an aversive environment. Both NSFT and BWB tests are strongly related to anxiogenic-like symptoms(Zemdegs et al., 2016), thus suggesting that GT juice nutritional supplementation constitutes an effective procedure in reversing HFD-induced depressive-like phenotype. However, outcomes from saccharin preference test were not in accordance with the previous ones, since we did not find noticeable differences after GT consumption in HFD rats. Indeed, HFD was reported to reduce preference for sweet solutions, in line with an augmented anhedonia which is typically considered a core reward symptom of major depression(Rabasa et al., 2016). Thus, our results support a protective activity of GT juice on depressive-like phenotype though still not able to modulate HFD-induced reward hypofunction, as demonstrated by some degree of anhedonia in the saccharin preference test. The eventual effect of GT on saccharine preference test could have also been masked by an alteration of palatability of sweet solutions.

An explanation for the observed modulation of anxiogenic-related behaviours by GT juice could lie in glucocorticoid levels since they are considered mediating factors for MetS-boosted cognitive decline(Hendrickx et al., 2005; Reagan, 2005), by an increased hypothalamic–pituitary–adrenal axis activity (Giammanco et al., 2018). In this context, it could be hypothesizeable that GT oral administration have modulated leptin secretion and circulating levels by modifying adipose tissue distribution and mass, though this hypothesis should be further assessed in future studies. Circulating leptin levels, indeed, have been found to compensate glucorticoid levels(Bocarsly et al., 2015) and could thus be responsible for an improved response to stress and anxiety. Consistently, the activation of inflammatory processes in HFD rodent models negatively affects the anxiogenic/depressive phenotype(Zemdegs et al., 2016). HFD has indeed been compared to what observed in various animal models of depression(David et al., 2009) in terms of decrease in local levels of brain-derived neurotrophic factor and related neurogenesis(Lindqvist et al., 2006; Park et al., 2010) or synaptodendritic connections(Arnold et al., 2014).

A further goal of our study was to unveil whether GT juice was able to impact on HFD detrimental effects on memory system, by exploring particularly declarative memory network through an OR task already employed in obesity models (Bocarsly et al. 2015). The data obtained revealed that HFD rats did not manage to recognize novel stimuli when presented after 1-hour exposure from familiar ones, whereas HFD rats treated with GT juice noticeably recovered their recognition index as normal controls. In detail, all rats spent the same amount of time in the investigation of the two identical objects during the training phase. During the 1-hour retention phase, only HFD/GT and NPD rats versus HFD rats displayed an increased RI that was higher than 50%, indicating a marked preference on the exploration of the novel object with respect to the time spent on the familiar one.

This test assesses declarative memory, the conscious non-spatial memory for facts and events(Ennaceur & Delacour, 1988; Squire & Zola, 1996). The dorsal hippocampus and the connected hierarchical networks including the perirhinal cortex, but also medial prefrontal cortex, are the main structures that are crucial novelty detector that integrate, encode and compare previously stored information with new incoming environmental stimuli(Chao et al., 2022; Clarke et al., 2010; Giglia et al., 2021; X. Lin et al., 2021; Sun et al., 2020). Thus, the higher preference for novelty displayed by HFD/GT group after 1 h retention interval could be due to a facilitation in the activity of these networks, as demonstrated by previous evidence on metabolic syndrome, diabetes and obesity that are correlated with altered cognitive tasks together with reducing synaptic markers and increasing microglia activation (Bocarsly et al., 2015; Gambino et al., 2020a; Hao et al., 2016; Lee & Yau, 2020).

With a view to get insight on possible molecular mechanisms involved in the neuro-active effects of GT, we investigated MAPK and PI3K signaling pathway activation in hippocampus, hypothalamus and prefrontal cortex. Activation of MAPKs induces adipose tissue inflammation and insulin resistance in obesity(Wen et al., 2022) and plays a key role in the neuroinflammatory response triggered by glial cells during brain pathological states; on the other hand, the MAP-ERK pathway participates in neuroplastic events, including long-term potentiation(Albert-Gascó et al., 2020). In this study, we unravel for the first time the upregulation of the MAPK-ERK pathway in the hypothalamus and in the prefrontal cortex of MetS rats, likely linked to the inflammatory processes that occur, both systemically and in the brain of HFD rats(Gambino et al., 2023; Jais & Brüning, 2017). Usually, under MetS conditions, visceral fat produces proinflammatory cytokines which can cross the blood–brain barrier, inducing neuroinflammatory phenomena(Lauridsen et al., 2017). In the hypothalamus, a key brain region for the regulation of energy homeostasis, the activation of inflammatory pathways leads to the uncoupling of caloric intake and energy consumption, and contributes to the development of obesity-associated insulin resistance and alterations of glucose metabolism(Bhusal et al., 2021; Jais & Brüning, 2017). Similarly, both the hippocampus and the prefrontal cortex of MetS rats are characterized by high levels of neuroinflammatory biomarkers(Treviño et al., 2022), which contribute to the development of cognitive deficits. The consumption of GT in HFD rats, which reduces the increase in MAPK-ERK activation, likely counteracts the detrimental brain vicious cycle between oxidative stress and inflammation, as already systematically assessed(Gambino et al., 2023), being involved in the recovery of behavior and cognitive functions described in this study.

Similarly to MAPK/ERK, dysregulated PI3K/Akt pathway leads to obesity and type 2 diabetes as the result of insulin resistance, and in turn, insulin resistance exacerbates the PI3K/Akt pathway dysfunction (Huang et al., 2018). The PI3K family is involved in the regulation of several physiological processes including cell growth and survival, synaptogenesis and differentiation, autophagy, chemotaxis, glucose and lipid metabolism, and its dysregulation leads to the development of neurodegeneration, obesity and obesity-related complications(Savova et al., 2023). In particular, at the brain level it was shown that this signalling pathway modifies insulin and leptin actions in the hypothalamus hence reshaping food intake homeostasis (Huang et al., 2018). In the brain of obese subjects in fact the transport of insulin through the blood brain barrier is impaired and, due to the inflammatory conditions, even insulin action is attenuated; this contributes to the development of brain insulin resistance and alterations in PI3K-Akt signaling(Huang et al., 2018). In accordance, we here describe a significant reduction in the activation of the PI3K/Akt pathway in HFD rat hippocampus.

Several reports have already documented the hippocampal alterations in MetS rats, strictly associated with deficits in cognitive functions, including impaired electrophysiological activity(Prieto-Gómez et al., 2020) and neurogenesis(Lindqvist et al., 2006), alterations in insulin signaling pathway, neurotrophic factor levels and increased apoptosis, oxidative, stress and neuroinflammation(Hersey et al., 2021; Morrison et al., 2010). In this study, GT consumption, which re-establishes the physiologic activation of PI3K/Akt signaling, likely reverses the altered hippocampal insulin signaling, as already systemically evidenced by peripheral metabolic investigations(Gambino et al., 2023).

However, since Akt signaling in the brain is involved in many other neuroplastic functions, its role in modulating neurotrophic effects in the hippocampal tissue following GT supplementation must be also considered at this stage. These preliminary biochemical investigations in the brain tissue of MetS rats seem to demonstrate the alterations of intracellular cascades associated with insulin resistance, neuroplasticity, neuronal survival and neuroinflammation, and the recovery effects associated with GT consumption (Arnold et al., 2014; Huang et al., 2018; Lee & Yau, 2020). Crucially, our biochemical outcomes perfectly fit with the GT-mediated behavioural improvement in cognitive function linked to the activity of discrete brain areas. However, further studies are necessary to characterize the differential patterns of activation of these cascades in specific brain areas, the causal relationship between them and specific brain functions, and the other effectors involved.

The composition of GT juice food matrix could be responsible for the intriguing effects emerged on cognitive alterations and associated biochemical modification at brain level. As a matter of fact, the phytonutrients present in GT juice have been individually correlated to neuroprotective mechanisms by several authors, though it cannot be underestimated the importance of the entire phytocomplex on these processes.

In particular, the flavonoid glycoside rutin was reported to be effective in vivo in hippocampal-dependent impairment of memory pathways in HFD model modulating the protein expression of key molecules implicated in metabolic pathways and synaptic trafficking (Cheng et al., 2016). Furthermore, data from a cell culture model of neurodegeneration revealed that rutin improves cell survival via regulation of Akt/AMPK signaling(Enogieru et al., 2021). According to literature reports, it exerts effects on the redox balance implicated in neuronal cell loss and removes the inflammatory component in neurodegeneration. This occurs by reducing the levels of pro-inflammatory markers tumor necrosis factor-α, interleukin (IL)-6, cyclooxygenase-2, IL-1β, and blocks the activation of nuclear factor kappa B (NF-κB)/MAPK(Habtemariam, 2016; Muvhulawa et al., 2022).

Beyond rutin, among the compounds found in GT juice that have been widely considered as cognitive-modulating, we should emphasize naringenin (Atoki et al., 2023). Foremost, this compound was evaluated in several animal models, HFD included, and was found to induce alone marked modifications in the molecular cascades mediated by Akt and MAPK-ERK, ultimately favoring neuronal survival in vitro and ameliorating cognitive dysfunction in rats(Nouri et al., 2019; Xu et al., 2015; Zhou et al., 2020). Also in an AD rat model, naringenin was able to control the PI3K/Akt/GSK-3 pathway and lower hyper-phosphorylation of Tau proteins, noticeably improving cognition (Yang et al., 2014). Additionally, naringenin binds to endogenous TRP receptors involved in many physiological processes that influence neuronal modulation and central oxy-inflammation (Gambino et al., 2022b; Gambino et al., 2020b; Liang et al., 2023; Waheed et al., 2014). Equally important for the evaluation of the behavioural effects observed after GT treatment could be the antidepressant-like activity of naringenin that has been reconducted to improved sensitivity of the receptors of norepinephrine and serotonin, to brain-derived neurotrophic factor activation and to a decrease in blood corticosterone (Atoki et al., 2023). Noteworthy, GT juice contains also β-carotene that was found to improve behavioural response and cognitive function in neurodegenerative disease models, and also to modulate metabolic disorders even at hypothalamic level (Abrego-Guandique et al., 2023; Hira et al., 2019). Last but not least, lycopene was extensively studied for its neuroprotective role in dysmetabolisms but also in neurological disorders, by modulating cascades related to oxy-inflamatory pathways, including PI3K/Akt(Chen et al., 2019; H.-Y. Lin et al., 2014; Yin et al., 2014). In the HFD rat model, lycopene acted on impaired dendritic morphology in hippocampal CA1 and consequent memory alterations(Z. Wang et al., 2016). These speculations on the role of the phytonutrients present in GT juice could support the effects observed, though it surely deserves *ad-hoc* investigations to unveil the eventual specific influence of each component.

## 5. Conclusion

The current research disclosed the phytonutrient composition and the neuro-active properties of a fascinating food product, GT juice, in a rat model of MetS. GT juice here emerged as a functional food thriving on several phytonutrients such as naringenin, rutin, lycopene and β-carotene that, taken together as a phytocomplex, exert a favourable modulating activity on brain processes. The nutritional supplementation demonstrated indeed beneficial effects counteracting the behavioural, cognitive and biochemical alterations induced by HFD in this animal model, in terms of response to anxiety, of reactivity, declarative memory and neuroinflammatory signalling pathways. Our outcomes would support further investigation on the potential of GT juice supplementation in oxi-inflammatory dysmetabolism and associated cognitive alterations also in humans. This research could thus pave the way to dietary-based approaches that exploit biologically-active components of functional foods for prevention and management of cognitive decline triggered by metabolic syndrome.

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**Informed Consent Statement**

Not applicable.

**Data Availability Statement**

The data presented in this study are available on reasonable request from the corresponding author.

## CRediT authorship contribution statement

**Giuditta Gambino:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft preparation, Writing – review & editing. **Monica Frinchi:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft preparation, Writing – review and editing. **Giuseppe Giglia:** Methodology, Investigation, Writing – original draft preparation, Writing – review and editing. **Miriana Scordino:** Investigation, Writing – review and editing. **Giulia Urone:** Investigation, Writing – review and editing. **Giuseppe Ferraro:** Writing – review & editing, Supervision, Resources, Investigation. **Giuseppa Mudò:** Writing – review & editing, Supervision, Resources, Investigation. **Pierangelo Sardo:** Writing – review & editing, Supervision, Resources, Investigation. **Danila Di Majo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Conceptualization. **Valentina Di Liberto:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105964.

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