

Special Issue Reprint

Nutraceuticals and Human Health and Disease

Edited by
Francesca Oppedisano

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Nutraceuticals and Human Health and Disease

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Guest Editor

Francesca Oppedisano



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Editorial

Nutraceuticals and Human Health and Disease

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A “possible beneficial role” for health is indicated by the coordinated and integrated activity of biologically active chemicals and nutritional components, as nutraceuticals [1]. Nutraceuticals are derived from either plant or animal sources, and, currently, researchers around the world are endeavoring to completely elucidate their mechanism of action, safety, and effectiveness by supporting their role with clinical data [1].

Nutraceuticals and functional foods can reduce the risk of the onset and worsening of numerous pathologies by acting in parallel with pharmacological therapy, as adjuvants in case of treatment failure, or in situations in which pharmacological therapy cannot be used.

Numerous studies have documented in the literature that natural compounds can affect the expression or activity of proteins and enzymes involved in different cellular and, specifically, mitochondrial functions. This interaction can have beneficial effects on altered metabolic pathways in a variety of pathologies [2,3].

In the Southern Italian region, the Mediterranean diet (Md), which is considered to be a part of the “intangible cultural heritage of humanity”, is linked to better health, particularly in terms of lower rates of diabetes mellitus, cancer, and—most significantly—coronary heart disease [4]. Specifically, Md has a positive impact on the development and progression of heart disease due to the decreased consumption of red meat and increased consumption of plant-based foods and olive oil [4]. For example, bergamot (*Citrus bergamia* Risso et Poiteau), thanks to its high concentration of polyphenols, is used in nutraceutical integration in numerous pathologies. BPF, obtained from the juice and albedo of bergamot, is a fraction rich in polyphenols known for its protective effects on human health, demonstrated through its antioxidant, anti-inflammatory, hypolipidemic, and hypoglycemic effects [4]. In fact, BPF seems to have a beneficial role in the pathophysiological mechanisms of diabetic cardiomyopathy, particularly in mitochondrial and sarcoplasmic dysfunctions [4]. BPF’s positive effects on oxidative metabolism and mitochondrial bioenergetics have recently been documented, adding to the list of biological targets for its favorable impacts [4]. In addition, BPF could be incorporated into pharmacological therapy for cancer and schizophrenia [4,5].

This Special Issue, “Nutraceuticals and Human Health and Disease”, is devoted to the function and future directions of nutraceuticals in human health, analyzed from a variety of perspectives, from efficacy studies to clinical trials, in terms of their analytical aspects and their positive effects on health issues.

To demonstrate the efficacy of nutraceuticals in human health, Rauch et al. (Contributor 1) studied and proposed the intragastric gavage of exogenous ketones as KEMCT (a mix of 1,3-butanediol-acetoacetate diester/ketone ester/KE and medium-chain triglyceride/MCT oil at a 1:1 ratio) to alleviate anxiety disorder symptoms. Min et al. (Contributor 2) evaluated the anti-Human coronavirus OC43 (HCoV-OC43) effects of auraptene (7-geranyloxy coumarin), a prenyloxy coumarin abundantly present in *Citrus* spp. fruits, demonstrating its efficacy as a therapeutic agent against human coronavirus.

Additionally, Pouchieu et al. (Contributor 3) evaluated the acute effects of a saffron extract (Safr'Inside™) and its primary volatile compound, safranal, on biological and psychological stress responses in a randomized, double-blind, placebo-controlled crossover study involving 19 healthy volunteers who underwent a laboratory stress procedure.

The effects of citrate on epithelial cells with intact cystic fibrosis (CF) transmembrane conductance regulator (CFTR) and defective $\Delta F508$ -CFTR were assessed by Borkenhagen and Prehm (Contributor 4). These studies showed that citrate is an activator of $\Delta F508$ -CFTR and increases export by defective $\Delta F508$ -CFTR into the extracellular matrix of epithelial cells.

The antiproliferative effect of fruits and vegetables on cancer, and, in particular, on breast cancer, has also been demonstrated by the studies of Rhman et al. (Contributor 5). In this research, Rhman et al. reported their results obtained on MCF-7 breast cancer cells treated with quercetin (Que) and naringenin (Nar). In particular, they demonstrated the synergistic effects of Que and Nar (CoQN) in inducing oxidative stress and apoptosis in these cells.

Furthermore, the main chemical and biological characteristics of alpha-lipoic acid (ALA) in glucose metabolism have been reported by Capece et al. (Contributor 6). In particular, the antioxidant activity of ALA and its role in the modulation of insulin sensitivity and secretion and in symptomatic peripheral diabetic polyneuropathy are described. In addition, Capece et al. have given a possible explanation for why subjects taking ALA are at risk of developing insulin autoimmune syndrome (IAS).

Therefore, as reported in the review by Puri et al. (Contributors 7), nutraceuticals, in addition to being important from a nutritional point of view, are assuming a central role in the prevention and treatment of various diseases, supporting classic pharmacological therapies, and also reducing radiotherapy- and chemotherapy-related side effects in cancer patients. To enhance these beneficial effects on human health, researchers are using nanotechnologies to improve micronized dietary product and nutraceutical supplement production.

Furthermore, it is known that the intestinal microbiota and its alterations can be involved in numerous diseases, such as metabolic syndrome and acne. Ilari et al. (Contributors 8), in their systematic review, search for microorganisms that may contribute to both metabolic syndrome and acne and report data from the literature relating to the efficacy of polyphenols in metabolic syndrome treatments. In particular, the authors focus their attention on the prebiotic characteristics of polyphenols in promoting intestinal eubiosis in individuals with metabolic syndrome. On the contrary, there are no studies in the literature relating to the specific efficacy of polyphenols for acne treatment.

In light of all this, knowledge of the biochemical relationships between known and unknown natural compounds and cellular metabolism in both health and sickness can aid in the creation of new and innovative treatment strategies.

Conflicts of Interest: The author declares no conflicts of interest.

List of Contributions:

1. Rauch, E.; Ari, C.; Kovács, Z. Dose-Dependent Beneficial Effect of Ketone Supplement-Evoked Ketosis on Anxiety Level in Female WAG/Rij Rats: Sometimes Less Is More. *Nutrients* **2023**, *15*, 4412. <https://doi.org/10.3390/nu15204412>.
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Article

Dose-Dependent Beneficial Effect of Ketone Supplement-Evoked Ketosis on Anxiety Level in Female WAG/Rij Rats: Sometimes Less Is More

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Abstract: While one-third of the population can be affected by anxiety disorders during their lifetime, our knowledge of the pathophysiology of these disorders is far from complete. Previously, it has been demonstrated in male animals that exogenous ketone supplement-evoked ketosis can decrease anxiety levels in preclinical rodent models, such as Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats. Thus, in this study, we investigated whether intragastric gavage of the exogenous ketone supplement KEMCT (mix of 1,3-butanediol-acetoacetate diester/ketone ester/KE and medium-chain triglyceride/MCT oil in 1:1 ratio) for 7 days can alter the anxiety levels of female WAG/Rij rats using the light–dark box (LDB) test. We demonstrated that a lower dose of KEMCT (3 g/kg/day) increased blood R-βHB (R-β-hydroxybutyrate) levels and significantly decreased anxiety levels (e.g., increased the time spent in the light compartment) in female WAG/Rij rats on the seventh day of administration. Although the higher KEMCT dose (5 g/kg/day) increased blood R-βHB levels more effectively, compared with the lower KEMCT dose, anxiety levels did not improve significantly. We conclude that ketone supplementation might be an effective strategy to induce anxiolytic effects not only in male but also in female WAG/Rij rats. However, these results suggest that the optimal level may be moderately, not highly, elevated blood R-βHB levels when the goal is to alleviate symptoms of anxiety. More studies are needed to understand the exact mechanism of action of ketone supplementation on anxiety levels and to investigate their use in other animal models and humans for the treatment of anxiety disorders and other mental health conditions.

Keywords: exogenous ketone supplement; anxiety; LDB test; female WAG/Rij rat; mental health

1. Introduction

It has been demonstrated that up to one-third of the population can be affected by anxiety disorders during their lifetime [1–3]. However, our knowledge about the mechanism of action of anxiety disorders is far from complete [1,3]. It has been demonstrated that changes, in particular GABA (gamma-aminobutyric acid)-ergic, serotonergic, glutamatergic, noradrenergic and adenosinergic systems in implicated brain areas, such as the hippocampus, prefrontal cortex and amygdala, may have a role in the pathological alterations resulting anxiety disorders [4–7]. Indeed, different drugs that can modify the above mentioned systems, such as selective serotonin reuptake inhibitors, selective serotonin–norepinephrine reuptake inhibitors and benzodiazepines, may be used to alleviate different symptoms of anxiety disorders [4,7,8]. As the efficacy of currently used anxiolytic medications is highly variable and anxiolytics may evoke several adverse side effects [8], further studies are needed to develop safer and more effective anxiolytic drugs and therapeutic approaches.

It has also been demonstrated previously that exogenous ketone supplements, such as ketone salts (KSs) and ketone esters (KEs), not only increase the blood levels of ketone bodies (ketosis) but also effectively decrease anxiety levels in different preclinical rodent models [3,9–15]. Moreover, the connection between the ketone supplement-evoked increase in blood ketone body β -hydroxybutyrate (β HB) levels and anxiolytic effects was also confirmed [3,9,12,14,16]. These results may suggest that ketone supplementation-generated ketosis may alleviate symptoms of anxiety disorders not only in rodent models but also in humans, and it may be a promising adjunctive treatment, but further studies are needed specifically for human applications and for the treatment of other mental health conditions [3,17,18]. However, in spite of having different ingestible ketone preparations commercially available that can effectively and safely increase blood ketone body levels, ketone therapy (e.g., oral administration of ketone supplements) in the treatment of neuropsychiatric disorders, such as anxiety, depression and bipolar disorder, has been mostly neglected [3,17,18].

While anxiolytic drugs are used in both genders in human therapy, the incidence of anxiety disorders was found to be higher in women than in men [1]. However, the ketone supplement-generated influence on anxiety disorders has been studied mainly on male preclinical animal models [9–15]. For example, the ketone supplementation-generated anxiolytic effect was demonstrated only by investigation of male Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats [9,14]. It is important to consider that it has been demonstrated in WAG/Rij rats [19] that a ketone supplement-evoked increase in blood R- β HB levels may be gender-dependent [20] and the sex of the animals may influence the results of behavioral tests [21]. Therefore, to obtain a better understanding of the potential differences between genders, multiple types of experiments need to be performed on both genders. Thus, as a first step, in the current study, we investigated whether administration of the ketone supplement KEMCT (mix of 1,3-butanediol-acetoacetate diester/ketone ester/KE and medium-chain triglyceride/MCT oil in 1:1 ratio) for 7 days can generate changes in the anxiety levels of female WAG/Rij rats using the light–dark box (LDB) test [22,23].

2. Materials and Methods

2.1. Animals

The animals were treated according to the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, section 243), European Communities Council Directive (86/609/EEC), as well as the EU Directive 2010/63/EU. The Animal Care and Experimentation Committee of the ELTE Savaria University Centre (Eötvös Loránd University) approved the experiments under license number VA/ÉBÁF-ÁO/00279-4/2021 by the National Scientific Ethical Committee on Animal Experimentation (Hungary).

Female WAG/Rij rats ($n = 24$; 8 months old, 171–204 g) were housed in groups (4 animals) and were kept under standard laboratory conditions. We provided free access to normal food (SSNIFF RM-Z+H rat breeding and maintenance diet, TOXI-COOP Ltd., Budapest, Hungary) and water, as well as air-conditioned room at 22 ± 2 °C. After the day of the last treatment, the rats were euthanized by isoflurane.

2.2. Light–Dark Box Test

The LDB test is frequently used to evaluate the anxiolytic influence of different drugs and treatments in rodent models [22,23]. The test is based on a conflict between the tendency of rodents to explore their environment and the predisposition of rodents to avoid brightly lit places [24,25]. The LDB apparatus was made from Plexiglas and consisted of two chambers. One chamber was transparent and remained uncovered during the test (light box/compartment), whereas the other chamber was black and was covered with a black lid (dark box/compartment). The two chambers were connected by a rectangular opening. The light box was illuminated by 80 lux (the light was 150 cm above the box), whereas inside the dark box, illumination was not observable (<5 lux) [23]. LDB tests were performed between 4.00 p.m. and 7.00 p.m.

During the LDB test, the animal was placed at the center of the light compartment facing away from the opening and was allowed to freely explore the apparatus for a period of 5 min. The following variables were measured: the time spent in the light compartment, the latency to exit the light compartment (time until first entry into the dark compartment), the latency to first reentry into the light compartment, the number of chamber transitions (entry was considered when all 4 paws of the rat were in the opposite chamber) and number of risk assessments (if animal looked out from the dark compartment, but it did not step into the light compartment with all 4 paws: aborted reentry into the light box) [23,25,26]. It has been demonstrated previously that decreased time spent in the light compartment, decreased latency to exit the light compartment, increased latency to first reentry into the light compartment, decreased number of chamber transitions and increased number of risk assessments are associated with an increase in anxiety-type behavior [24,25,27,28]. The behavior of the animals during the experiment was video recorded in the light compartment. Between sessions, the apparatus was cleaned using 30% ethanol, followed by water.

2.3. Experimental Design

We demonstrated previously that intragastric administration (gavage) of KEMCT for 7 days was well tolerated, did not evoke side effects (e.g., diarrhea) and was able not only to increase but also to maintain blood R- β HB level (ketosis) in WAG/Rij rats [29–31]. In this study, the KEMCT was a mix of 1,3-butanediol-acetoacetate diester (ketone ester/KE) (developed at University of South Florida, Tampa, FL, USA, and Savind Inc., Urbana, IL, USA) [32], as well as medium-chain triglyceride (MCT) oil in 1:1 ratio (containing ~60% caprylic triglyceride, ~40% capric triglyceride, Bloomingdale, IL, USA).

Animals were moved to an anteroom and were treated by gavage on all experimental days. Female WAG/Rij rats were assigned to 3 groups. In order to facilitate adapting the animals to the gavage method, we administered water by intragastric gavage (3 g/kg/day, group 1 and group 2; 5 g/kg/day, group 3) for 5 days (5 days adaptation period; group 1–group 3). Then, animals were gavaged by water (3 g/kg/day) (control, group 1, $n = 8$) or KEMCT (3 g/kg/day: group 2, $n = 8$; or 5 g/kg/day: group 3, $n = 8$) on 7 consecutive days. On the seventh day of water (group 1/control) or KEMCT (group 2 and group 3) gavage, LDB tests were performed one hour after gavage treatment.

To determine the effect of KEMCT on blood glucose and R- β HB levels, measurements were taken on the last (fifth) adaptation day (baseline level), as well as first and last (seventh) treatment days about 70 min after treatment (on the last/seventh day: about 5 min after LDB test; group 1–group 3). To measure blood glucose, as well as R- β HB levels, a commercially available blood glucose and ketone monitoring system (Precision Xtra, Abbott Laboratories, Abbott Park, IL, USA) was used [9,31]. For the test, blood was taken from the rats' tail veins.

We also measured the body weight of the rats before treatments started (fifth adaptation day: baseline) and after last (seventh) treatment.

2.4. Statistics

Data presented are the mean $\pm$ standard error of the mean (S.E.M.). Changes in blood glucose and R- β HB levels, LDB parameters (e.g., time spent in light box) and body weight were compared to baseline (body weight), to the water-gavaged animals (control, group 1; LDB test) and to both of them (baseline and control; blood glucose and R- β HB levels). For data analysis, GraphPad Prism version 9.2.0 (using a two-way ANOVA with Tukey's multiple comparisons test and Dunnett's multiple comparisons test) was used. We considered the results significant when $p < 0.05$.

3. Results

3.1. Effect of KEMCT Administration on Anxiety Levels

The lower dose of KEMCT (3 g/kg/day; group 2) significantly increased the time spent in the light compartment and the latency to exit the light compartment, whereas the

number of chamber transitions significantly decreased on day 7 of the KEMCT treatment compared with the control (group 1) (Figure 1A,B; Table 1). In response to the high KEMCT dose (5 g/kg/day; group 3), the number of chamber transitions significantly decreased on the seventh day of KEMCT gavage (Figure 1B; Table 1). Other variables of the LDB test, such as the latency to first reentry into the light compartment and the number of risk assessments, were not changed significantly after administration of low (3 g/kg/day; group 2) or high (5 g/kg/day; group 3) KEMCT doses compared with the control (group 1) (Figure 1A,B; Table 1).

Table 1. Effect of KEMCT (3 g/kg/day and 5 g/kg/day; group 2 and group 3, respectively) administration for 7 days on anxiety levels in female WAG/Rij rats compared with water-gavaged animals (control, group 1). Abbreviations: KEMCT, mix of ketone ester (1,3-butanediol-acetoacetate diester/KE) and medium-chain triglyceride (MCT) oil in 1:1 ratio; ns, non-significant; * $p < 0.05$; ** $p < 0.01$.

	Water (Control; Group 1; Mean $\pm$ S.E.M.)	3 g/kg KEMCT (Group 2; Mean $\pm$ S.E.M.; Level of Significance/ p -Value)	5 g/kg KEMCT (Group 3; Mean $\pm$ S.E.M.; Level of Significance/ p -Value)
Time spent in light compartment (s)	119.4 $\pm$ 7.34 -	235.5 $\pm$ 24.09 **/0.0025	169.8 $\pm$ 29.0 ns/0.2106
Latency to exit light compartment (s)	55.4 $\pm$ 9.19 -	163.3 $\pm$ 39.43 */0.0271	115.5 $\pm$ 28.75 ns/0.2582
Latency to first reentry to the light compartment (s)	24.8 $\pm$ 4.89 -	23.0 $\pm$ 8.29 ns/0.9877	42.0 $\pm$ 13.43 ns/0.3527
Number of chamber transitions	7.1 $\pm$ 0.72 -	3.8 $\pm$ 1.13 */0.0351	3.6 $\pm$ 0.75 */0.0284
Number of risk assessments	7.3 $\pm$ 0.99 -	4.4 $\pm$ 1.55 ns/0.2234	4.9 $\pm$ 0.92 ns/0.3509

3.2. Effect of KEMCT Administration on Blood Levels of R- β HB and Glucose as Well as Body Weight

After administration of water gavage (control, group 1), we did not find significant differences in blood levels of R- β HB and glucose compared with baseline (Figure 1C,D; Table 2).

A significant increase in blood R- β HB levels was observed after administration of both lower (3 g/kg/day; group 2) and higher (5 g/kg/day; group 3) doses of KEMCT on the first and seventh days of the treatments compared with baseline and the control (group 1) (Figure 1C; Table 2). Moreover, gavage of a higher KEMCT dose (5 g/kg/day) was able to induce significantly higher blood R- β HB levels compared with a lower KEMCT dose (3 g/kg/day) on the seventh day of the treatment (Figure 1C). A significantly higher R- β HB level was also demonstrated after the administration of a higher KEMCT dose (5 g/kg/day) on the seventh day of administration compared with its first administration (Figure 1C).

A similar and significant decrease was demonstrated in blood glucose levels after administration of both doses of KEMCT (3 g/kg/day and 5 g/kg/day, group 2 and group 3, respectively) on the first and seventh days of the treatments compared with baseline and the control (group 1) (Figure 1D; Table 2).

A significant change in body weight was not observed after the seventh day of gavage administration of water (control, group 1) or at low (3 g/kg/day; group 2) or high (5 g/kg/day; group 3) doses of KEMCT compared with baseline (Figure 1E; Table 2).

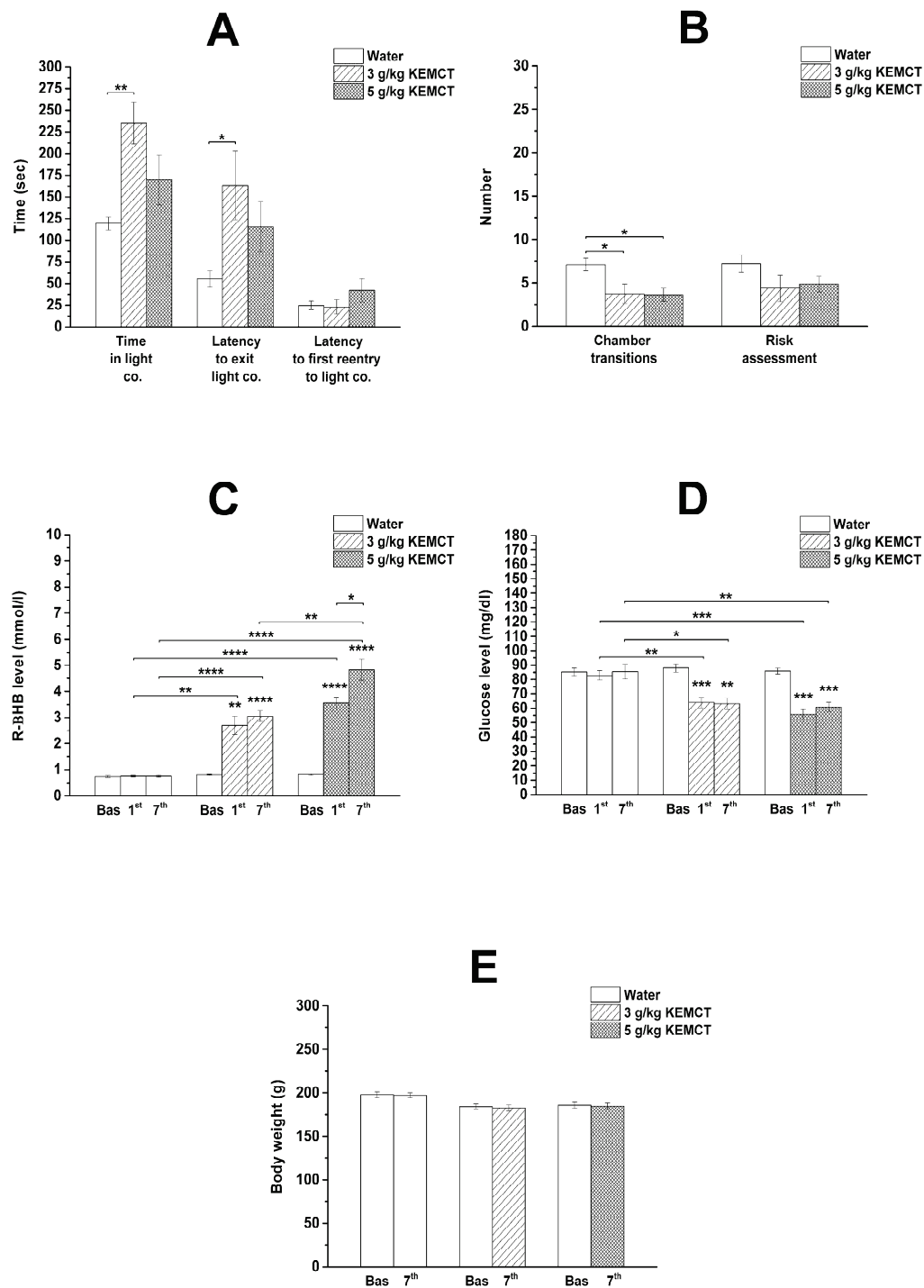


Figure 1. Effect of low KEMCT dose (3 g/kg/day; group 2) and high KEMCT dose (5 g/kg/day; group 3) on anxiety levels (A,B), blood R-βHB (C) and glucose levels (D), as well as body weight (E) compared with baseline (E), water-gavaged animals (control, group 1) (A,B) or both of them (baseline and control; C,D). Abbreviations: Bas, baseline; co., compartment; KEMCT, mix of ketone ester (1,3-butanediol-acetoacetate diester/KE) and medium-chain triglyceride (MCT) oil in 1:1 ratio; R-βHB, R-beta-hydroxybutyrate; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 2. Influence of water (control, group 1) and KEMCT (3 g/kg/day and 5 g/kg/day; group 2 and group 3, respectively) administration on blood R- β HB and glucose levels as well as body weight in female WAG/Rij rats compared with baseline. Abbreviations: KEMCT, mix of ketone ester (1,3-butanediol-acetoacetate diester/KE) and medium-chain triglyceride (MCT) oil in 1:1 ratio; ns, non-significant; R- β HB, R-beta-hydroxybutyrate; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

	Blood R- β HB Level (mmol/L; Mean $\pm$ S.E.M.; Level of Significance/ p - Value)	Blood Glucose Level (mg/dL; Mean $\pm$ S.E.M.; Level of Significance/ p - Value)	Body Weight (g; Mean $\pm$ S.E.M.; Level of Significance/ p - Value)
Group 1 (Control)			
Baseline	0.74 $\pm$ 0.04 -	85.25 $\pm$ 2.71 -	197.75 $\pm$ 3.31 -
First gavage	0.75 $\pm$ 0.03 ns/0.9329	82.88 $\pm$ 3.32 ns/0.5448	- -
Seventh gavage	0.75 $\pm$ 0.03 ns/0.9719	85.50 $\pm$ 5.18 ns/0.9979	197.25 $\pm$ 2.72 ns/0.9587
Group 2 (3 g/kg KEMCT)			
Baseline	0.80 $\pm$ 0.03 -	88.00 $\pm$ 2.87 -	184.25 $\pm$ 3.03 -
First gavage	2.70 $\pm$ 0.36 **/0.0027	64.00 $\pm$ 3.54 ***/0.0002	- -
Seventh gavage	3.05 $\pm$ 0.21 ****/<0.0001	63.25 $\pm$ 4.00 **/0.0057	182.75 $\pm$ 3.59 ns/0.4633
Group 3 (5 g/kg KEMCT)			
Baseline	0.81 $\pm$ 0.03 -	85.88 $\pm$ 1.99 -	186.00 $\pm$ 3.38 -
First gavage	3.56 $\pm$ 0.22 ****/<0.0001	55.75 $\pm$ 3.54 ***/0.0002	- -
Seventh gavage	4.83 $\pm$ 0.40 ****/<0.0001	60.88 $\pm$ 3.28 ***/0.0003	184.75 $\pm$ 3.58 ns/0.6092

4. Discussion

Following our previous study on male WAG/Rij rats, in this study performed on females, we demonstrated that administration of a lower KEMCT dose (3 g/kg/day) for 7 days not only increased blood R- β HB levels and decreased blood glucose levels but also significantly decreased some measures of anxiety levels in female WAG/Rij rats (specifically, the time spent in the light compartment and the latency to exit the light compartment increased). Although a higher KEMCT dose (5 g/kg/day) similarly decreased blood glucose levels and more effectively increased blood R- β HB levels, compared with the low KEMCT dose-generated effects, it was not effective in significantly reducing anxiety levels.

We demonstrated in female WAG/Rij rats that the time spent in the light compartment and the latency to exit the light compartment increased after administration of a lower dose of the KEMCT (3 g/kg/day) (Figure 1A). After the gavage of the higher KEMCT dose (5 g/kg/day), only the number of chamber transitions changed (decreased) significantly. However, a non-significant increase was observed, not only in the time spent in the light compartment and the latency to exit the light compartment, but also in the latency to first reentry into the light compartment after the gavage of the higher KEMCT dose. Moreover, there was a non-significant decrease in risk assessments after the administration of both KEMCT doses. Thus, these results suggest that the administration of a lower KEMCT dose may be able to induce anxiolytic effects, but a higher KEMCT dose did not decrease anxiety levels effectively (Figure 1A,B). Nevertheless, the number of chamber transitions

decreased after the gavage of the lower KEMCT dose (3 g/kg/day) (Figure 1B), suggesting a discrepancy with the anxiolytic influence of the lower KEMCT dose described above. We speculated that this controversial result may be in association with the KEMCT-generated high increase in both the time spent in the light compartment and the latency time to exit the light compartment compared with the results of the control group (group 1) (Figure 1A). Namely, based on the data in Figure 1A and Table 1, the percentages of the session time spent in the light compartment for the control group (group 1) and KEMCT-treated group (3 g/kg/day, group 2) were 39.8 and 78.5, respectively. Moreover, the latency to exit the light compartment was 2.95 times longer after the gavage of 3 g/kg of KEMCT compared with the control group (theoretically, more time spent in the light compartment, less time and moderate tendency to enter the dark compartment and, as a consequence, chamber transition). However, in relation to the LDB test, it was demonstrated that the most reliable and consistent sign of decreased anxiety is the increased time spent in the light compartment [24,25]. Thus, as the time spent in the light compartment significantly increased in group 2, we could conclude that the administration of a lower KEMCT dose (3 g/kg/day) had an anxiolytic effect in female WAG/Rij rats.

In spite of that, a ketone supplement-evoked increase in blood β HB levels may correlate with a ketone supplement-generated anxiolytic effect [3,9,12,14,16], and the higher KEMCT dose (5 g/kg/day) enhanced more effectively the blood levels of R- β HB compared with the lower KEMCT dose-generated effects, only the gavage of the lower dose of KEMCT (3 g/kg/day) increased the time spent in the light compartment significantly (Figure 1A,C). As there was no significant improvement in the time spent in the light compartment after administration of the higher KEMCT dose, these results suggest that generating higher R- β HB levels by administration of a higher KEMCT dose might not be the most effective approach to induce anxiolytic effect in females. Moreover, it has been demonstrated previously that KEMCT (2.5 g/kg/day, gavage for 7 days) generated higher R- β HB levels than the same dose of KEKS (mix of KE and KS). In spite of this last result, KEKS increased the time more effectively until immobility in isoflurane-anesthetized WAG/Rij rats compared with KEMCT [30], while KEKS and KEMCT similarly increased the latency to fall on an accelerating rotarod test in WAG/Rij rats [33]. All of these results suggest that a proper range of R- β HB levels may be needed for different applications and to alleviate symptoms of different diseases, such as anxiety, and to improve physiological functions, and higher doses might not always work most effectively. Therefore, we conclude that inducing very high levels of blood R- β HB may not always be necessary and optimal to enhance the efficacy of treatment for various applications.

We did not find a correlation between the decrease in blood glucose concentration in response to different doses and anxiety levels in rats. However, we demonstrated a similar decrease in blood glucose levels after administration of both doses of KEMCT (3 g/kg/day and 5 g/kg/day) for 7 days (Figure 1D). Given that only the lower KEMCT dose (3 g/kg/day) had an anxiolytic effect (Figure 1A,B) and the administration of another ketone supplement KSMCT (mix of KS and MCT oil) did not decrease blood glucose concentrations significantly but significantly mitigated anxiety levels on the seventh treatment day in male rats [14], we hypothesized that decreased blood glucose levels likely do not have the main role in the KEMCT-evoked alleviating influence on anxiety levels. It has been demonstrated previously that widespread neuronal death occurs only when blood glucose levels decrease below 1 mM (~18 mg/dL) in both mice and rats [34], while neuroprotective effects in very low glucose level situations may be connected to the astrocyte–neuron lactate shuttle [35–37]. Given that after the administration of the KEMCT, the lowest glucose level was 55.75 ± 3.54 mg/dL in our study (Table 2), we suggest that the effect of KEMCT on anxiety levels likely was not associated with hypoglycemia-evoked cell (neuronal) damage.

It has been demonstrated that following oral consumption or intragastric gavage, ketone esters, such as 1,3-butanediol-acetoacetate diester, are entirely hydrolyzed in the small intestine by esterases, and 1,3-butanediol is converted to acetoacetate and β HB in the liver by alcohol and aldehyde dehydrogenase [38–40]. Moreover, MCTs can be hy-

drolyzed to medium-chain fatty acids by lipases in the gastrointestinal tract, which are also converted to ketone bodies in the liver [41]. Finally, ketone bodies, such as β HB, can exit the liver, be transported to the central nervous system through the bloodstream and be utilized for ATP (adenosine triphosphate) synthesis in neurons [42,43]. In relation to the mechanism of action of KEMCT on anxiety levels, it was suggested that enhanced levels of ketone bodies, such as β HB, may increase the level of adenosine in the central nervous system [44]. Indeed, it has been demonstrated that ketone bodies enhance not only the intracellular concentration of ATP in neurons but also the neuronal release of ATP, as well as the extracellular level of adenosine by the ectonucleotidases-generated catabolism of ATP [42]. Moreover, previous *in vivo* and *in vitro* studies show in rodents that after the digestion of ketone supplements [38–40], increased levels of blood β HB may be able to enhance adenosine concentration in different brain areas, such as the striatum [44–47]. Increased neuronal levels of adenosine can enhance the activation of A1-type adenosine receptors (A1Rs), which may open ATP-sensitive potassium (K_{ATP}) channels and generate neuronal hyperpolarization of central nervous system neurons, resulting in a decrease in excitatory neurotransmitter release [42,48–50]. Accordingly, it was demonstrated that β HB can decrease glutamate release and neuronal excitability [51], likely via enhanced activity of A1Rs. This influence may evoke an anxiolytic effect through, for example, attenuation of glutamate-evoked hyperexcitability of brain areas (e.g., prefrontal cortex) implicated in the emergence of anxiety disorders [7,52,53]. Thus, ketone supplement-induced ketosis could alleviate levels of anxiety, likely through enhanced activity of neuronal A1Rs [5,42,54,55]. These results suggest that similarly to the KSMCT-generated ketosis in male WAG/Rij rats [14], the KEMCT-evoked increase in R- β HB levels in the present study may also generate an anxiolytic effect via the adenosinergic system in female WAG/Rij rats. Low basal extracellular levels of adenosine [56] and likely a slight increase in adenosine levels preferentially activate neuronal A1Rs, resulting in inhibitory effects in the central nervous system, such as a decrease in glutamate release [57]. Nevertheless, a higher increase in adenosine levels can activate A2A-type adenosine receptors (A2ARs) in the A1R–A2AR heteromer, blocking the functions of A1Rs and generating enhanced glutamate release [57,58]. Thus, it is possible that administration of higher doses of ketone supplements (e.g., 5 g/kg KEMCT/day) could generate higher levels of not only blood R- β HB (Figure 1C) but also adenosine compared with the effects evoked by lower doses of ketone supplements, in which higher adenosine levels may be enough for the preferential activation of neuronal A2ARs [59]. Increased activation of A2ARs may enhance glutamatergic neurotransmission [57], and glutamate-evoked hyperexcitability in the implicated brain areas of anxiety disorders may result in a mitigated (abolished) anxiolytic effect compared with lower doses of KEMCT-generated alleviating influence (Figure 1A,B), likely through A1Rs.

Administration of KEMCT, theoretically, may modulate not only the adenosinergic and glutamatergic but also other neurotransmitter systems (e.g., GABAergic and serotonergic system) of different brain areas through, for example, increased ketone body levels, resulting in a decrease in anxiety [7,60,61]. Moreover, R- β HB may also exert its effects on functions and diseases of the central nervous system through G-protein coupled hydroxycarboxylic acid receptor 2 (HCAR2 or GPR109 receptor) and free fatty acid receptor 3 (FFAR3) [43,62]. However, further studies are needed to understand the exact role of neurotransmitter systems, receptors and other factors (e.g., other ketone bodies, such as acetoacetate, which were not measured in this study) by which ketone supplements, such as KEMCT, are able to generate an anxiolytic effect.

There are several limitations of the current study. Those limitations include that we investigated the KEMCT-evoked effect on anxiety levels by using only one age group (8 months old) of female WAG/Rij rats, and only one method, the LDB test, was used. However, the current study aimed to further validate and extend our previous studies on the effects of ketone supplementation on anxiety levels in similarly aged male WAG/Rij rats [9,14]. While the LDB test is a commonly used method for the investigation of anxiety levels in rats [22,63,64], and the LDB test is frequently used alone to investigate anxiety-

like behavior [25,28,65], additional studies using other age groups and methods will be needed to further evaluate the current results. It also needs to be considered that the results of the behavioral testing may be affected by the strain and estrous cycle of animals, as well as the drug delivery method [21], and these potential influencing factors were not considered in this study. These influences will need to be taken into consideration in future studies (e.g., using other strains and behavioral tests, such as the elevated plus maze/EPM test) in order to increase confidence in the presented results and to obtain a more comprehensive understanding of the influence of ketone supplementation on anxiety levels. Moreover, further studies are needed to find the most effective formulations and the optimal doses of ketone supplements to mitigate anxiety levels, to investigate the effect of ketone supplementation-evoked increases in not only β HB levels but also acetoacetate levels in relation to ketosis-evoked anxiolytic influences. It will be important to extend the studies to reveal the effect of chronic treatment using ketone supplements on anxiety levels, as well as to measure how long the anxiolytic influence would last in response to chronic treatment. The current study did not measure changes in other neurotransmitter systems in response to ketone supplementation, which will also be important to better understand the exact mechanisms of action.

5. Conclusions

The current results extended our previous studies performed on male WAG/Rij rats by using the LDB test on female WAG/Rij rats. Therefore, we can conclude that ketone supplementation may evoke an anxiolytic effect not only in male, but also in female WAG/Rij rats. Our results also show that there may be a dose-dependent influence of ketone supplementation on central nervous system diseases, such as anxiety. We found that the alleviating effect of ketosis on anxiety levels cannot necessarily be increased by higher levels of blood ketone bodies. Consequently, we conclude that inducing an optimal range of blood R- β HB (ketosis) levels may be needed for the effective treatment of anxiety disorders, and high levels might not be optimal, at least in females, for this application. These results strengthen our hypothesis that ketone supplement-evoked nutritional ketosis may be a useful therapeutic tool to alleviate symptoms of anxiety disorders; however, more detailed studies are needed for specific applications, for example, for diabetic or therapy-resistant human patients or for other mental health conditions.

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Institutional Review Board Statement: The study was approved by the Animal Care and Experimentation Committee of the ELTE Savaria University Centre (protocol code VA/ÉBÁF-ÁO/00279-4/2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: Patent US10980764B1 C. Ari, D.P. D’Agostino, “Exogenous ketone supplements for reducing anxiety-related behavior”. C. Ari is co-owner of Ketone Technologies LLC and owner of Fortis World LLC. These interests have been reviewed and managed by the University in accordance with its Institutional and Individual Conflict of Interest policies. All authors declare that there are no additional conflicts of interest.

Abbreviations

A1R, A1-type adenosine receptors; A2AR, A2A-type adenosine receptors; ATP, adenosine triphosphate; β HB, beta-hydroxybutyrate; GABA, gamma-aminobutyric acid; KE, ketone ester; KEKS, a mix of KE and KS; KEMCT, a mix of KE and MCT oil; KS, ketone salt; KSMCT, a mix of KS and MCT oil; LDB, light–dark box; MCT, medium-chain triglyceride; WAG/Rij, Wistar Albino Glaxo/Rijswijk

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Article

Auraptene Has Antiviral Activity against Human Coronavirus OC43 in MRC-5 Cells

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Abstract: Auraptene (7-geranyloxy coumarin) is the abundant prenyloxy coumarin found in the fruits of *Citrus* spp. Auraptene has a variety of pharmacological and therapeutic functions, such as anticancer, antioxidant, immunomodulatory, and anti-inflammation activities, with excellent safety profiles. In this study, we evaluated the anticoronaviral activity of auraptene in HCoV-OC43-infected human lung fibroblast MRC-5 cells. We found that auraptene effectively inhibited HCoV-OC43-induced cytopathic effects with 4.3 μ M IC₅₀ and 6.1 μ M IC₉₀, resulting in a selectivity index (CC₅₀/IC₅₀) of >3.5. Auraptene treatment also decreased viral RNA levels in HCoV-OC43-infected cells, as detected through quantitative real-time PCR, and decreased the expression level of spike proteins and nucleocapsid proteins in virus-infected cells, as detected through the Western blot analysis and immunofluorescence staining. Time-of-addition analysis showed auraptene's inhibitory effects at the post-entry stage of the virus life cycle; however, auraptene did not induce the antiviral interferon families, IFN- α 1, IFN- β 1, and IFN- λ 1. Additionally, auraptene-treated MRC-5 cells during HCoV-OC43 infection decreased the MMP-9 mRNA levels which are usually increased due to the infection, as auraptene is a previously reported MMP-9 inhibitor. Therefore, auraptene showed antiviral activity against HCoV-OC43 infection, and we suggest that auraptene has the potential to serve as a therapeutic agent against human coronavirus.

Keywords: HCoV-OC43; auraptene; human coronavirus; MRC-5; antiviral; matrix metalloproteinase; MMP-9; MMP-2; *Citrus trifoliata*

1. Introduction

Coronaviruses (CoVs) are the enveloped positive-sense single-stranded RNA viruses belonging to the *Nidovirales* order of the *Coronaviridae* family, which is divided into four genera, namely *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*, along with the seven strains of human infection CoVs [1]. Among the seven human coronaviruses (HCoVs), HCoV-NL63 and HCoV-229E are included in the *Alphacoronavirus* genus. Severe acute respiratory syndromes, (SARS)-CoV, and SARS-CoV-2, and Middle East respiratory syndrome (MERS)-CoV, as well as HCoV-OC43 and HCoV-HKU1, are included in the *Betacoronavirus* genus [2]. The coronavirus disease 2019 (COVID-19) pandemic, caused by SARS-CoV-2 infection, engendered a global health crisis that led to 770 million confirmed cases and 7 million deaths over three years as of the end of May 2023 [3]. Despite the development of several highly effective COVID-19 vaccines, many people are still vulnerable to virus infection and developing the disease due to the high rate of transmissibility and the frequent occurrence of concerning variants of SARS-CoV-2. The Food and Drug Administration approved Veklury[®] (remdesivir) as the first antiviral treatment for COVID-19 and Lagevrio[™] (molnupiravir) and Paxlovid[™] (ritonavir-boosted

nirmatrelvir) for emergency use authorization during the COVID-19 pandemic, but additional antiviral therapeutics to treat and prevent the disease are still needed. Among all CoVs, HCoV-OC43 was isolated in the 1960s and is the most prevalent HCoV globally [4]. HCoV-OC43 infection usually causes a common cold; however, it can progress to bronchitis or pneumonia, comprising around 10% of all respiratory tract infections [5]. Experimental models of HCoV-OC43 infection in human lung cells are useful for evaluating the biological and anticoronaviral activity of various candidate agents because they can be safely conducted in a biosafety level (BSL)-2 facility, unlike the highly pathogenic members of the *Betacoronavirus*, SARS-CoV, MERS-CoV, and SARS-CoV-2, the experimental models of which must be conducted in BSL-3 facilities [6,7].

Monoterpene coumarin ether, also known as auraptene (7-geranyloxycoumarin), was first discovered and characterized in *Citrus aurantium* L. (Rutaceae), known as bitter orange, in 1930 [8]. It is the best known prenyloxycoumarin and commonly found in the fruits of *Citrus* spp., such as oranges, lemons, and grapefruits [9]. A toxicity study of auraptene demonstrated its excellent safety profile, as it fell within normal ranges for histopathological, hematological, biochemical parameters. Furthermore, no mortality was observed. Auraptene exhibits a variety of pharmacological and therapeutic properties, such as anti-cancer, neuroprotective, cardioprotective, gastrointestinal protective, immunomodulatory, anti-inflammatory, antioxidant, antibacterial, antiprotazoal, and antifungal activities [10]. Notably, auraptene has shown anticancer activity against various types of cancer by targeting multiple cellular signaling pathways involved in proliferation, apoptosis, growth factors, cytokines, and transcription factors. Additionally, it inhibits the biological function of target proteins, such as matrix metalloproteinases (MMPs), peroxisome proliferator-activated receptors, and acetylcholinesterase [11,12]. Recently, auraptene was reported to have antiviral activity against the influenza A virus, H1N1 [13], as well as to inhibit enterovirus 71 infection at the attachment and entry steps of their life cycle, targeting the viral envelope proteins, VP1 and VP4 [14]. However, the anticoronaviral activity of auraptene remains unclear. Thus, we examined the anticoronaviral activity and mode of action of auraptene against HCoV-OC43 in the human lung cell line, MRC-5. We found that auraptene effectively inhibits HCoV-OC43 infection in MRC-5 cells, suggesting that it could be a potential anticoronaviral therapeutic agent.

2. Materials and Methods

2.1. Compound

Auraptene (PubChem CID: 1550607, and molecular weight: 298.4) isolated from the peel of *Poncirus trifoliata* (L.) Raf. (also known as *Citrus trifoliata*) was acquired from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, China) and 20 mM stock solutions were created in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and stored at -80°C in a deep freezer for long-term storage.

2.2. Cells and Virus

Human fetal lung fibroblast MRC-5 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in minimal essential medium (Corning Incorporated, Corning, NY, USA, Cat. No. 10-009-CV) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. HCoV-OC43 (ATCC, Manassas, VA, USA) was propagated in MRC-5 cells at 33°C in a 5% CO_2 incubator and titrated, as previously described [15].

2.3. Cytopathic Effect Reduction Assay

MRC-5 cells were seeded at 1×10^4 cells/96-well plate (Thermo Fisher Scientific Inc., Waltham, MA, USA) overnight. MRC-5 cells were infected with HCoV-OC43 ($10^{3.5}$ TCID₅₀/100 μL) and treated with the serially diluted compound at 33°C incubator for 4 days. A colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay kit (Promega Corporation, Madison, WI, USA)

was used to quantify viable cells at 4 days post-infection following the manufacturer's instructions. Then, absorbance at 490 nm was measured using a GloMax[®] Microplate Reader (GM3000, Promega). The absorbance of vehicle-treated cells was set as 100% viable cells and the absorbance of infected and vehicle-treated cells was set as 0% viable cells. Final DMSO concentration was maintained at <0.1%.

2.4. HCoV-OC43 Viral RNA Copy Number Analysis

Viral RNAs were harvested from the culture media supernatant of HCoV-OC43-infected MRC-5 cells using a QIAamp viral RNA mini kit (Qiagen N.V., Hilden, Germany), and viral RNAs from cell lysate of HCoV-OC43-infected MRC-5 cells were isolated using the RNeasy[®] Mini Kit (Qiagen), following manufacturer's instructions. These RNAs were used to synthesize and amplify cDNA using a One Step TB Green[®] PrimeScript[™] RT-PCR kit (Takara Bio Inc., Kusatsu, Japan) and CFX Opus 96 Real-time PCR system (Bio-Rad Lab., Hercules, CA, USA) according to manufacturer's instructions with the following primers for the HCoV-OC43 Nucleocapsid protein: (sense primer) 5'-AGCAACCAGGCTGATGTCAATACC-3' and (antisense primer) 5'-AGCAGACCTTCCTGAGCCTTCAAT. A standard curve of HCoV-OC43 viral RNA was used to calculate the viral RNA copy number, as previously described [15].

2.5. Western Blot Assay

Cells were lysed in Glo lysis buffer (Promega), total cell lysates were separated on a 10% SDS-PAGE gel (Bio-Rad Lab), and proteins were transferred to a PVDF membrane (Bio-Rad Lab). The transferred membranes were incubated with 5% skim milk in TBST (TBS with 0.5% Tween20) for 30 min at 25 °C and washed three times with cold TBST. Thereafter, the anti-HCoV-OC43 spike protein antibody (Catalog Number CSB-PA336163EA01HIY, Cusabio, Houston, TX, USA), anti-HCoV-OC43 nucleocapsid protein antibody (Cat. No. MAB9012, Merck & Co., Inc., Rahway, NJ, USA), or anti-β-actin antibody (Cat. No. 3700S, Cell Signaling Technology, Danvers, MA, USA) was incubated at 4 °C overnight. The membranes were then washed with cold TBST and interacted with secondary antibodies conjugated with horseradish peroxidase (Abcam PLC, Cambridge, UK) for 1 h at 25 °C. Protein bands were observed using a Clarity Max Western ECL substrate (Bio-Rad) and the Chemidoc[™] MP Gel Imaging System (Bio-Rad).

2.6. Immunofluorescence Staining Assay

MRC-5 cells were grown on poly-L-lysine-coated coverslip, fixed with 4% paraformaldehyde for 10 min and rinsed with cold PBS. Then, cells were permeabilized in 0.2% Triton X-100-contained PBS for 10 min and blocked with 3% BSA in permeabilizing buffer. Cells were incubated with anti-HCoV-OC43 spike protein antibody (CusaBio), or anti-HCoV-OC43 nucleocapsid protein antibody (Merck) at 4 °C overnight. Cells were washed with cold PBS three times and incubated with AlexaFluor488-conjugated goat anti-rabbit IgG antibody (Cat. No. A11001, Thermo Fisher Scientific Inc.) or AlexaFluor555-conjugated goat anti-mouse IgG antibody (Cat. No. A21428, Thermo Fisher, Waltham, MA, USA) for 1 h at room temperature. These cells were washed with PBS and mounted using SlowFade[™] Gold Antifade Mountant with DAPI (Invitrogen, Waltham, MA, USA) and visualized using an Olympus IX71 fluorescence microscope (Olympus Corporation, Tokyo, Japan) and the cellSens program (Olympus).

2.7. Time-of-Addition Assay

MRC-5 cells were grown overnight at a density of 1×10^4 cells/96-well culture plate. As pretreatment, the cells were pretreated with the indicated concentration of auraptene for 4 h before HCoV-OC43 infection and then the compound was removed. Cells were infected with the virus and incubated for four days. For cotreatment, the compound was added with the virus for 4 h; then, cells were washed and incubated with the virus for four days. As posttreatment, cells were infected with the virus and after 4 h, the compound

was added with the virus during four days. At 4 days postinfection, a cytopathic effect reduction assay was performed using an MTS assay kit (Promega).

2.8. Quantitative Real-Time PCR (qRT-PCR)

Total RNA of cell lysates was isolated using the RNeasy[®] Mini kit (Qiagen) and used to synthesize and amplify cDNA via the TB Green[®] PrimeScript[™] RT-PCR Kit (Takara Bio) and the CFX Opus 96 Real-time PCR system (Bio-Rad Lab). The following primers were used: for IFN- α 1, 5'-GTGCTCAGCTGCAAGTCAAG-3' and 5'-TATCCAGGCTGTGGGTCTC-3'; for IFN- β 1, 5'-TAGCACTGGCTGGAATGAG-3' and 5'-GTTTCGGAGGTAACCTGTAAG-3'; for IFN- λ 1, 5'-GTCACCTTCAACCTCTTCCG-3' and 5'-TCAGACACAGGTTCCCATCG-3'; for MMP-2, 5'-TCTCCTGACATTGACCTTGGC-3' and 5'-CAAGGTGCTGGCTGAGTAGATC-3'; for MMP-9, 5'-TTGACAGCGACAAGAAGTGG-3' and 5'-GCCATTACGTCGTCCTTAT-3'; and for β -actin, 5'-GGAAATCGTGCGTGACATCA-3' and 5'-ATCTCCTGCTCGAAGTCCAG-3' [15,16].

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software V9.5.1 purchased from GraphPad Software, Inc. (San Diego, CA, USA). Data are represented as means $\pm$ standard errors of the mean (SEMs). Probability (p) values were obtained through one-way or two-way ANOVA followed by Bonferroni's test, as described in the figure legends. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

3. Results

3.1. Auraptene Inhibits the HCoV-OC43-Induced Cytopathic Effects

To investigate the anticoronaviral activity of auraptene (Figure 1A), we examined its effect on HCoV-OC43-induced cytopathic effects in a human lung cell line, MRC-5 cells. Auraptene did not induce cytotoxic effects under 10 μ M, although approximately 10% of cell death was induced by 15 μ M of auraptene, suggesting 50% of cytotoxic concentration (CC_{50}) of >15 μ M. When MRC-5 cells were infected with $10^{4.5}$ TCID₅₀/mL of HCoV-OC43, virus infection induced around 89% of cell death due to cytopathic effects at 4 days postinfection. However, treatment with serially diluted concentrations of auraptene reduced the HCoV-OC43-induced cytopathic effects dose-dependently (Figure 1B). The 50% and 90% inhibitory concentration (IC_{50}) and (IC_{90}) values were 4.3 μ M and 6.1 μ M, respectively, as obtained via nonlinear regression analysis, resulting in a selectivity index (CC_{50}/IC_{50}) of >3.5. Thus, we used up to 10 μ M of auraptene for further experiments. In addition, we confirmed the complete protection of 10 μ M auraptene from the HCoV-OC4-induced cytopathic effects based on cellular morphology. The data revealed that treatment with 10 μ M auraptene effectively prevented cell death caused by HCoV-OC43-induced cytopathic effects (Figure 1C, the third image, HCoV-OC43). Thus, the morphology of virus-infected cells treated with auraptene (Figure 1C, the fourth image, HCoV w/ Auraptene) remained intact, resembling the morphology of non-infected cells (Figure 1C, the first image, MOCK) and auraptene-treated cells (Figure 1C, the second image, Auraptene). Therefore, these data showed that auraptene efficiently protected from HCoV-OC43-induced cytopathic effects.

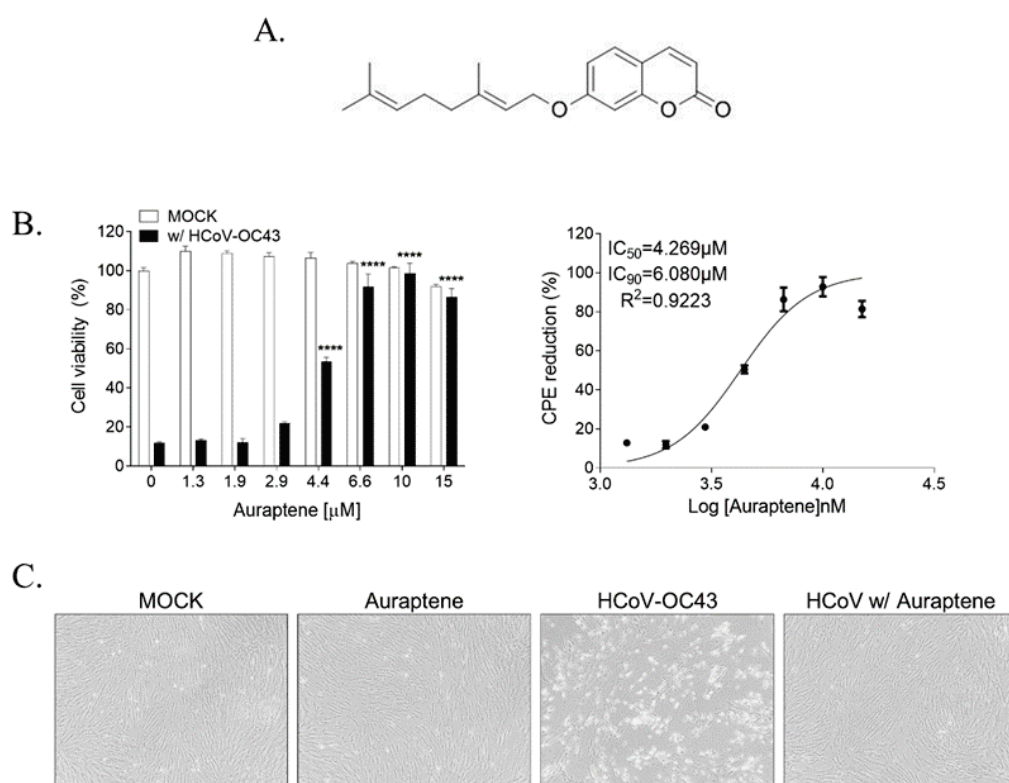


Figure 1. HCoV-OC43-induced cytopathic effects were inhibited by auraptene treatment in human lung fibroblast cells, MRC-5. **(A)** Chemical structure of auraptene. **(B)** MRC-5 cells were infected with $10^{4.5}$ TCID₅₀/mL HCoV-OC43 and treated with serially diluted concentrations of auraptene from 1.3 to 15 μ M. At 4 days postinfection, an MTS assay was conducted to detect cell viability (left graph). The IC₅₀ and IC₉₀ of auraptene were 4.3 μ M and 6.1 μ M, respectively, as calculated via nonlinear regression analysis (right graph). Data were analyzed using a one-way analysis of variance followed by Bonferroni's multiple comparisons test ($F_{7,16} = 120.5$, $p < 0.0001$; **** $p < 0.0001$ vs. 0 μ M of auraptene). **(C)** Optical microscope images of mock, 10 μ M auraptene-treated cells, HCoV-OC43-infected cells, or virus-infected and 10 μ M auraptene-treated cells at 4 days postinfection. Data are presented as mean $\pm$ SEM and represent three independent experiments.

3.2. Auraptene Inhibits HCoV-OC43 Viral RNA Replication and Viral RNA Expression

To examine the effect of auraptene on viral replication, intracellular and extracellular viral RNA levels were measured in HCoV-OC43-infected and 10 μ M auraptene-treated MRC5 cells using qRT-PCR. The level of extracellular viral RNA increased up to 5×10^7 viral RNA copy number/ μ L, however, auraptene treatment decreased it to 8×10^6 viral RNA copy number/ μ L at 4 days postinfection. In addition, the intracellular viral RNA level peaked at 1.4×10^{10} viral RNA copy number/ μ L at 2 days postinfection, and decreased with auraptene treatment to 1.2×10^9 viral RNA copy number/ μ L at 2 days postinfection (Figure 2A). Moreover, the Western blot analysis showed that viral proteins spike (S, 145 kDa) and nucleocapsid protein (N, 50.4 kDa) could be detected in HCoV-OC43-infected cells but not in 10 μ M auraptene-treated MRC5 cells between 2 and 4 days postinfection (Figure 2B). Immunofluorescence staining analysis also showed that spike (S) and nucleocapsid protein (N) could be detected in HCoV-OC43-infected cells between 1 and 3 days postinfection but not in 10 μ M auraptene-treated MRC5 cells (Figure 2C). Therefore, these data showed that auraptene inhibited viral replication and the expression level of viral proteins, suggesting the antiviral activity of auraptene against HCoV-OC43 infection.

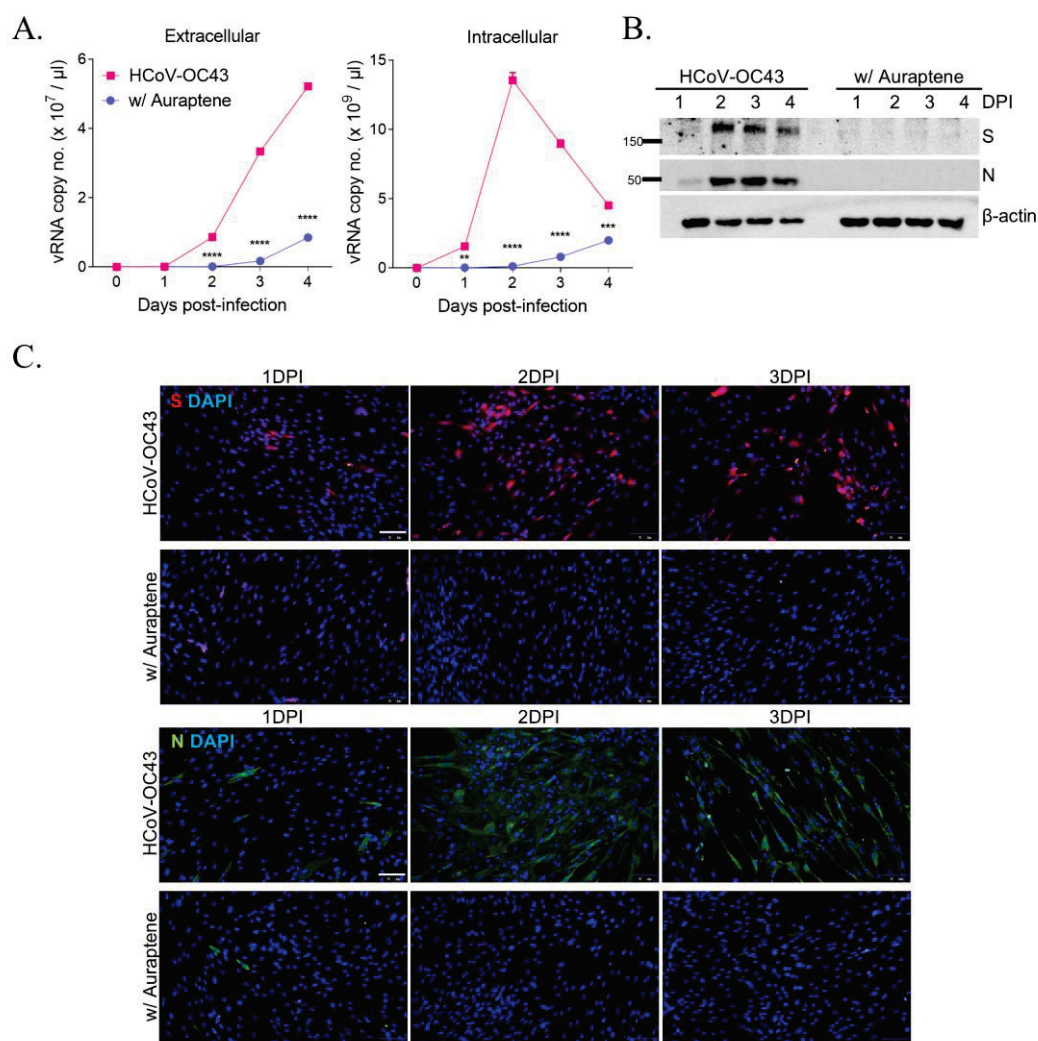


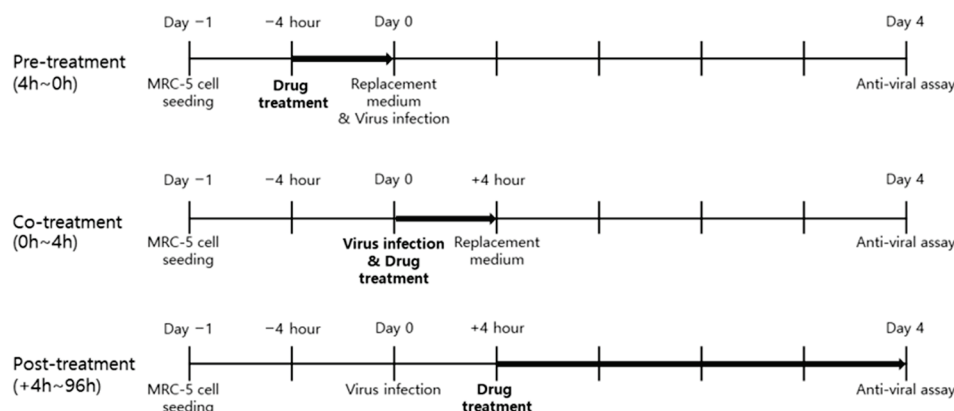
Figure 2. HCoV-OC43 replication and the expression of viral protein were inhibited by 10 μ M auraptene treatment. **(A)** Extracellular (left graph) and intracellular viral RNA levels (right graph) in HCoV-OC43-infected MRC-5 cells treated with (circle) or without (rectangle) 10 μ M auraptene assessed using qRT-PCR at 1, 2, 3, and 4 days postinfection. Data were analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test (extracellular: time effect, $F_{4,8} = 3050$, $p < 0.0001$; auraptene effect, $F_{1,8} = 5590$, $p < 0.0001$; time $\times$ auraptene interaction, $F_{4,8} = 1690$, $p < 0.0001$; intracellular: time effect, $F_{4,8} = 273.4$, $p < 0.0001$; auraptene effect, $F_{1,8} = 1126$, $p < 0.0001$; time $\times$ auraptene interaction, $F_{4,8} = 281.4$, $p < 0.0001$; ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. HCoV-OC43 at the same day postinfection). Data are presented as mean $\pm$ SEM. **(B)** Expression of viral spike (S) proteins and nucleocapsid (N) protein in MRC-5 cells with HCoV-OC43 infection and treatment of vehicle or 10 μ M auraptene at 1, 2, 3, and 4 days postinfection according to the Western blot analysis. The β -actin band was detected as a loading control. Data are representative of three independent experiments. **(C)** Immunofluorescence staining images at 1, 2, and 3 days postinfection. HCoV-OC43 spike protein (red) and nucleus visualized with DAPI (blue) (upper panel), and N protein (green) and nucleus visualized with DAPI (blue) (lower panel) in MRC-5 cells virus-infected with vehicle or 10 μ M auraptene treatment. Scale bar = 100 μ m. The experiments were performed independently ≥ 3 times.

3.3. Auraptene Inhibited HCoV-OC43 Infection at the Post-Entry Infection Stage

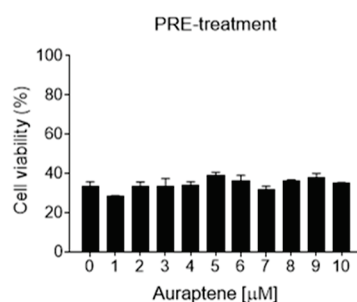
To define the mechanism of auraptene's antiviral effects, a time-of-addition assay was conducted (Figure 3A). In the pretreatment experiment, MRC-5 cells were pretreated with 10 μ M auraptene for 4 h, washed, and then infected with HCoV-OC43 for 4 days. In the cotreatment experiment, MRC-5 cells were cotreated with 10 μ M auraptene and

HCoV-OC43 infection for 4 h. At 4 days postinfection, auraptene did not inhibit HCoV-OC43 infection based on cytopathic effects reduction data in the pre- and cotreatment experiments (Figure 3B,C). In the posttreatment assay, MRC-5 cells were infected with HCoV-OC43 and then treated with 10 μ M auraptene at 4 h postinfection. Auraptene was found to inhibit virus-induced cytopathic effects in the posttreatment assay (Figure 3D). These data suggested that auraptene inhibited HCoV-OC43 infection after 4 h of infection, which corresponds to the post-entry stage.

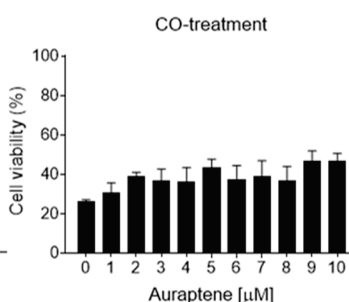
A.



B.



C.



D.

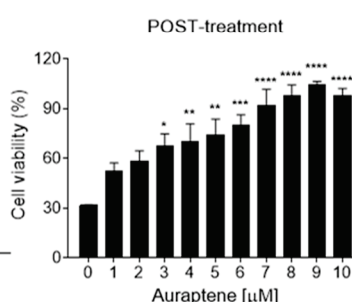


Figure 3. Time-of-addition assay of auraptene during HCoV-OC43 infection in MRC-5 cells. (A) Scheme for the time-of-addition assay. (B–D) Cell viability of pretreatment (B), cotreatment (C), and posttreatment (D) assay assessed using MTS at 4 days postinfection. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test (PRE-treatment: $F_{10,22} = 1.978$, $p = 0.0877$; CO-treatment: $F_{10,22} = 1.232$, $p = 0.3251$; POST-treatment: $F_{10,22} = 10.61$, $p < 0.0001$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs. 0 μ M of auraptene). Data are presented as mean $\pm$ SEM and representative of three independent experiments.

3.4. Auraptene Did Not Induce the Antiviral IFN Gene Expression

To determine whether auraptene induced antiviral interferon genes (IFN- α 1, IFN- β 1, and IFN- λ 1), their expression level was evaluated using qRT-PCR. HCoV-OC43 infection induced IFN- α 1, IFN- β 1, and IFN- λ 1 in MRC-5 cells time-dependently at 1, 2, 3, and 4 days postinfection. However, treatment with 4, 7, or 10 μ M auraptene did not increase the expression level of these antiviral interferon genes in HCoV-OC43-infected cells (Figure 4). These data suggested that auraptene treatment itself did not induce antiviral IFN genes; instead, their expression was decreased due to the lower viral RNA level in auraptene-treated MRC-5 cells during HCoV-OC43 infection, as shown in Figure 2A. Therefore, auraptene protected HCoV-OC43-infected MRC-5 cells but not through the induction of the antiviral IFN genes, IFN- α 1, IFN- β 1, and IFN- λ 1.

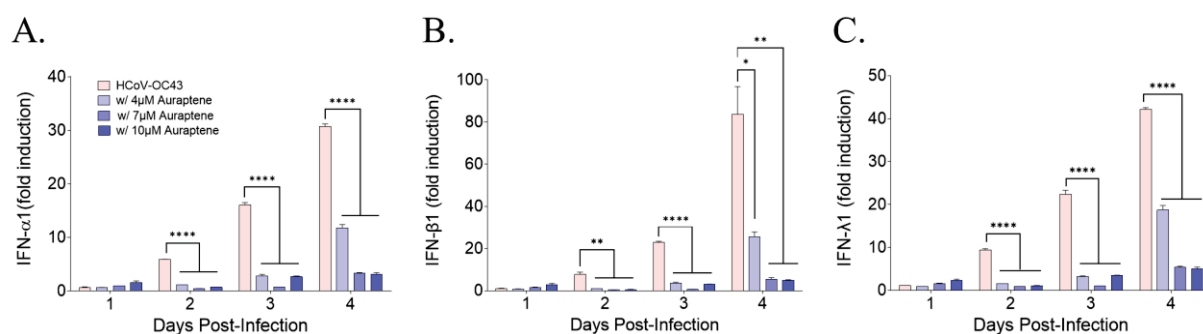


Figure 4. IFN-related antiviral response was not induced by auraptene treatment during HCoV-OC43 infection. (A–C) Level of IFN-α1 (A), IFN-β1 (B), and IFN-λ1 (C) mRNA induction quantified in HCoV-OC43-infected MRC-5 cells with vehicle (pink bar) or 4, 7, or 10 μM auraptene (blue bar) treatment using qRT-PCR at 1, 2, 3, and 4 days postinfection. Data were normalized to β-actin gene expression level. Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test (IFN-α1: 2 days postinfection, $F_{3,4} = 18177$, $p < 0.0001$; 3 days postinfection, $F_{3,4} = 713.7$, $p < 0.0001$; 4 days postinfection, $F_{3,4} = 962.1$, $p < 0.0001$; IFN-β1: 2 days postinfection, $F_{3,4} = 46.39$, $p < 0.0014$; 3 days postinfection, $F_{3,4} = 804.6$, $p < 0.0001$; 4 days postinfection, $F_{3,4} = 31.04$, $p < 0.0031$; IFN-λ1: 2 days postinfection, $F_{3,4} = 670.4$, $p < 0.0001$; 3 days postinfection, $F_{3,4} = 472.7$, $p < 0.0001$; 4 days postinfection, $F_{3,4} = 810.5$, $p < 0.0001$; * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ vs. HCoV-OC43 at the same day postinfection). Data are presented as means ± SEMs and represent three independent experiments.

3.5. Auraptene Decreased MMP-9 mRNA Levels, which Increased with HCoV-OC43 Infection in MRC-5 Cells

Auraptene has been reported to suppress matrix metalloproteinase (MMP)-2, MMP-7, and MMP-9 expression in human colon cancer cells [17], as well as MMP-2 and MMP-9 activity, to inhibit the migration and invasion of cancer cells [18]. Thus, we examined whether HCoV-OC43 infection induced MMP-2 and MMP-9 mRNA expression using qRT-PCR at 1, 2, 3, and 4 days postinfection. HCoV-OC43 infection increased MMP-9 mRNA expression in a time-dependent manner but not MMP-2 (Figure 5). Moreover, when administering auraptene at 4, 7, or 10 μM during HCoV-OC43 infection, MMP-9 mRNA expression was reduced in a dose-dependent manner at 4 days postinfection compared to virus-infected MRC-5 cells. These data showed that HCoV-OC43 infection induced MMP-9 but not MMP-2 mRNA expression in MRC-5 cells, and that MMP-9 mRNA expression was reduced in auraptene-treated MRC-5 cells.

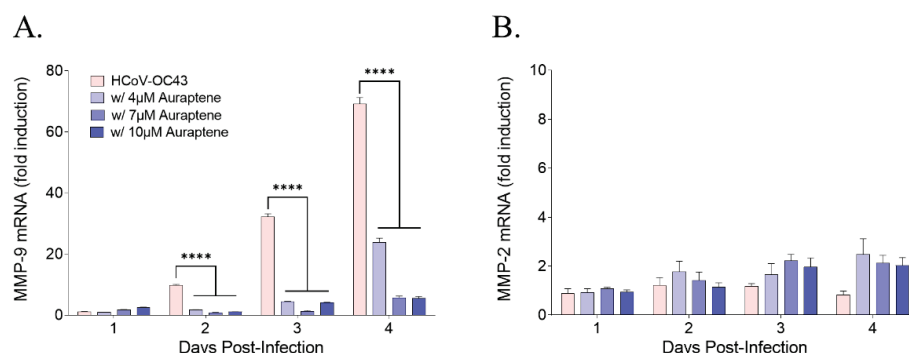


Figure 5. Auraptene treatment decreased the induced MMP-9 expression by HCoV-OC43 infection. (A,B) Level of MMP-9 (A) and MMP-2 (B) mRNA quantified in HCoV-OC43-infected MRC-5 cells treated with vehicle (pink bar) or 4, 7, or 10 μM auraptene (blue bar) using qRT-PCR at 1, 2, 3, and 4 days postinfection. Data were normalized to β-actin gene expression level. Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparisons test (MMP-9: 2 days postinfection, $F_{3,4} = 1093$, $p < 0.0001$; 3 days postinfection, $F_{3,4} = 811.7$, $p < 0.0001$; 4 days postinfection,

$F_{3,4} = 527.3$, $p < 0.0001$; **** $p < 0.0001$ vs. HCoV-OC43 at the same day postinfection). Data are presented as means $\pm$ SEMs and represent three independent experiments.

4. Discussion

Auraptene is the most abundant prenyloxycoumarin in nature and commonly exists in the fruits of *Citrus* spp. [9]. Auraptene exhibits a variety of pharmacological and therapeutic properties, mediating host signaling pathways and inhibiting the functions of target proteins [10]. In this study, we showed auraptene's antiviral activity against HCoV-OC43 infection in human lung fibroblast cells, MRC-5, by effectively inhibiting virus-induced cytopathic effects and the expression level of viral RNA and viral proteins during HCoV-OC43 infection.

Previously, auraptene was shown to have antiviral activity against influenza virus A, H1N1 [13]. Although auraptene has previously been shown to inhibit enterovirus 71 infection at the attachment and entry step of the viral life cycle by targeting capsid proteins, VP1 and VP2, using a reverse genetic system [14], the time-of-addition assay of auraptene against HCoV-OC43 in our study showed that auraptene inhibited HCoV-OC43 infection at the post-entry stage. These results suggest that auraptene exhibits distinct modes of action in different viral infections.

Among the well-known modes of action of auraptene as an anti-tumor agent, it suppressed the expression of MMP-2, MMP-7, and MMP-9 proteins in colon cancer cells and colonic mucosa from colitis mice [17] and also repressed MMP-2 and -9 activity to inhibit the migration and invasion of cervical and ovarian cancer cells [18]. Moreover, auraptene inhibited the *Porphyromonas gingivalis* bacterial infection which causes periodontal diseases by reducing the secretion and activity of MMP-8 and MMP-9 [19]. Similar to these previous studies, we found that auraptene treatment suppressed MMP-9 expression in HCoV-OC43-infected MRC-5 cells, and this suppression occurred in a dose-dependent manner, despite the induction of MMP-9 by HCoV-OC43 infection in a time-dependent manner.

In our study, we showed for the first time that HCoV-OC43 infection induced MMP-9 but not MMP-2 mRNA expression in MRC-5 cells. Virus infections have been shown to modulate MMP-9 expression. For instance, MMP-9 protein was increased by West Nile virus infection in the circulation and brain and was involved in its entry into the central nervous system (CNS) [20]. Similarly, human immunodeficiency virus Tat protein was found to upregulate MMP-9 expression, which can accelerate the trafficking of leukocytes into the CNS, resulting in the acquired immune deficiency syndrome dementia complex [21]. Dengue virus and Japanese encephalitis virus were both found to increase MMP-2 and MMP-9 expression and increase blood-brain barrier permeability, resulting in brain damage [22,23]. The upregulated MMP9 expression by Zika virus promotes virus entry into the testes by disrupting the blood–testis barrier [24]. Epstein–Barr virus oncoprotein induces MMP-9 expression, which may contribute to tumor invasion and metastasis [25].

In addition, MMP-9 is reported to modulate virus replication. Respiratory syncytial virus (RSV) infection induces MMP-9 expression in vitro and in vivo, whereas MMP-9 deletion decreases RSV replication [26]; however, MMP-9 also recruited neutrophils resulting in the RSV clearance as antiviral activity in vivo [27]. A higher level of MMP-9 has been reported in influenza A virus-infected murine lung, whereas virus-infected MMP-9 deficient mice showed lower viral titer, being protected from lung disease by an effective immune response [28]. These data indicate that MMP-9 could be involved in HCoV-OC43 infection and replication. Moreover, Hepatitis B virus infection induces MMP-9 expression in immune cells, thereby evading host immunity by binding MMP-9 to IFN receptor I and blocking IFN signaling. This mechanism enables the virus to maintain a persistent infection [29]. Based on these results, it is possible that HCoV-OC43 infection also induces the expression of type I interferon genes, but the virus-induced MMP-9 proteins could potentially block the IFN signal, thereby evading the host's antiviral response in MRC-5

cells, which could be blocked by auraptene treatment. However, to validate this hypothesis, further studies are required.

Recently, it was reported that SARS-CoV-2 infection induced MMP proteins. SARS-CoV-2 infection in K18-hACE2-transgenic mice (K18-hACE2) increased the expression of MMP-8, MMP-9, and MMP-14 proteins in the lung, suggesting that SARS-CoV-2 infection is associated with the activity of the MMP family which may cause tissue damage [30]. Furthermore, MMP-9 levels increased while MMP-2 levels decreased in COVID-19 patients, which is consistent with HCoV-OC43 infection in MRC-5 cells. Moreover, the increased serum MMP proteins levels in COVID-19 patients may be useful biomarkers of severe COVID-19 [31,32]. Therefore, HCoV-OC43 infection may induce the expression of MMP-9 proteins, which can modulate virus replication and pathophysiology. However, the treatment with auraptene has the potential to inhibit these effects, making it a promising therapeutic agent.

5. Conclusions

In this study, we presented the antiviral effect of a natural prenyloxycoumarin, auraptene, against human coronavirus HCoV-OC43 infection by inhibiting virus-induced cytopathic effects, viral RNA replication, and viral protein expression. A time-of-addition assay suggested that auraptene inhibited HCoV-OC43 infection at the post-entry stage of the virus life cycle. Furthermore, auraptene decreased the induced MMP-9 expression caused by HCoV-OC43 infection in human lung fibroblast cells. Thus, auraptene could be a future and potential anticoronaviral therapeutic agent. Further research is needed to clarify the molecular mechanism of auraptene in detail to allow its development into a therapeutic treatment with an excellent safety profile.

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Article

Acute Effect of a Saffron Extract (Safr'Inside™) and Its Main Volatile Compound on the Stress Response in Healthy Young Men: A Randomized, Double Blind, Placebo-Controlled, Crossover Study

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Abstract: According to animal studies, saffron and its main volatile compound safranal may reduce biological and behavioral signs of acute stress. However, little is known about its impact in humans. This study investigated the acute effect of a saffron extract and safranal on the biological and psychological stress responses in healthy men experiencing a laboratory stress procedure. In this double-blind, placebo-controlled, randomized, cross-over study, 19 volunteers aged 18–25 received a single dose of 30 mg saffron extract (Safr'Inside™), 0.06 mg synthetic safranal, or a placebo on three visits separated by a 28-day washout. Thirteen minutes after administration, participants were exposed to the Maastricht acute stress test (MAST). Salivary cortisol and cortisone were collected from 15 min before the MAST (and pre-dose), 3 min before the MAST, and then 15, 30, 45, 60, and 75 min after the MAST, and stress and anxiety were measured using visual analogic scales. Compared to the placebo, stress and anxiety were significantly toned down after Safranal and Safr'Inside™ administration and coupled with a delay in the times to peak salivary cortisol and cortisone concentrations ($p < 0.05$). Safr'Inside™ and its volatile compound seem to improve psychological stress response in healthy men after exposure to a lab-based stressor and may modulate the biological stress response.

Keywords: saffron extract; safranal; terpene; stress; anxiety; corticotropic axis

1. Introduction

Stress may be defined as “a particular relationship between the person and the environment that is appraised by the person as taxing or exceeding their resources and endangering his or her wellbeing” [1]. Exposure to stress is known to trigger a complex interplay of nervous, endocrine, and immune mechanisms [2]. Playing a central role, the hypothalamus–pituitary–adrenal (HPA) axis is activated, resulting in an adaptive increased cortisol secretion. The latter was investigated via salivary cortisol secretion peaks between 20 and 40 min after exposure to the stressful event [3]. Conversely, if this exposure

is actually or perceived as intense, repetitive, or prolonged, the stress response may become maladaptive and may induce deleterious cognitive, emotional, physical, and behavioral symptoms [4].

In modern life, people may be exposed daily to different sources of stress, such as constant digital connection, heavy workload, changes in sleep wake schedules, or fear of climate change. It has been shown that chronic stress is associated with an increase in the global prevalence of mental and physical health problems, such as anxiety and depression [5–7], but also cardiovascular diseases [8,9]. In addition, it is now known that the COVID-19 pandemic-related stress has affected the brain and mental health. Indeed, COVID-19 has led to a 27.6% increase in cases of major depressive disorder and a 25.6% increase in cases of anxiety disorders worldwide in 2020 [10]. Becoming aware of the side effects from conventional anxiolytics such as benzodiazepines or other psychotropic drugs, people are seeking more safe and natural alternatives in order to cope with the adverse effects of stress, such as yoga or dietary supplements [11,12]. Indeed, the European stress management supplements market is expected to grow at a compound annual growth rate of 2.3% through the 2017–2030 period [13]. This “over-the counter” medication may be effective in the management of psychological and physiological stress responses, yet broad evidence based on clinical trials or experimental data is lacking [14]. Combining pharmacological and non-pharmacological interventions, such as nutraceuticals, phytochemicals, or psychological interventions, to manage stress-related problems may be the most effective approach, taking into account individual patients’ characteristics [15,16].

Among natural alternatives, saffron extract produced from the dried stigma of *Crocus sativus* L. appears to be a promising candidate. Saffron stigma contains volatile compounds, mainly terpenes such as safranal, as well as non-volatile ones such as crocins, crocetins, picrocrocins, and flavonoids [17]. Interestingly, saffron extract may reduce the plasma levels of corticosterone in response to acute stress in rodent studies. This suggests a potential modulation of the HPA axis following a single dose of such an extract but the effect of its isolated constituents, i.e., crocins and safranal, are inconsistent [18–20]. To our knowledge, only two clinical studies have investigated the biological and psychological anti-stress effect of saffron in chronically stressed women. The first demonstrated that women affected by menstrual distress and who inhaled saffron for 20 min experienced significant decreased salivary cortisol coupled with reduced self-perceived anxiety compared to those receiving the placebo, suggesting a potential anti-stress and anxiolytic effect likely induced by the volatile compound safranal [21]. Indeed, the means of salivary cortisol was about 0.11 ug/dl after saffron inhalation compared to 0.18 ug/dl in the placebo group ($p < 0.0051$). The second clinical study showed a beneficial effect of a single dose of Safr’Inside™ on another biological response to stress, namely heart rate variability (root mean square of the successive differences (RMSSD)), after an induced laboratory acute stressor (the observed multitasking stressor) in healthy adults experiencing low mood [22]. Indeed, participants who received placebo had a decreased RMSSD during the laboratory stressor, whereas participants receiving Safr’Inside™ experienced no change in RMSSD ($p = 0.003$). Although the effect of saffron on the HPA axis was assessed, the participants had anticipated the stressor, rendering the results uninterpretable. Otherwise, Safr’Inside™ was well tolerated and associated with reduced depression scores and improved social relationships after 8 weeks of supplementation.

Therefore, the aim of the present study was to investigate the acute effect of a saffron extract and its isolated main volatile compound tested as synthetic safranal on the biological and psychological responses induced by an acute validated laboratory stress procedure in healthy young men.

2. Materials and Methods

2.1. Participants

Twenty healthy men, aged 18–25 years, with a body mass index between 18.5 and 25.0 kg/m², who had not smoked since at least 3 months, with usual weekdays waking

hours 6.00–9.00 am, were recruited between September 2020 and December 2021 at the SANPSY Unit located in the Teaching University Hospital, Bordeaux, France (see Figure 1 for the Consolidated Standards of Reporting Trials (CONSORT) diagram). The participants were recruited using the investigational center’s participant database and the study was advertised by local newspapers and social networks. Participants with high blood pressure (BP > 140/90 mmHg); a history of or currently suffering from mental disorders, infectious diseases, diabetes mellitus, allergic diseases, cardiovascular diseases, unbalance thyroid diseases; or subjects taking anxiolytic, antidepressants, or any other treatment likely to affect salivary cortisol measures (such as steroids, including ointments) were not eligible. In addition, exclusion criteria likely to bias salivary cortisol measures were: the occurrence of a life event 2 weeks before enrollment or a planned life event during the study period (death, divorce, change in professional activities, surgery, travel involving jet lag, etc.), shift workers or those working in extreme conditions (in cold rooms), those engaged in high level of physical activities, those who regularly consumed >3 coffee cups in the morning, those who regularly consumed >3 alcoholic drinks per day, those who used dietary supplements in the 2 weeks before enrolment, and those using illicit drugs. In order to enroll participants likely to experience a biological stress response after the acute stress protocol [23], only subjects displaying an increase in salivary cortisol from 15 to 30 min post-stressor greater than $2 \times$ the coefficient of variation (i.e., >16%) of the cortisol assays at the screening visit were eligible.

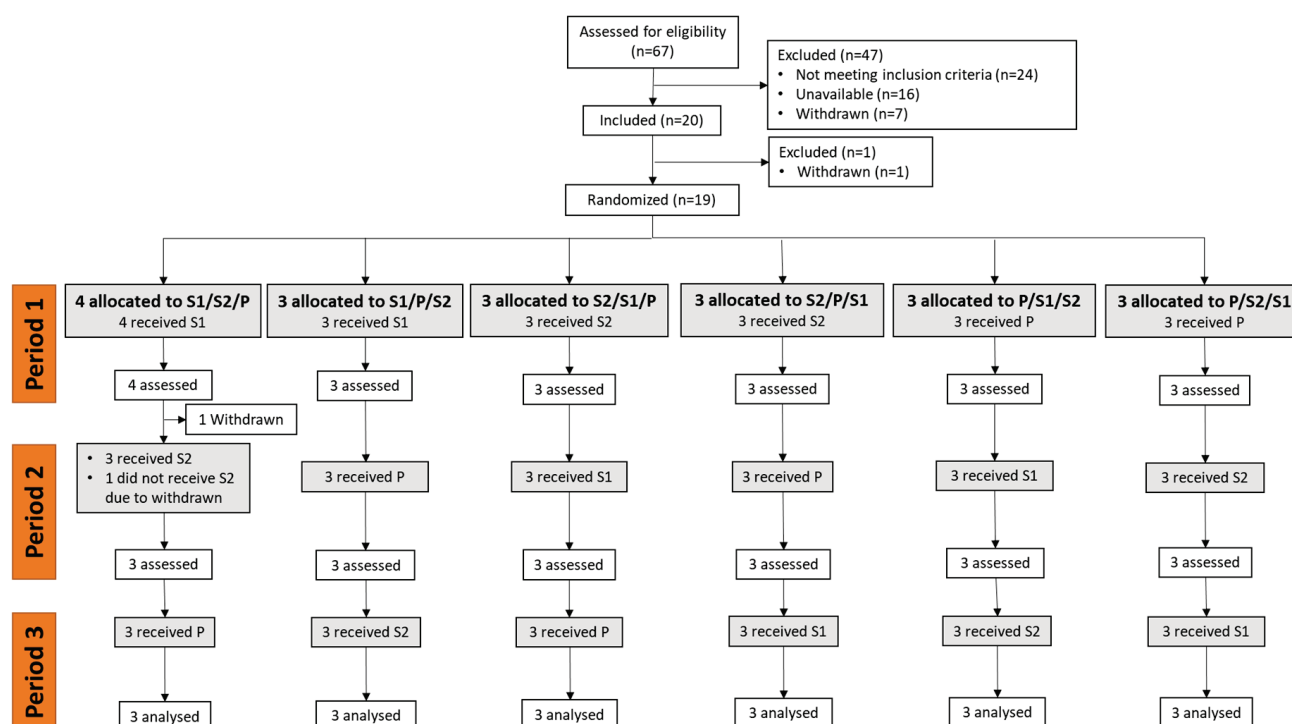


Figure 1. Consolidated Standards of Reporting Trials (CONSORT) flowchart diagram. S1: saffron extract; S2: safranal; P: placebo.

2.2. Tested Products

Participants were randomly allocated to receive a single dose of 30 mg of a full-spectrum standardized saffron extract (Safr’Inside™, Activ’Inside, Beychac et Caillau, France) obtained according to the patent EP3490575B1-WO2017EP69200, which contains crocins (mainly trans-4-GG, trans-3-Gg; cis-4-GG, trans-2-G) > 3%, safranal > 0.2%, picrocrocins derivatives (mainly picrocrocins, HTCC) > 1%, and kaempferol derivatives (mainly kaempferol-3-sophoroside-7-glucoside, kaempferol-3-sophoroside) > 0.1%, analyzed using the U-HPLC method; 0.06 mg synthetic safranal (≥90% stabilized, Sigma-Aldrich, Darmstadt, Germany), corresponding to the dose provided by 30 mg Safr’Inside™; or a placebo

containing maltodextrin. Each product (saffron extract, synthetic safranal, or placebo) was presented as a single hard-shell capsule with same appearance (color, size, odor) containing 100 mg of powder and was administered sublingually during the testing visits (V1, V2, and V3). Each capsule was provided on the visit day in opaque pill dispensers.

2.3. Study Design and Protocol

This study adopted a randomized, double-blind, placebo-controlled, cross-over (3 arms) design. Each participant went through one screening visit (V0) followed by three testing visits (V1, V2, and V3), during which they consumed either the saffron extract, the safranal or the placebo capsule. Following criteria checks for inclusion (V0), eligible participants provided lifestyle and demographic data, and their weight, height, heart rate, and blood pressure were measured. Participants were asked not to change their dietary, sleeping, and exercise habits throughout the whole study. In addition, they were requested not to consume saffron-based food or beverages, alcohol, and not to practice moderate to vigorous physical activities within the 24 h preceding a testing visit. Then, they performed a training session on the MAST. Saliva samples were collected before and immediately after the MAST and every 15 min for 1 h. At the end of the visit, participants were provided with sleep and food diaries and study instructions. Participants that had no reactive cortisol response 15 min after the MAST were not eligible.

The V1 visit was planned 21 $\pm$ 2 days after the inclusion visit, while the V2 and V3 visit were planned following a washout period (28 $\pm$ 2 days). During each testing visit, participants arrived at the research unit at an agreed time in early afternoon that remained consistent across all testing visits. A standardized lunch free from saffron, dark chocolate, caffeine, herbs, and alcohol was provided. Sleep and food diaries were checked. The absence of recent consumption of cannabis and alcohol were confirmed via a urine tetrahydrocannabinol test and a breath alcohol test. The assessment timeline during study visits is shown in Supplementary Figure S1. One hour after a standardized lunch, participants rinsed their mouth 15 min before the collection of a saliva sample and the rating of their self-perceived stress and anxiety using visual analogic scales (VAS). Stress and anxiety were rated by making a vertical mark on a 100 mm straight horizontal line orientated from the left (“not at all”) to the right (“very much”). Participants were randomly assigned to a treatment sequence order. Randomization was based on a computer-generated list provided by staff not involved in the clinical assessment or in the data analyses. The randomization was equilibrated by block size of 6. The capsule of the tested product was opened by the participant and totally poured under his tongue. Saliva samples and self-perceived stress and anxiety levels were obtained 15 min after product intake. The MAST was administered to the participants. Finally, saliva samples and self-perceived stress and anxiety were collected after the end of the MAST and every 15 min for 1 h.

2.4. Maastricht Acute Stress Test

The MAST is a validated experimental stress procedure that has been widely used as a safe and reliable measure to activate both acute physiological and subjective measures of the stress response in healthy adults [24,25]. A 5 min preparation phase allowed the participant to read the instructions for the upcoming tasks (on a PowerPoint presentation). During the 10 min acute stress phase, participants were required to place their hand in cold water at 2 °C and complete an arithmetic task (counting backward by 13, 17, 19, or 23) for alternating time intervals. The sequence (order and duration) of the various hand immersion trials and mental arithmetic tasks was the same for all participants but differed according to visit.

2.5. Salivary Cortisol and Cortisone

Salivary samples were obtained using a Salivette kit (Sarstedt, Nümbrecht, Germany) to measure cortisol and cortisone concentrations. Once collected, samples were stored at 4 °C before being analyzed. Salivary cortisol and cortisone were measured by LC-MS/MS

(Prominence liquid chromatography system; Shimadzu, Nakagyo, Japan; and 5500 Qtrap detector, Sciex, Framingham, MA, USA). A liquefying agent (Sputasol; Thermo Fisher Scientific, Waltham, MA, USA) was added to 400 µL of saliva sample, which was then incubated for 30 min at 37 °C. Next, solid-phase extraction with a hydrophilic lipophilic balance (WatersTM) was performed before injecting the extract into the LC-MS/MS system. The coefficients of variability for the cortisol and cortisone assays were 8 and 9%, respectively.

2.6. Statistical Analyses

As no clinical study has investigated the effect of saffron extract in response to an acute laboratory stressor on salivary cortisol response, the sample size calculation was based on the effect size of a lemon balm food supplement on salivary cortisol in healthy adults 1 h post-treatment [26]. A sample size of 18 subjects was hypothesized to detect an effect size of 1.4 on salivary cortisol response, with a two-sided 5% significance level and a power of 80%. Considering a 10% drop-out rate, 20 volunteers were included in order to obtain 18 completions. Among the 20 included participants, 1 participant withdrew before randomization and 1 participant discontinued the study after visit 1, leading to missing data at visit 2 and 3 for all biological and psychological parameters.

Demographic and lifestyle characteristics at baseline (V0) and pre-treatment values of VAS stress, VAS anxiety, salivary cortisol and cortisone collected during testing visits were compared according to the sequence using a linear mixed model (PROC MIXED). The model included sequence, period, and treatment as fixed effects and participants within sequence as the random effects. In order to confirm that participants found the MAST stressful, VAS anxiety, VAS stress, salivary cortisol, and cortisone were compared before, during, and after the MAST using the linear mixed model as above and using the time of sample collection as additional fixed factor.

The incremental area under the curve (AUC) for VAS stress, VAS anxiety, salivary cortisol, and cortisone were calculated using the trapezoid rule, discarding the area beneath the pre-treatment values, considered the baseline value [27]. As AUC, maximum concentration (Cmax) and time to reach Cmax (Tmax) did not exhibit the normal distribution of residuals regardless of transformation, the Friedman ANOVA test was used to analyze the treatment effect with post hoc analyses using Tukey's method when appropriate. Due to biological non-response to the stressor, 5 datapoints were judged to be abnormal regarding Tmax and Cmax and were excluded from these analyses.

All statistical tests were 2-sided, and a *p*-value < 0.05 was considered statistically significant. All data were analyzed using SAS version 9.4 (SAS Institute, Cary, NC, USA). Figures were computed with GraphPad Prims (version 9.4.0, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Inclusion and Population Characteristics

The study population involved 19 healthy males with a mean age of 22.4 ± 2.0 years. All participants reported being healthy, not consuming any food supplements, medications, or illicit drugs that would interfere with the test product. All baseline values were within the physiological range (Table 1). The participants usually consumed less than an average of one cup of coffee and alcoholic drink per day. Participants displayed a 94% (± 59) mean increase in salivary cortisol from 15 to 30 min after the end of the MAST, showing a reactive cortisol response. Test capsules were well tolerated, and no adverse effects were reported during the study. Except for one participant that dropped out after the 1st test visit without giving any reason, all subjects completed the trial.

No statistically significant differences were observed at baseline among the six sequence groups with respect to demographic, lifestyle, or psychological and physiological characteristics. Therefore, randomization was considered successful.

Table 1. Baseline characteristics of the population (N = 19).

	Mean	SD
Age (years)	22.4	2.0
Body mass index (kg/m ²)	22.1	1.6
Systolic blood pressure (mmHG)	113.5	8.0
Diastolic blood pressure (mmHG)	72.8	6.4
Heart rate (bpm)	73.0	14.7
Coffee consumption (units/day)	0.6	0.9
Alcohol consumption (units/day)	0.2	0.5
Normal sleep duration (hours)	8.0	1.0
Salivary cortisol change from 15 to 30 min after the MAST (%)	94	59

3.2. Validation of the MAST

The analyses of the VAS revealed that the participants, irrespective of treatments, experienced a peak of subjective anxiety and stress 15 min after the MAST (Figure 2a,b), which was coupled to a peak of salivary cortisol and cortisone 30 min after the MAST (Figure 2c,d), which is indicative of a psychological and hormonal stress response (effect of time $p < 0.0001$ for all outcomes).

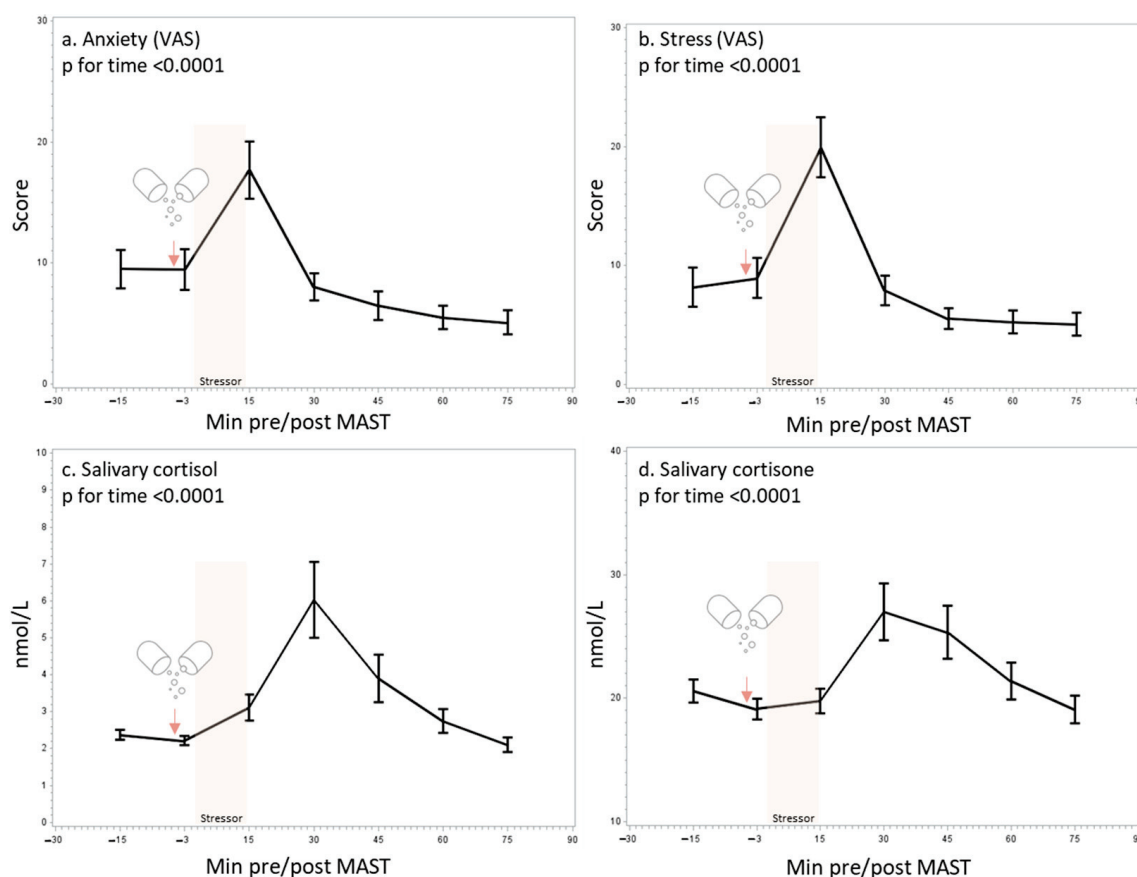


Figure 2. Effect of the Maastricht acute stress test (MAST) on self-perceived anxiety (a) and stress (b) using a visual analog scale (VAS), salivary cortisol (c), and salivary cortisone (d). Mean and SEM are shown.

3.3. Self-Perceived Anxiety and Stress

With regard to anxiety VAS, there was a significant difference in VAS anxiety response (AUC_{t2t4} under the anxiety peak) between the three treatments ($p = 0.04$, Friedman's test). The subjective anxiety response was lower in the saffron extract and safranin groups

compared to the placebo group (Figure 3a,b). The multiple pairwise comparison showed a significant difference between the safranal and placebo groups ($p = 0.01$, Tukey's test).

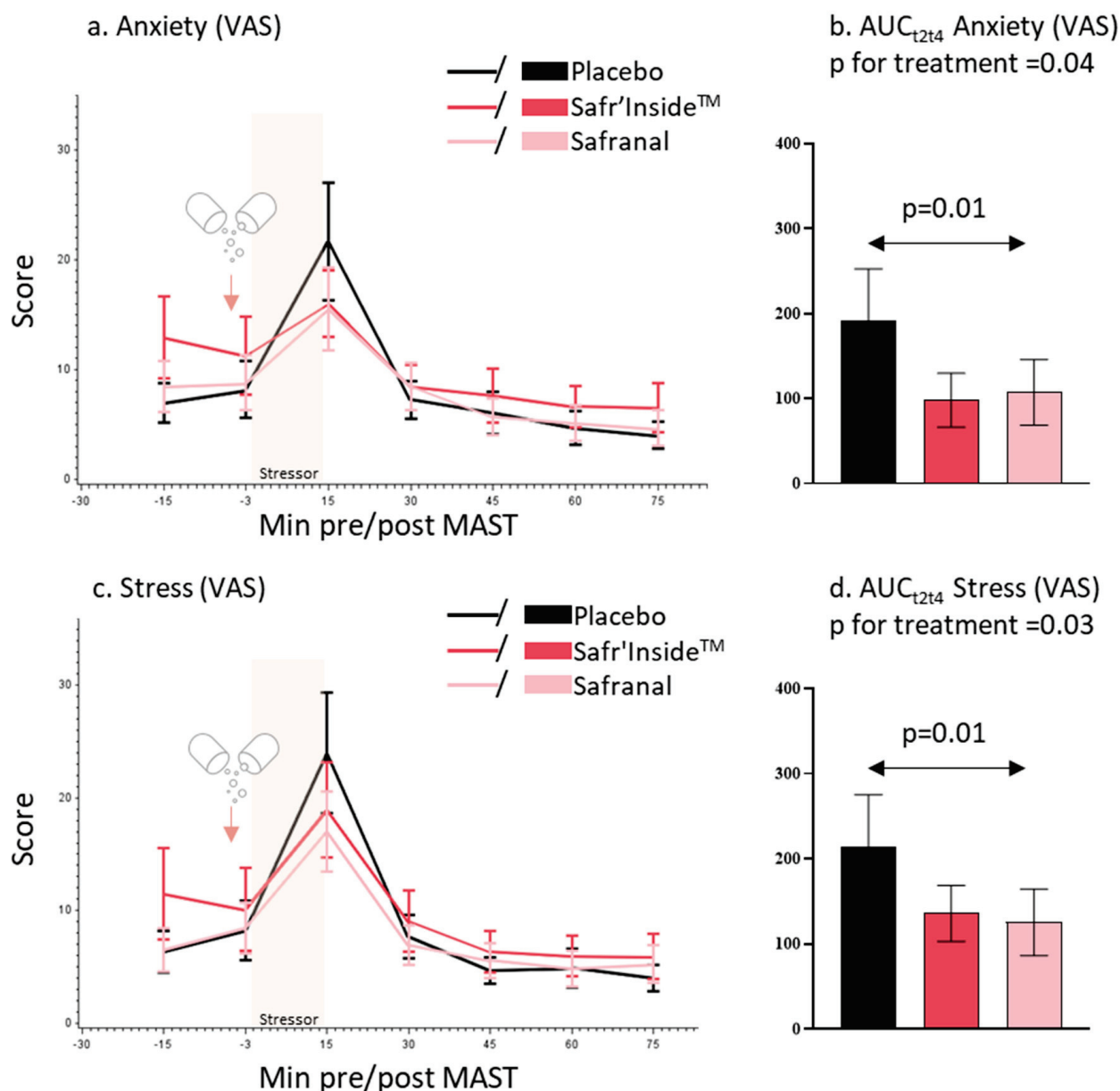


Figure 3. Mean and SEM of self-perceived anxiety (a) and stress (c) using visual analog scale (VAS) by minutes pre/post MAST and treatment. Mean and SEM of area under the curve (AUC_{t2t4}) for VAS anxiety (b) and stress (d) by treatment.

With regard to stress VAS, there was a significant difference in VAS stress response (AUC_{t2t4} under the stress peak) between the three treatments ($p = 0.03$, Friedman's test). The subjective stress response was lower in the saffron extract and safranal groups compared to the placebo group (Figure 3c,d). The multiple pairwise comparison showed a significant difference between safranal and placebo groups ($p = 0.01$, Tukey's test).

3.4. Salivary Cortisol and Cortisone Concentration

With regard to salivary cortisol, there was a significant treatment effect regarding the time to reach maximal salivary cortisol concentration (T_{max}) ($p = 0.04$, Friedman's test). The participants receiving an acute saffron extract supplementation had a higher T_{max}

for salivary cortisol compared to placebo in response to the acute stress ($p = 0.04$, Tukey's test) (Figure 4a). There was a significant treatment effect on salivary cortisone peaking time ($T_{\max}$) ($p = 0.04$, Friedman's test) (Figure 4b). The participants receiving an acute saffron extract supplementation had a longer salivary cortisone $T_{\max}$ compared to placebo in response to the acute stress ($p = 0.04$, Tukey test) (Figure 4b). No other effects of treatment were observed on salivary cortisol ($C_{\max}$, AUC_{t1t7}) nor salivary cortisone ($C_{\max}$, AUC_{t1t7}) (Table 2).

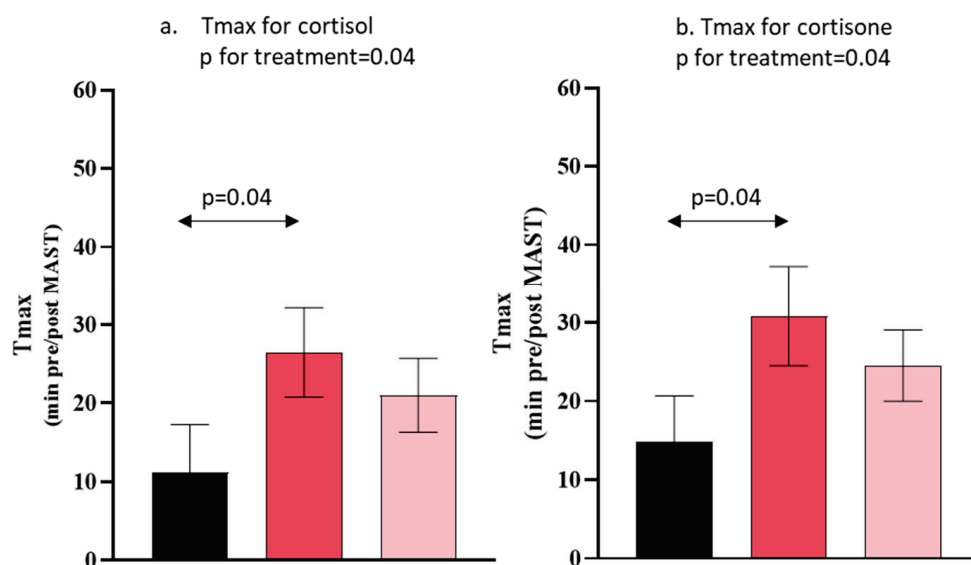


Figure 4. Mean and SEM of $T_{\max}$ for salivary cortisol and cortisone by treatment. Placebo (in black), Safr'Inside™ (in red) and Safranal (in pink).

Table 2. Salivary cortisol and cortisone parameters according to the treatment group.

	Placebo (n = 17)	Safr'Inside™ (n = 19)	Safranal (n = 18)	p-Value
Salivary cortisol				
AUC_{t1t7} (nmol/L*min)	58 (26)	67 (33)	58 (26)	0.17
$C_{\max}$ (nmol/L)	6.9 (1.9)	7.4 (2.4)	6.4 (1.6)	0.90
$T_{\max}$ (minutes pre/post MAST)	11 (6.0)	26 (5.7)	21 (4.7)	0.04
Salivary cortisone				
AUC_{t1t7} (nmol/L*min)	148 (56)	193 (78)	161 (70)	0.10
$C_{\max}$ (nmol/L)	32 (4.0)	32 (5.2)	31 (4.5)	0.52
$T_{\max}$ (minutes pre/post MAST)	15 (5.8)	31 (6.4)	24 (4.5)	0.04

Data are expressed as mean (SEM). AUC_{t1t7} , area under the curve from 15 min pre-MAST to 75 min post-MAST; $C_{\max}$, maximum concentration; $T_{\max}$, time to reach $C_{\max}$; p -value for treatment effect derived from the Friedman's test.

4. Discussion

The current study is the first double-blind, randomized, crossover study that explored the effect of a single dose of a proprietary saffron extract (Safr'Inside™) or its main volatile compound safranal, on the biological and psychological stress responses in healthy young men after experiencing a physically and psychologically challenging laboratory stress test (MAST), compared to a placebo group. As expected, both biological and psychological parameters responded to the MAST with stressor-induced changes. The levels of self-perceived stress and anxiety using VAS were toned down after Safr'Inside™ and safranal administration compared to the placebo. In addition, we observed a significant effect of the saffron extract on the glucocorticoid stress response, showing a delay in the salivary cortisol and cortisone responses in participants receiving the saffron extract compared to those receiving the placebo.

Irrespective of the treatment, the participants found the MAST to indeed be stressful, as suggested by the increase in perceived anxiety and stress 15 min after completing the stressor and to a lesser extent in the salivary cortisol and cortisone 30 min post-stressor. As expected, the HPA axis stress response related to cortisol secretion was in line with previous studies using the MAST in healthy young men [24,28]. However, the basal salivary cortisol concentration of these study's participants was clearly lower than observed in other healthy populations using different acute stress protocols [23,24,28,29]. This may be partly explained by the method of cortisol assays. Indeed, in our study, salivary cortisol was quantified by the method of choice by LC-MS/MS, which has higher sensitivity and specificity than conventional immunoassays used in clinical practices [30]. Thus, other studies using immunoassays to assay salivary cortisol suffered from some degree of cross-reactivity of assays with cortisone, leading to higher apparent values of cortisol. In addition, this low basal level of salivary cortisol strongly suggests that the participants tested here were indeed healthy and relaxed.

To our knowledge, no clinical study has investigated the efficacy of an acute saffron extract or safranin neither using oral nor sublingual administration in an acute experimental stress setting on psychological and biological markers, including salivary cortisol and its metabolite, cortisone. Salivary cortisol is commonly used as a surrogate marker of serum free cortisol in psychophysiological stress research with its tremendous advantage of non-invasive sample collection. Recently, salivary cortisone has been identified as a promising stress marker that showed a high discriminatory power and significant associations with subjective stress measures [31]. Indeed, salivary cortisol is rapidly, locally, and irreversibly converted to cortisone, as the salivary glands exhibit high levels of 11 β -hydroxysteroid dehydrogenase 2 (11 β -HSD2). This results in 2–6 times higher concentrations of salivary cortisone compared to cortisol in saliva, consistent with what is observed in our study.

Interestingly, the saffron extract and safranin increased the peaking time to reach the maximum concentration of salivary cortisol and cortisone after acute stress in comparison to the placebo, suggesting a delayed glucocorticoid secretion. However, the multiple pairwise comparison revealed a significant difference between the saffron extract and placebo groups. The anxiolytic effect of saffron was previously demonstrated in patients with mild to moderate depression [32–34], but the chronic supplementation of saffron extracts may not consistently modify feelings of anxiety in healthy individuals self-reporting low mood [22,35]. The delay in glucocorticoid secretion after saffron administration highlighted its modulatory role on the HPA response to an acute stress exposure. Only one randomized clinical study has investigated the efficacy of anti-stress saffron aromatherapy, but the study was conducted in chronically stressed female students suffering from menstrual troubles without including any experimental acute stressors [21], leading comparisons difficult. After 20 min of exposure, saffron odor significantly decreased salivary cortisol in both follicular and luteal phases (by almost 1.7 and 0.9 nmol/L, respectively), compared to ethanol inhalation, and was also associated with reductions in the symptoms of anxiety. As this is a preliminary study conducted in healthy non-stressed men, it will be necessary to confirm the clinical significance of the HPA axis modulation in a larger sample including both sexes suffering from natural “day to day” stress.

It is well known that stress activates the HPA axis, leading to the plasma corticosterone increase as a response in mice [36]. In male mice exposed to electroshock stress, several studies showed that different doses of saffron extracts via intraperitoneal administration prevented elevations in plasma corticosterone but findings regarding the administration of isolated components, such as crocin or safranin, were inconsistent across research [19,20]. Although the biological mechanisms by which saffron delayed the stress-induced glucocorticoid secretion needs further investigations, our findings are supported by mechanistic studies: saffron may inhibit corticosterone secretion in stressed mice by reducing the gene expression of the corticotrophin-releasing hormone in hypothalamus [37] and by blocking NMDA glutamate and/or sigma opioid receptors located in the adrenal cortex [20,38].

Participants receiving Safr'Inside™ or safranal felt less stressed and anxious than those receiving placebo on a short period of 30 min after the stressor onset. The pairwise comparison revealed that these feelings were significantly different between safranal and placebo. As Safr'Inside™ is composed of more than hundreds of metabolites, including safranal, this latter may be subjected to a “matrix effect”, contrary to pure safranal. This matrix effect may accelerate or delay the absorption of safranal, leading to a maximum effect on perceived stress and anxiety either few minutes earlier or later than pure safranal. In addition, as these feelings were not continuously collected, it is likely that the maximum perceived effect occurred in the 15 min period separating two assessments of VAS. Although no clinical study has explored the anxiolytic and anti-stress effect of synthetic safranal, its anxiolytic effect was supported by some preclinical studies [18,19]. However, experimental design may not be comparable due to discrepancies between the tested doses, administration route, and timing of outcome collection. Indeed, Hooshmandi et al. showed that intraperitoneal (i.p.) pretreatment with safranal can inhibit behavioral signs of stress in male Wistar rats, although these signs were explored more than 20 days after electroshock stress induction. In addition, i.p. safranal administration demonstrated anxiolytic activity in a dose-dependent manner in mice experiencing the elevated-plus maze test, the most commonly employed animal behavioral test to measure anxiety-like behavior. As safranal exerts anxiolytic effects similar to those of anxiolytic medications (diazepam), researchers hypothesized that it may act by interacting with the benzodiazepine binding site at the GABA_A receptor, although no study has really demonstrated this neurobiological effect so far [18,36].

The primary strength of this study includes the use of both objective and subjective outcomes measures to assess the anti-stress effect of a saffron extract and its main volatile compound, safranal, in healthy non-stressed men experiencing a validated laboratory stress procedure (MAST). Secondly, as the anti-stress effect should be collected quickly after product intake, we administered the tested product via the sublingual route to provide the immediate onset of pharmacological effect [39].

However, despite several positive findings from this study, some limitations should be acknowledged, suggesting directions for future research. Firstly, as greater acute HPA responses have been found in adult men as compared to adult women [40], we investigated the stress response only in healthy young men to increase the likelihood of detecting the anti-stress effect of the tested products. We excluded women due to the influence of sex hormones, which fluctuate with the menstrual cycle, and the influence of oral contraceptives on cortisol levels. The generalizability of our results to other participant demographics, such as women or chronically stressed people, thus remains to be tested in future research. Secondly, while a linear mixed model considering the sequence, period, time of collection, and treatment would have been the statistical method of choice, the restricted sample size in combination with a non-Gaussian distribution of physiological and psychological parameters only allowed for a more basic statistical approach.

5. Conclusions

This study reports that a single dose of Safr'Inside™ in healthy young men delayed the typical peak of salivary cortisol and cortisone appearing in response to a physical and psychosocial stressor, in comparison to a placebo. This interesting finding therefore supports the biological role of Safr'Inside™ in the stress management. In addition, we also showed, for the first time in a human study, that Safr'Inside™ and its main volatile compound safranal may reduce the level of perceived stress and anxiety, although the underlying biological mechanism needs further investigations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu15132921/s1>, Figure S1: Assessment timeline during study visits.

Author Contributions: Conceptualization, P.P. and J.B.; methodology, P.P., J.B. and J.-B.C.; formal analysis, C.P.; investigation, P.P.; data curation, J.B. and J.-B.C.; project administration, P.P.; writing—original draft preparation, C.P., N.C. and L.C.; writing—review and editing, C.P.; visualization, C.P. and L.P.; supervision, P.P.; funding acquisition, D.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and the French Public Health Code. The study protocol was approved by the Ethics committee for people’s protection (CPP EST IV, 28/09/2019; ID-RCB: 2018-A02178-47). The trial was registered at ClinicalTrials.gov (NCT04523870).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: C.P., L.P. and D.G. work for Activ’Inside and provided the tested products.

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Recovery of $\Delta F508$ -CFTR Function by Citrate

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Abstract: Treatment of cystic fibrosis relies so far on expensive and sophisticated drugs. A logical approach to rescuing the defective $\Delta F508$ -CFTR protein has not yet been published. Therefore, virtual docking of ATP and CFTR activators to the open conformation of the CFTR protein was performed. A new ATP binding site outside of the two known locations was identified. It was located in the cleft between the nucleotide binding domains NBD1 and NBD2 and comprised six basic amino acids in close proximity. Citrate and isocitrate were also bound to this site. Citrate was evaluated for its action on epithelial cells with intact CFTR and defective $\Delta F508$ -CFTR. It activated hyaluronan export from human breast carcinoma cells and iodide efflux, and recovered $\Delta F508$ -CFTR from premature intracellular degradation. In conclusion, citrate is an activator for $\Delta F508$ -CFTR and increases export by defective $\Delta F508$ -CFTR into the extracellular matrix of epithelial cells.

Keywords: cystic fibrosis; CFTR; citrate; hyaluronan

1. Introduction

Cystic fibrosis (CF) is one of the most common inherited diseases, afflicting 1 in approximately 2500 white individuals [1]. The primary cause of morbidity and mortality in CF is chronic lung infection and deterioration of lung function. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel expressed at the apical membrane of epithelial cells in the airways, pancreas, testis, and other tissues [2,3]. The most common CFTR mutation producing CF is deletion of phenylalanine at residue 508 ($\Delta F508$) in its amino acid sequence. The $\Delta F508$ -CFTR protein is misfolded and retained at the endoplasmic reticulum, where it is degraded rapidly. Small-molecule activators of defective $\Delta F508$ -CFTR folding/cellular processing (“correctors”) and channel gating (“potentiators”) may provide a strategy for therapy of CF that corrects the underlying defect. A number of small-molecule $\Delta F508$ -CFTR potentiators and correctors have been identified [4]. They were found by high-throughput screening for activation of the chloride channel.

We discovered that hyaluronan is exported from mesenchymal fibroblasts by MRP5 (multidrug resistance associated protein 5) [5] and from epithelial cells by CFTR [6]. Hyaluronan has an important role in epithelial clearing of the bronchial surface by facilitating ciliary beating [7,8]. In an attempt to evaluate interaction between CFTR and hyaluronan transmembrane transport, we synthesized a new class of drug-like compounds (Hylout4) that mimic the non-reducing end of hyaluronan and discovered that they activated chloride export from bronchial epithelial cells and hyaluronan export from breast cancer cells [9]. The compounds together with other known modulators were docked to the open and closed conformation of $\Delta F508$ -CFTR [10] to identify possible optimized activators for $\Delta F508$ -CFTR. Molecular modelling and physiological experiments showed that citrate serves as an activator of hyaluronan transport in $\Delta F508$ -CFTR.

2. Materials and Methods

2.1. Materials

Mouse-anti-CFTR-IgM was from Acris Antibodies, Hiddenhausen, Germany. Other chemicals were from Sigma-Aldrich Chemical Corporation (St. Louis, MO, USA).

2.2. Computational Studies

In silico docking studies were carried out to evaluate the affinity and binding interactions by molecular docking simulation using the AutoDock 4.2 and PyRx software—Python Prescription 0.8 to coordinates of the open and closed CFTR conformation [10–12]. ATP, citrate and docosahexaenoic acid (DHA) were docked as fully ionized molecules to enable ionic interaction with basic amino acids. The theoretical K_I -values of binding are listed in Table 1.

Table 1. Virtual docking of activators and inhibitors to the open conformation of CFTR.

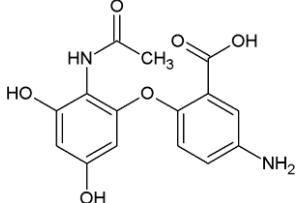
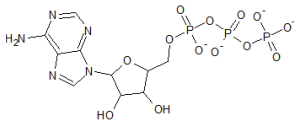
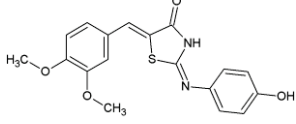
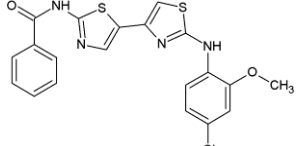
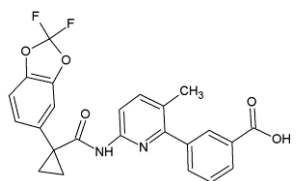
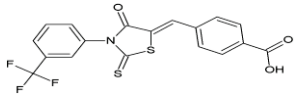
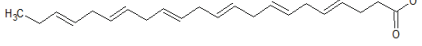
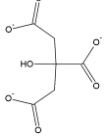
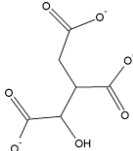
Name	Structure	Calculated Affinity K_m (μM)	Experimental Activation/ Inhibition EC ₅₀ (μM)	Ref.
Hylout4		7	100 for HA	[9]
ATP		0.022	41 for Cl [−]	[13]
CFTRact-06		1.4	1 for HA	[6]
Corr-4a		0.01	1 for Cl [−]	[14]
VX809		0.01	0.081 for Cl [−]	[15]
CFTR _{inh} 127		0.22	0.3 for Cl [−]	[16]
DHA		17		[17]
Citrate		2.7	6 for HA	

Table 1. Cont.

Name	Structure	Calculated Affinity Km (μM)	Experimental Activation/ Inhibition EC50 (μM)	Ref.
Isocitrate		1.1		

Docking was performed with the AutoDock software 1.5.6.rc3. The affinities of the best fits are listed.

2.3. Cell Lines

The cell line HMT3552 has been described previously [18]. The wildtype CFTR (16HBE14o[−]) and the mutant ΔF508-CFTR (CFBE41o[−]) cell line were from Dr. D.C. Gruenert [19].

2.4. Western Blotting of CFTR

Western blotting was performed as described [9].

2.5. Determination of Hyaluronan Export

Hyaluronan export of HMT3552 cells was performed as described [20].

2.6. Iodide Efflux

Iodide efflux experiments were performed as described [21].

3. Results

3.1. Virtual Docking

In an attempt to identify optimized CFTR activators, we docked Hylout4 [9] and several other known activators and inhibitors to the open and closed conformation of CFTR [10,12]. In preliminary docking calculations, we found that the outward-facing CFTR configuration [10,11] bound Hylout4 with higher affinities than the inward-facing ATP free configurations. The predicted Hylout4 binding site was a cleft between the nucleotide binding domains NBD1 (433 to 634) and NBD2 (1225 to 1415). The CFTR activators corr-4a, CFTR_{act}-06, VX809, the inhibitor CFTR_{inh}-172 and dietary supplement docosahexaenoic acid (DHA) also bound to this cavity with the highest affinity (Table 1). It comprised the amino acids R170, A171, E267, W465, F494, R553, K968, K1060, W1063, K1292, G1342, and K1351. The most striking feature of this site was the close neighbourhood of six basic amino acids (underlined) and the preference of lysines that have long flexible arms for ligand binding by ionic interactions and hydrogen bonds. The distances of positive charges in the open conformation ranged from 5 Å to 16 Å and in the closed conformation from 12 Å to 32 Å.

Evaluating other potential binding compounds, we included substrates of the intermediate metabolism and found that citrate and isocitrate showed considerable affinity for this binding site (Table 1, Figure 1).

Figure 1a shows the location of citrate in the cleft between the nucleotide binding domains. Figure 1b shows that citrate and the phosphate groups of ATP are located at the same position in close vicinity of F508, which was 11 Å apart from R553.

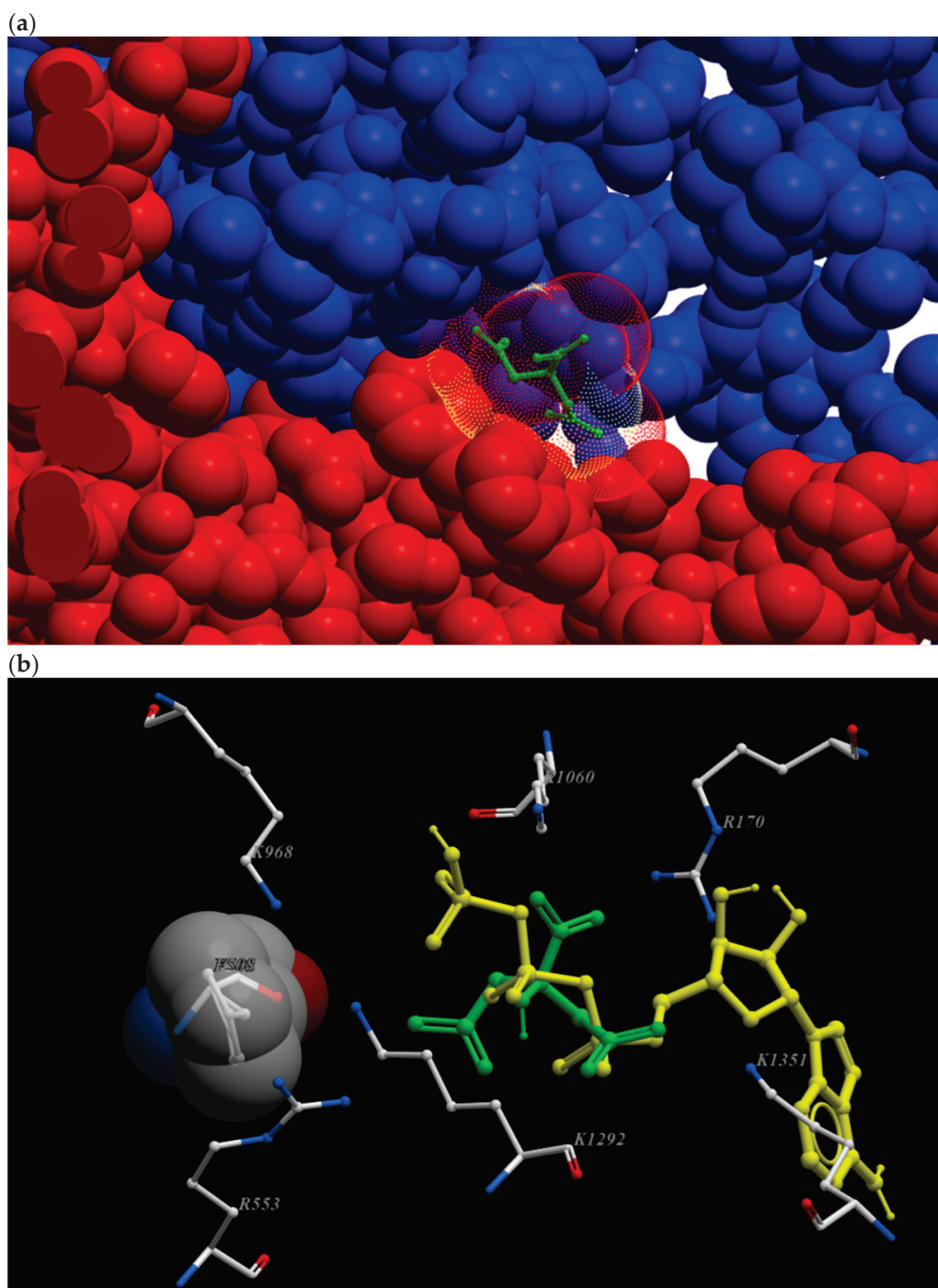


Figure 1. Binding site of citrate and ATP to the open conformation of CFTR. (a) Citrate (green) binds to a cleft between the nucleotide binding domains NBD1 (red) and NBD2 (blue). (b) Citrate (green) and ATP (yellow) bind to basic amino acid residues in the vicinity of F508.

3.2. Activation of Hyaluronan Export

Since hyaluronan is exported from human breast carcinoma cells by CFTR [5], citrate and isocitrate were tested for their influence on hyaluronan export in cell culture. Figure 2 shows that both compounds activated hyaluronan export in a concentration-dependent manner. Citrate showed a higher increase of hyaluronan export than isocitrate. Therefore, citrate was chosen for further evaluation.

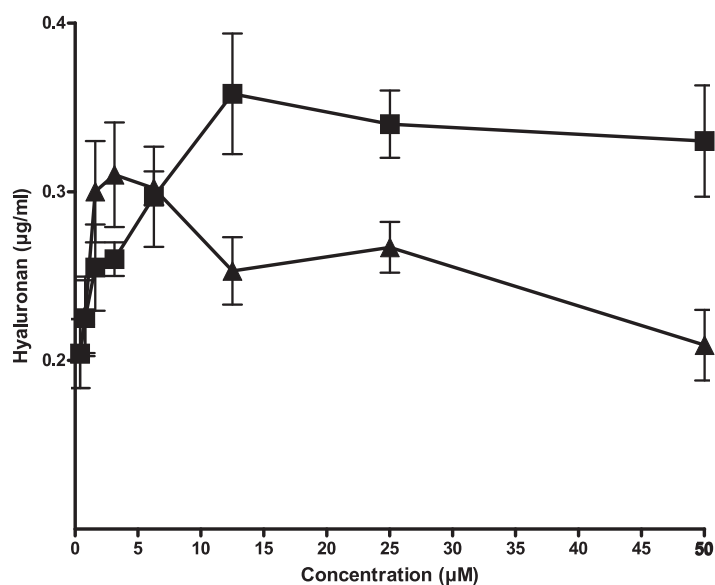


Figure 2. Activation of hyaluronan export by citrate (■) and isocitrate (▲) from HMT3552 cells. The error bars indicate the means of two determinations.

3.3. Iodide Efflux

In addition to chloride and hyaluronan, CFTR also exports iodide that can be measured by an iodide selective electrode. Iodide efflux from 16HBE14o- and CFBE41o- cells was assessed with increasing citrate concentrations. Citrate activated immediately iodide efflux from 16HBE14o- as well as from CFBE41o- cells (Figure 3), indicating that citrate recovered the activity of $\Delta F508$ -CFTR.

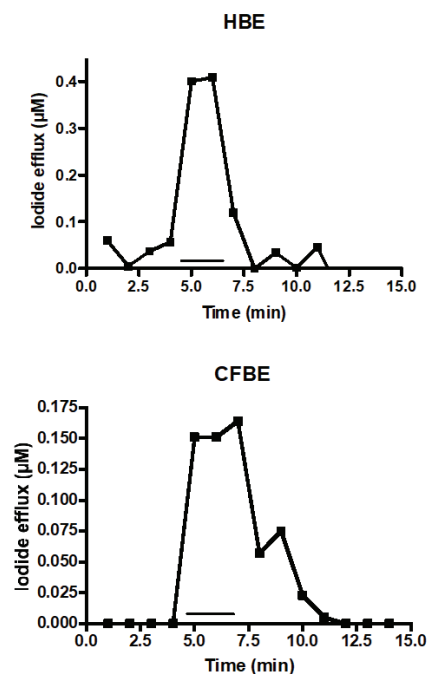


Figure 3. Citrate stimulated iodide efflux. CFTR can also export iodide instead of chloride. 16HBE14o- or CFBE41o- were loaded with iodide and the iodide concentration was measured by an iodide-sensitive electrode in 1 min intervals. The bar indicates the time period of exposure to 100 µM of citrate.

3.4. Recovery of $\Delta F508$ -CFTR Cell Surface Expression

The $\Delta F508$ -CFTR mutation impairs maturation and destabilizes the protein in post-Golgi compartments. Figure 4 shows that citrate increased $\Delta F508$ -CFTR cell surface expression, indicating that citrate recovered cellular processing of $\Delta F508$ -CFTR.

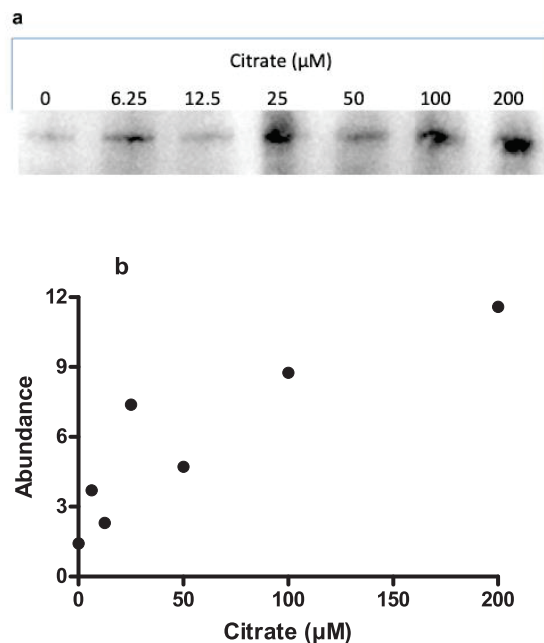


Figure 4. (a) Citrate recovered from $\Delta F508$ -CFTR cell surface expression as analysed by Western blotting. (b) relative abundance of cell surface-expressed $\Delta F508$ -CFTR.

4. Discussion

It is surprising that virtual docking of ATP has so far been conducted on computed CFTR fragments [22], but not the whole structural model, in spite of the long history of intensive pharmaceutical research. It is even more surprising that the highest docking affinity for ATP and other activators of CFTR function did not reside in the two known ATP binding sites, but in a cleft between the nucleotide binding sites NBD1 and NBD2. This site has been claimed to open the CFTR channel due to salt bridges between opposing basic and acidic residues [23]. Remarkably, the external phosphate of ATP overlaps with the positions of citrate and the carboxyl group of DHA. Thus, our docking results confirm the notion of salt bridges between the NBD1 and NBD2 domains; however, we propose that the salt bridges are mediated by anionic activators.

These basic amino acids are probably responsible for ionic nucleotide binding and stabilization of the open CFTR conformation. The ATP binding affinity to this binding site is very high with $K_D = 22$ nM in comparison to normal intracellular nucleotide triphosphate concentrations in the mM range, but such high ATP concentrations are required for reliable gating [24,25]. The ATP binding sites had previously been located by site-directed CFTR mutations to W401 in NBD1 and Y1219 in NBD2 [26] which are only 9.8 Å and 11.4 Å apart from our predicted amino acids R170 and K968, respectively. Virtual docking also revealed that the most known CFTR activators bound to this cavity by bridging both nucleotide binding domains and thus appear to function as ATP agonists stabilizing the open conformation. This mechanism of action has been shown for the CFTR activator VX-770, which opens the $\Delta F508$ -CFTR in an ATP-independent manner [27] and decouples gating from ATP hydrolysis [28]. It is interesting to note that also the inhibitor CFTR_{inh}-172 had high affinity to the ATP binding site. It could be an ATP antagonist which displaces ATP, destabilizing the open conformation.

The conspicuous proximity of six basic amino acids led us to investigate other possible ligands. We tested citrate and isocitrate and found considerable affinity of $K_D = 2.7 \mu\text{M}$ and $K_D = 1.1 \mu\text{M}$, respectively. These theoretical affinities were again much higher than the normal cytosolic concentrations of $80 \mu\text{M}$ to $220 \mu\text{M}$ for citrate and about $10 \mu\text{M}$ for isocitrate [29,30], indicating that high saturation density is again necessary for gating. The levels of both ATP and citrate are indicators of the cellular energy level [31].

Accordingly, our cell culture experiments showed that citrate activated hyaluronan export from breast carcinoma cells, stimulated iodide efflux from epithelial cells carrying normal CFTR as well as ΔF508 -CFTR and recovered it from premature intracellular degradation. It can thus be regarded as a potentiator as well as a corrector for the defective ΔF508 -CFTR protein.

Part of the cytosolic citrate is exported into the circulation, giving rise to citrate in plasma and urine. It is known that urinary citrate secretion from cystic fibrosis patients was significantly lower [32,33]. A striking similarity of citrate and hyaluronan is their high Ca^{2+} -chelating property with $K_D = 171 \mu\text{M}$ [34] and $K_D = 1.4 \text{ mM}$ [35], respectively, as compared to the normal plasma concentration of about 2 mM . Cystic fibrosis patients have abnormal Ca^{2+} homeostasis [36], and chelate formation of cytosolic citrate and Ca^{2+} could reduce the effective Ca^{2+} concentration which is required for normal ΔF508 -CFTR trafficking [37].

The function of citrate as Ca^{2+} chelator and as hyaluronan export activator may explain some of the observed metabolic disturbances of cystic fibrosis patients such as the associated diabetes [38] and lithiasis [33]. Diabetes favours a shift from the aerobic energy production by the citric acid cycle and respiration towards gluconeogenesis, causing withdrawal of metabolites from the citric acid cycle. Low citrate in other tissues such as kidney again decreases extracellular hyaluronan required for Ca^{2+} complexation and solubilization, causing lithiasis in cystic fibrosis patients [39–42]. Patients thus remain in a vicious cycle, unless they are treated by citrate as a food additive for stone solubilization [43–50]. Citrate is freely available in the supermarket and also a nutritional additive for treatment of lithiasis.

Citrate has been patented as an ingredient of inhalation solutions to reduce the viscosity of the sticky mucous in cystic fibrosis patients [51]. It was speculated that citrate might correct all other symptoms of cystic fibrosis by systemically recovering the function of ΔF508 -CFTR.

Citrate and isocitrate are intermediates of the citric acid cycle within mitochondria. They are nutrients taken up by mammalian cells and form two separated citrate pools: mitochondrial and cytosolic. The intracellular citrate level depends on nutrition: A carbohydrate-rich nutrition induces the citrate lyase to reduce the citrate concentration by transformation into fatty acids. A protein-rich nutrition induces anaplerotic reactions to increase intracellular citrate concentrations. A protein-rich diet has frequently been evaluated over the past decades with no final conclusion, but it seemed to be underestimated [52] probably because activation of hyaluronan export by citrate by the regulatory balance of carbohydrate-rich and protein-rich diets was not taken into account. Indeed, a low glycemic index diet has already been shown to be beneficial for cystic fibrosis patients [53,54].

Frequent and severe infections of cystic fibrosis patients by *Pseudomonas aeruginosa* are often responsible for morbidity in CF patients. These bacteria can upregulate an isocitrate lyase as a virulence factor for persistent infection [55], which further depletes citrate levels and thus inactivates hyaluronan export. Sufficient hyaluronan shedding is required for efficient ciliary beating and debris removal from airways [8]. Thus, a protein-rich and low carbohydrate diet supplemented with DHA and citrate could have benefits similar to expensive drugs and lucrative treatments.

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Article

Synergism Potentiates Oxidative Antiproliferative Effects of Naringenin and Quercetin in MCF-7 Breast Cancer Cells

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Abstract: Breast cancer (BC) is the most frequently diagnosed type of cancer as of 2020. Quercetin (Que) and Naringenin (Nar) are predominantly found in citrus fruits and vegetables and have shown promising antiproliferative effects in multiple studies. It is also known that the bioactive effects of these flavonoids are more pronounced in whole fruit than in isolation. This study investigates the potential synergistic effects of Que and Nar (CoQN) in MCF-7 BC cells. MCF-7 cells were treated with a range of concentrations of Que, Nar or CoQN to determine cell viability. The IC₅₀ of CoQN was then used to investigate caspase 3/7 activity, Bcl-2 gene expression, lipid peroxidation and mitochondrial membrane potential to evaluate oxidative stress and apoptosis. CoQN treatment produced significant cytotoxicity, reduced Bcl-2 gene expression and increased caspase 3/7 activity compared to either Nar or Que. Furthermore, CoQN significantly increased lipid peroxidation and reduced mitochondrial membrane potential (MMP) compared to either Nar or Que. Therefore, CoQN treatment has potential pharmacological application in BC chemotherapy by inducing oxidative stress and apoptosis in MCF-7 BC cells. The results of this study support the increased consumption of whole fruits and vegetables to reduce cell proliferation in cancer.

Keywords: quercetin; naringenin; breast cancer; oxidative stress; apoptosis

1. Introduction

By 2020, breast cancer (BC) was the most frequently diagnosed type of cancer and the fifth leading cause of cancer-related deaths globally in women [1]. Despite technological advancements in the diagnosis and treatment, BC continues to burden the healthcare systems due to increased prevalence associated with socioeconomic disadvantages and continued exposure to risk factors [2]. BC mainly originates in the lobules or ducts of the breast and can be non-invasive or invasive, spreading to the lymph nodes and metastasizing to other parts of the body [3]. Its treatment requires a multidisciplinary approach involving surgery, radiation, chemotherapy, endocrine therapy and human epidermal growth factor receptor 2 (HER2)-targeted therapy depending on staging and molecular subtype [4]. Despite advancements in therapeutic approaches to BC, drug side effects due to narrow therapeutic indices and radiation burden reduce compliance. Therefore, novel therapeutic agents targeting BC cells with less cytotoxicity are required.

Polyphenolic flavonoids are plant-derived bioactive chemical substances [5] with anticancer, antioxidant, anti-inflammatory, antidiabetic and cardioprotective properties [6]. Flavonoids have been shown to exert their chemotherapeutic effects in various cancers by multiple pathways, including increased oxidative stress and apoptosis leading to reduced cell proliferation, invasion and migration [7]. Quercetin (Que) and Naringenin (Nar) are significant flavonoids found in many plant sources, including onions, broccoli, berries and citrus fruits [8,9]. Nar is the aglycone form of the flavanone glycoside, naringin [9]. The pharmacological effects of Que or Nar have previously been investigated in vitro in

MCF-7 and MDA-MB-231 cell lines and in vivo using xenografted or genetically modified BC mice models with promising results [10–12]. However, the administration of flavonoids usually requires high concentrations to produce a cytotoxic effect in cancer cells due to their low efficacy [13]. Co-administered flavonoids could have synergistic effects at lower concentrations and enhance selective toxicity in cancer cells [14–16]. We posit that plant bioactive chemical ingredients are more effective in their natural composition than when isolated. Compounds which are constituents of a particular plant can influence biological processes in a synergistic manner.

The intrinsic pathway of apoptosis (programmed cell death) involves non-receptor mediated intracellular signaling generated upon stimuli. In contrast, the extrinsic pathway requires binding an extracellular ligand to death receptors anchored in the cellular membrane [17]. Anti-apoptotic protein, Bcl-2, plays an essential role in preventing intrinsic pathway-induced apoptosis by inhibiting caspase cascade activation [18]. Bcl-2 protein expression is usually upregulated in cancerous cells resulting in increased proliferation and reduced apoptosis [19]. Flavonoids have been reported to exert their antiproliferative effects by downregulation of Bcl-2. This leads to the activation of the proapoptotic proteins of the Bcl-2 family, Bax and Bak, allowing their aggregation in the mitochondrial membrane. Subsequently, cytochrome complex (cyt-c) is released from the mitochondrion into the cytoplasm, which activates the apoptotic peptidase activating factor (Apaf1) protein. This activates caspase 9, which initiates the caspase cascade via the intrinsic pathway, leading to the induction of apoptosis [20] (Figure 1).

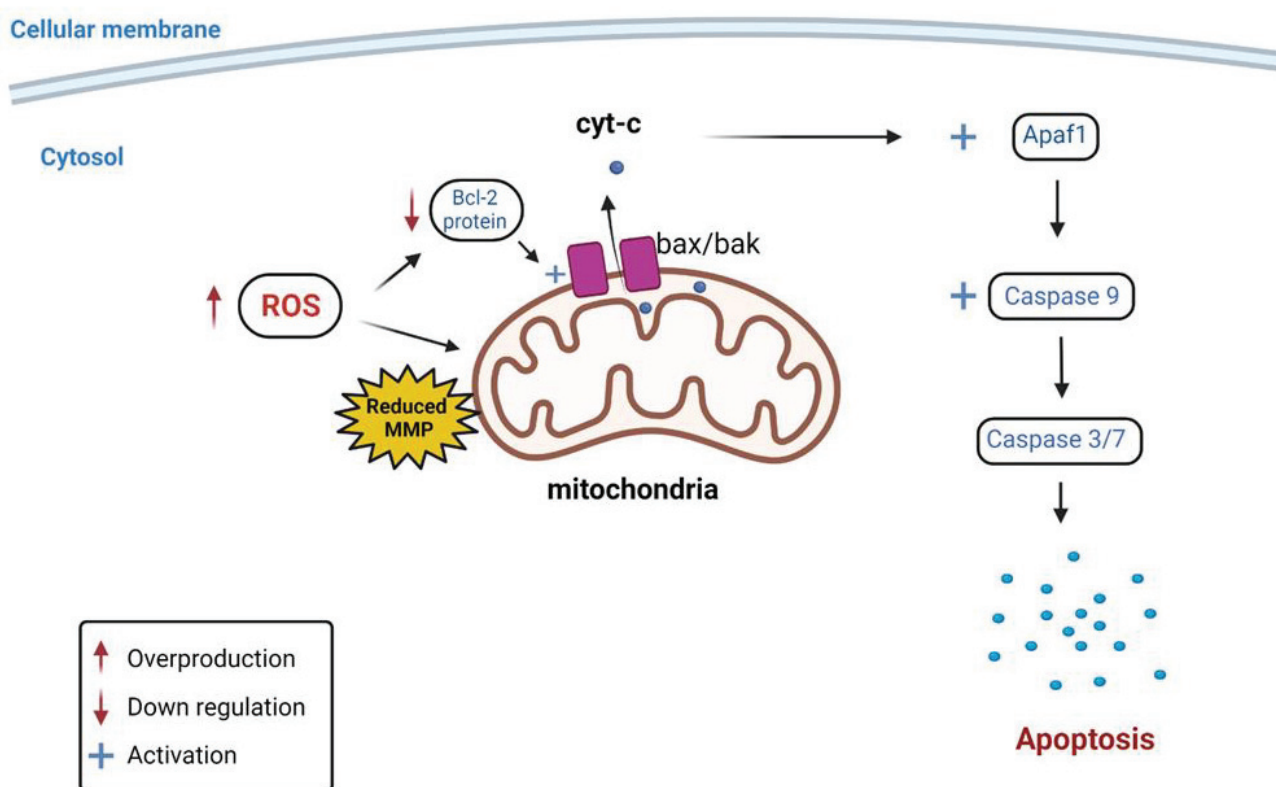


Figure 1. Schematic illustration of the effect of increased ROS production on Bcl-2 protein, MMP, caspase activity and intrinsic apoptotic pathway activation. ROS = Reactive oxygen species; MMP = Mitochondrial membrane potential; cyt-c = Cytochrome c; Apaf1 = Apoptotic peptidase activating factor 1; ↑ = Overproduction; ↓ = Down regulation; + = Activation.

Reactive oxygen species (ROS) are harmful free radicals, including hydroxyl radicals ($\bullet\text{OH}$) and superoxide radicals ($\text{O}_2^{\bullet-}$), or aid in the formation of free radicals, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$). They are counteracted and neutralized

by enzymatic or non-enzymatic antioxidants [21]. An imbalance between ROS production and antioxidant activity results in oxidative stress and damage to cellular components, such as lipid membranes and nuclear and mitochondrial DNA [22]. Flavonoids have been shown to exert pro-oxidant effects in various types of cancer cells by several mechanisms, including auto-oxidation generating free radicals, the depletion of antioxidant stores and the inhibition of enzymatic antioxidant activity [23]. Oxidative stress can also cause a reduction in mitochondrial membrane potential (MMP) due to mitochondrial membrane damage. This leads to mitochondrial dysfunction, reduced Bcl-2 activity and the release of cyt-c into the cytoplasm, which activates the caspase cascade through the intrinsic apoptotic pathway [24,25] (Figure 1). Therefore, increased lipid peroxidation and reduced MMP are valuable indicators of oxidative stress-mediated apoptosis.

Therefore, this study aimed to examine the synergistic effect of Que and Nar co-administration (CoQN) compared to either flavonoid alone in MCF-7 BC cell lines. Cell viability, lipid peroxidation, mitochondrial dysfunction and apoptosis of MCF-7 BC cells were used as efficacy indices.

2. Materials and Methods

2.1. Cell Culture

MCF-7 cell lines were purchased from Cellonex[®] (Johannesburg). Cell lines were grown to 80% confluency in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% (*v/v*) fetal bovine serum, 1% (*v/v*) penicillin-streptomycin antibiotics, 1% (*v/v*) L-glutamine and 1% (*v/v*) HEPES buffer in 25 cm² flasks. The cell cultures were maintained at 37 °C in a humidified 5% CO₂ incubator. The media was changed every 24 to 48 h. All the work was carried out under aseptic techniques and cell culture protocol.

2.2. Cell Treatments

Que was purchased from Sigma-Aldrich Pty Ltd. (Johannesburg, South Africa; Pcode: 1002181950; CAS number: 117-39-5; Lot ID: SLBM7336V). Nar was purchased from Sigma-Aldrich Pty Ltd. (Johannesburg, South Africa; Pcode: 1002456338; CAS number: 67604-48-2; Lot ID: MKBW8640V). Dimethyl sulfoxide (DMSO) was used to prepare 10 mg/mL stock concentrations of Que and Nar. The cells were then exposed to either Que or Nar only or combined at concentrations required for assays with a final DMSO concentration of less than 0.01%.

2.3. Cell Viability Assays

Methylthiazol tetrazolium bromide (MTT) assay was used to determine MCF-7 cell viability. MCF-7 cells (1.5×10^4 /well) were seeded in a 96-well plate and incubated for 24 h. Cells were then treated with serial (0–250 µg/mL) concentrations of Que or Nar or a combination at a concentration range of 0–50 µg/mL. Three ratios (50:50, 40:60, and 60:40 for Nar:Que) were used in the combination treatments to assess the most effective ratio. After 24 h of treatment, 20 µL of 5 mg/mL MTT solution and 100 µL of cell culture media were added to the cells and incubated for 4 h. Afterwards, the MTT solution was aspirated and 100 µL of DMSO was added to solubilize the formazan dye. A microplate reader was used to measure absorbance at 570 nm, and cell viability was calculated as a percentage compared to the control.

2.4. Apoptosis Detection

Caspase 3/7 fluorometric assay (Biocom[®], Johannesburg, South Africa) was used to determine apoptosis induction. MCF-7 cells were seeded in a 96-well plate (1.0×10^4 /well) and incubated for 24 h. The cells were then treated with Que, Nar or both at a concentration of 44.31 µg/mL for 24 h. The cells were then treated with 100 µL of caspase loading solution for caspase 3/7 detection and incubated for 1 h at room temperature. After that, fluorescence was measured using a microplate reader at an excitation/emission wavelength of 535/620 nm. Results were expressed as relative fluorescence units (RFU).

2.5. Real-Time qPCR Analysis

Real-Time qPCR was used to assess the expression of anti-apoptotic Bcl-2 mRNA. Total RNA was extracted from MCF-7 cells previously treated with Nar, Que or both at a concentration of 44.31 µg/mL, respectively, using Quick-RNA Miniprep Kit (Zymo Research Ltd., Johannesburg, South Africa) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from extracted RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Real-Time qPCR was carried out for Bcl-2 with β-actin as an internal control using the following primers (Biotec®, Johannesburg, South Africa): Bcl-2 (F: 5'-GGTGGTGGAGGAAGCTCTTCA-3'; R: 5'-ATGCCGGTTCAGGTACTCAG-3'), β-actin (F: 5'-GGAGATTACTGCCCTGGCTCCTA-3'; R: 5'-GACTCATCGTACTCCTGCTTGCTG-3').

RT-qPCR amplification and measurement were performed using SSo advanced SYBR Green Supermix (BioRad®, Hercules, CA, USA) on BioRad qPCR thermocycler in the following conditions: 30 s at 95 °C for polymerase activation followed by 40 cycles of 5 s at 95 °C for denaturation and 15 s at 55 °C for annealing and extension. Gene expression was then analyzed and normalized against the internal control. Results were displayed relative fold change compared to the control ($2^{-\Delta\Delta Ct}$) [26].

2.6. Thiobarbituric Acid Reactive Substances Assay

Thiobarbituric acid reactive substances (TBARS) assay was used to determine lipid peroxidation. MCF-7 cells were treated with Nar, Que or both at a concentration of 44.31 µg/mL. After that, 2% H₃PO₄ was added to cell suspension (2×10^5 cells) in respective treatment tubes, whereas 0.1 M malondialdehyde (MDA) was used as a positive control. After that, 7% H₃PO₄ was then added, followed by 400 µL of 0.1 mM thiobarbituric acid (TBA)/Butylated hydroxytoluene. Subsequently, 1 M HCl was added, and the tubes were heated in a water bath for 15 min. Butanol (1.5 mL) was then added, after which the absorbance of the resultant supernatant was measured in a 96-well using a microplate reader at 532 nm and 600 nm. MDA concentrations were then calculated using the extinction coefficient of the MDA-TBA adduct ($155 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.7. Mitochondrial Depolarization Detection

MMP was determined using JC-10 fluorometric assay kit (Sigma-Aldrich Ltd., St. Louis, MO, USA) according to the manufacturer's instructions. MCF-7 cells were seeded in a 96-well plate (1.5×10^4 /well) in triplicates following treatment with Que, Nar or CoQN at 44.31 µg/mL, respectively, for 24 h. After that, 50 µL of JC-10 loading dye was added to each well and incubated for 30 min in the dark. Red and green fluorescence were then measured using a microplate reader at an excitation/emission wavelength of 540/590 and 490/525, respectively. The ratio of red to green fluorescence was calculated to determine the MMP. Results were expressed as a percentage of control of the red/green fluorescence ratio.

2.8. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism version 9.4.1., GraphPad Software, San Diego, CA, USA. The concentration–response inhibition equation using non-linear regression was used to analyze the IC₅₀ in the MTT assay. Statistical significance was assessed by unpaired t-test with Welch's correction for 2 variable comparisons of each treatment (Que, Nar and CoQN) against the control in the results obtained from the caspase 3/7 assay, Real-Time qPCR, TBARS assay and mitochondrial depolarization assay. Treatment samples (Que, Nar and CoQN) were used in triplicate and experiments were repeated three times for all assays performed. A *p* value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. MCF-7 Cell Viability

The MTT assay was used to assess the toxicity of Nar, Que or CoQN to MCF-7 BC cells and to determine cellular metabolic activity indicated by mitochondrial dehydrogenase activity. The administration of Que or Nar at a concentration range of 0–250 $\mu\text{g/mL}$ resulted in decreased toxicity to MCF-7 cells compared to CoQN-treated cells. Neither Nar nor Que showed a dose-dependent response, with extrapolated IC_{50} values of 468 $\mu\text{g/mL}$ and 91.10 $\mu\text{g/mL}$, respectively (Figure 2A). At the 60:40 (N:Q) combination ratio, the calculated IC_{50} (44.31 $\mu\text{g/mL}$) and dose-dependence were significantly superior to either 50:50 or 40:60 ratios, respectively (Figure 2B). Hence, the 60:40 combination ratio was used in subsequent experiments.

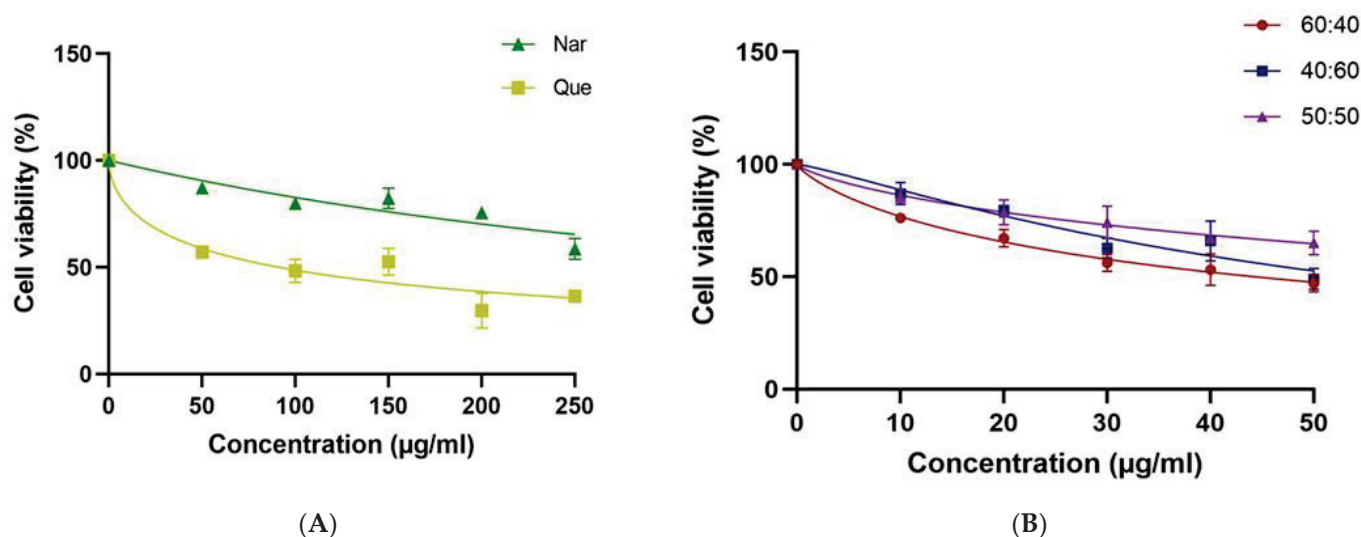


Figure 2. Dose-dependent MCF-7 cell viability as determined MTT assay after the cells were exposed to (A): different concentrations of Nar or Que, respectively, and (B): different combination ratios of Nar and Que, respectively.

3.2. Apoptosis Induction

Caspase 3/7 fluorometric assay was used to compare treatment effects with 44.31 $\mu\text{g/mL}$ of Que, Nar or both (CoQN) on the apoptosis of MCF-7 BC cells. CoQN significantly ($p = 0.0052$) increased executioner caspase 3/7 activity compared to control or either Nar or Que, signifying increased cell apoptosis (Figure 3).

The gene expression of Bcl-2 in MCF-7 cells was determined by qPCR following the RNA isolation and cDNA synthesis of all treatment groups to measure the apoptosis pathway. CoQN significantly ($p < 0.05$) reduced the mRNA expression of the anti-apoptotic Bcl-2 gene compared to either control or Que alone, signifying the activation of the intrinsic apoptotic pathway (Figure 4). Bcl-2 gene expression in Nar-treated cells was similar to CoQN.

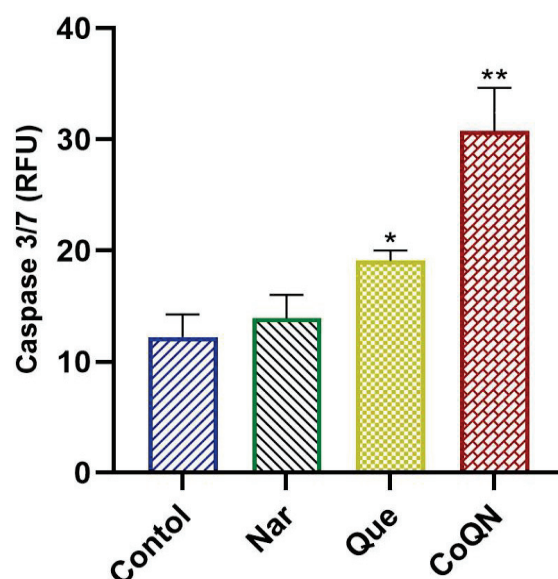


Figure 3. Caspase 3/7 fluorometric assay after MCF-7 BC cells were exposed to 44.31 $\mu\text{g/mL}$ of Nar, Que or both, respectively. * $p < 0.05$ relative to control. ** $p < 0.01$ relative to control. RFU = Relative fluorescence units; Ex/Em = Excitation/Emission; BC = Breast cancer; Nar = Naringenin; Que = Quercetin. CoQN = Co-administration of quercetin and naringenin.

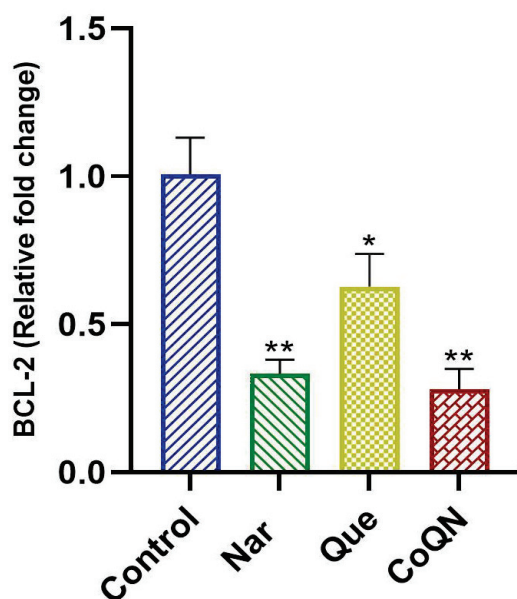


Figure 4. BCL-2 gene expression as determined by qRT-PCR after MCF-7 BC cells were exposed to 44.31 $\mu\text{g/mL}$ of Nar, Que or CoQN, respectively. * $p < 0.05$ relative to control. ** $p < 0.01$ relative to control. BC = Breast cancer; Nar = Naringenin; Que = Quercetin. CoQN = Co-administration of quercetin and naringenin.

3.3. Lipid Peroxidation

The TBARS assay was used to assess ROS production by quantifying the end-product of lipid peroxidation, MDA, in MCF-7 cells following 24-h treatment with 44.31 $\mu\text{g/mL}$ of Que, Nar or both (CoQN) relative to control. The CoQN treatment significantly ($p = 0.024$) increased MDA concentrations compared to the control, Que or Nar, respectively (Figure 5). Treatments with Que or Nar did not show a significant increase in MDA concentration compared to the control (Figure 5).

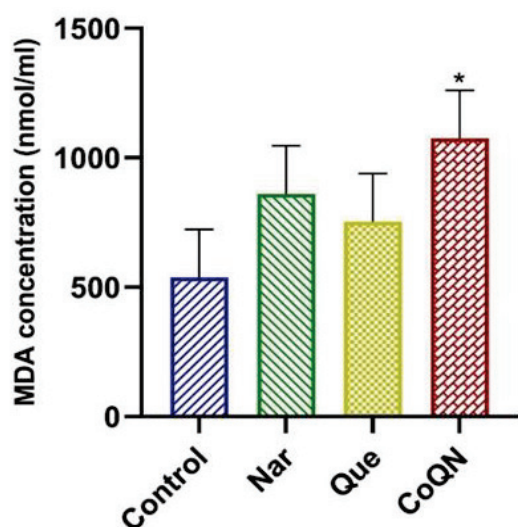


Figure 5. Lipid peroxidation measured by MDA concentrations after MCF-7 BC cells were exposed to 44.31 $\mu\text{g/mL}$ of Nar, Que or CoQN, respectively. * $p < 0.05$ relative to control. MDA = Malondialdehyde; BC = Breast cancer; Nar = Naringenin; Que = Quercetin. CoQN = Co-administration of quercetin and naringenin.

3.4. Mitochondrial Depolarization

Mitochondrial depolarization in MCF-7 cells was determined by assessing the MMP using the JC-10 dye following treatment with 44.31 $\mu\text{g/mL}$ of Que, Nar or CoQN, respectively. Treatment with CoQN or Nar significantly ($p < 0.05$) decreased MMP compared to controls and Que (Figure 6), suggesting mitochondrial depolarization leading to induction of apoptosis.

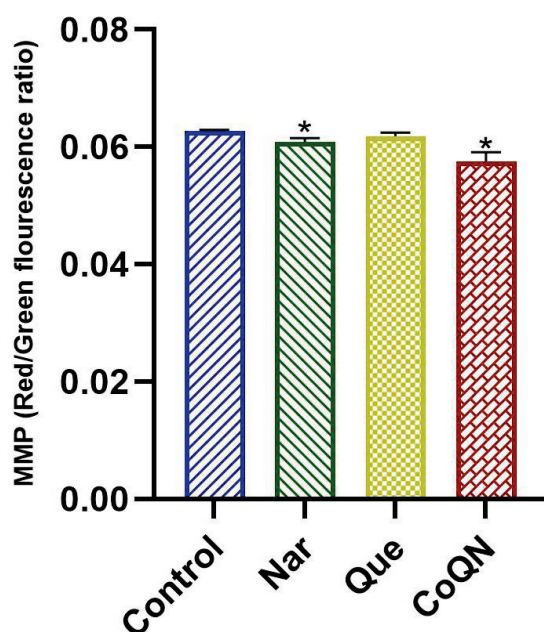


Figure 6. MMP measured by red/green fluorescence ratio after MCF-7 BC cells were exposed to 44.31 $\mu\text{g/mL}$ of Nar, Que or CoQN, respectively. * $p < 0.05$ relative to control. MMP = Mitochondrial membrane potential; BC = Breast cancer; Nar = Naringenin; Que = Quercetin. CoQN = Co-administration of quercetin and naringenin.

4. Discussion

Despite advancements in cancer therapeutics, current chemotherapeutic agents are still undesirable due to cytotoxicity and low selective toxicity [27]. Therefore, novel approaches, including combination therapies for targeting cancerous cells with minimal side effects, are required.

Previous studies have reported that Nar or Que exert promising chemotherapeutic effects in MCF-7 BC cells by reducing cell viability and inducing apoptosis [28–31]. However, high concentrations were required to achieve a minimum inhibitory response. Moreover, both Que and Nar have poor water solubility, which could impact their bioavailability for cellular uptake and further reduce efficacy [32,33]. In previous studies, a high concentration of 780–880 μM (212–239 $\mu\text{g/mL}$) of Nar was needed to reduce cell viability by 50% (IC_{50}) in MCF-7 BC cells [31]. This study used DMSO to dissolve Que and Nar to improve cellular uptake and penetration. An IC_{50} of 468 $\mu\text{g/mL}$ and 91.1 $\mu\text{g/mL}$ were determined for Nar and Que, respectively. However, our results showed that CoQN at a 60:40 ratio produced a significant synergistic effect on MCF-7 cell viability at a lower concentration (44.3 $\mu\text{g/mL}$) after a 24-h incubation period compared to 40:60, 50:50 ratios and either flavonoid alone, respectively Figure 2A,B. This indicates the synergistic potency of the combination of the two flavonoids.

Chemotherapeutic cytotoxicity by increased apoptosis is an effective method in cancer treatment leading to the reduction of cell proliferation [19]. Caspases are cysteine proteases that play a vital role in apoptosis execution through intrinsic and extrinsic pathways. Caspase 3/7 are known as executioner caspases, whereas caspases 9 and 8 are responsible for caspase induction by the intrinsic and extrinsic apoptotic pathways [34]. Previous studies have reported apoptosis induction by caspase activation following treatment with Nar or Que in different cancers, including BC [11,35–39]. To evaluate the synergistic effects of CoQN on apoptosis induction in MCF-7, the level of caspase 3/7 activity was compared to either Que or Nar alone. Our findings demonstrated an increase in caspase 3/7 activity in MCF-7 after a 24-h treatment period with Nar and Que alone compared to untreated cells (Figure 3). However, CoQN produced significantly higher caspase 3/7 activation compared to either Que or Nar alone at the same concentration (44.3 $\mu\text{g/mL}$), leading to more profound apoptosis induction and reduced cell proliferation (Figure 3).

Furthermore, the anti-apoptotic Bcl-2 protein activity was investigated by measuring its mRNA expression in the different treatment groups. The downregulation of Bcl-2 is a valuable mechanism for apoptosis induction via the intrinsic pathway [18,34]. Que and Nar have previously been reported to exert cytotoxic effects by regulating several members of the Bcl-2 family of proteins, including Bax, Bcl-xl and Bcl-2, resulting in intrinsic pathway-mediated apoptosis in neoplastic cells [40–47]. Our study shows that Que and Nar cause a significant decrease in Bcl-2 mRNA expression in MCF-7 cells compared to untreated cells (Figure 4). However, our study also showed that Nar or CoQN produced the significant downregulation of Bcl-2 expression compared to either Que or controls, suggesting a synergistic effect leading to intrinsic pathway-mediated apoptosis in MCF-7 BC cells (Figure 4).

The overproduction of intracellular ROS by endogenous or exogenous sources is one of the major driving forces leading to apoptosis induction [48]. ROS are mainly produced in mitochondria as metabolic by-products in biological systems [48]. Enzymatic antioxidants, such as catalase and superoxide dismutase (SOD), break down and remove free radicals. In contrast, non-enzymatic antioxidants, such as carotenoids and glutathione, convert free radicals into hydrogen peroxide (H_2O_2) and then water, in the presence of metal cation cofactors [21]. Malignant cells lack some antioxidants, such as catalase, which is present in non-cancerous cells and cannot, therefore, neutralize free radicals.

Furthermore, flavonoids have been shown to exhibit pro-oxidant effects in cancer cells [49]. Some flavonoids can complex with metal cations, leading to the increased production of superoxide ions through Fenton reactions, which induces apoptosis and halts cell proliferation [23]. Quercetin can also undergo auto-oxidation resulting in the

formation of the radical, Quercetin-O●, which further leads to free radical accumulation [50] or can diminish glutathione stores and inhibit the enzymatic antioxidant, thioredoxin reductase [51,52]. Naringenin has also displayed pro-oxidant effects in malignant cells by inhibiting glutathione peroxidase and the ROS-dependent activation of ERK1/2 [53,54]. The overall increase in ROS production induces lipid peroxidation and MDA formation, which damages cellular components, such as membrane lipids, and mitochondrial DNA, leading to apoptosis and reduced cell proliferation [55].

The resultant oxidative stress-induced apoptosis has been reported in several neoplastic cells, including BC, hepatocellular carcinoma, cervical cancer and skin cancer [12,56–59]. In our study, neither Que nor Nar at a lower concentration (44.3 µg/mL) showed a significant increase in MDA production, indicating no significant increase in lipid peroxidation and ROS production. However, CoQN administration at the same concentration showed a significant increase in lipid peroxidation, signifying a synergistic effect resulting in increased ROS production (Figure 5). This agrees with a recent study which reported that high ROS production with Que leads to synergistic chemotherapeutic effects with tamoxifen in MCF-7 cells [56]. Nar and curcumin co-delivery in nanoparticles in MCF-7 cells also increased ROS production and apoptosis induction [60]. Furthermore, increased ROS production and lipid peroxidation has previously been linked to the downregulation of Bcl-2 expression associated with subsequent caspase 3/7 activation leading to apoptosis [61–63]. This can be connected to the observed significant reduction in Bcl-2 expression and increased caspase 3/7 activity observed with CoQN treatment (Figures 3 and 4).

Another marker of potential apoptosis induction is MMP reduction, indicating mitochondrial depolarization [64]. Excess intracellular ROS production is one of the major factors leading to mitochondrial dysfunction and associated decrease in MMP [65]. Antineoplastic effects of flavonoids have previously been reported [58,59,66–68]. Therefore, MMP expression was assessed in MCF-7 cells following Que, Nar or CoQN exposure. Results showed that CoQN caused a slightly significant decrease in MMP compared to either Que or Nar, respectively (Figure 6). The synergistic activity of Que with curcumin has previously been reported in a study by Zhang et al. in gastric cell lines, demonstrating a reduction in MMP with subsequent apoptosis induction and reduced cell proliferation [69]. Similarly, Nar and curcumin caused oxidative stress-induced mitochondrial depolarization resulting in apoptosis induction in MCF-7 cells [60]. Therefore, we propose that the observed reduction in MMP following CoQN exposure results from the synergistic pro-oxidant effects of Que and Nar in MCF-7 cells indicated by the significant increase in ROS production. We used 60:40 ratio for Que:Nar, which closely mirrors their relative native concentration ratios in citrus fruits [70] and the enhanced antiproliferative effects could be a manifestation of their biological effects in the native fruit. This approach would give an opportunity for formulation of these flavonoids or their synthetic analogs into effective biopharmaceuticals.

Therefore, it can be concluded that CoQN shows promising synergistic antiproliferative effects in MCF-7 cells compared to either Nar or Que, respectively; by increasing lipid peroxidation, inducing mitochondrial depolarization, anti-apoptotic Bcl-2 suppression and concomitant activation of caspase 3/7. Flavonoids are known to be non-toxic to non-malignant cells [11,71,72]; hence more studies are suggested to confirm their antineoplastic role in this regard.

5. Conclusions

The co-administration of the flavonoids Que and Nar shows promising synergistic antiproliferative effects in MCF-7 BC cells compared to either alone by significantly increasing oxidative stress, reducing MMP and inducing apoptosis, leading to cytotoxicity and a reduction in cell viability. Our results here support the notion that the increased consumption of fruits and vegetables rich in these flavonoids is associated with a reduced incidence of many types of cancers. We posit that these flavonoid combinations further

be developed as chemotherapeutic agents against breast cancer. These flavonoids or their synthetic analogs could further be investigated and delivered in vivo via appropriate vehicles in nanoparticles delivery system as biopharmaceuticals.

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Review

Alpha-Lipoic Acid and Glucose Metabolism: A Comprehensive Update on Biochemical and Therapeutic Features

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Abstract: Alpha-lipoic acid (ALA) is a natural compound with antioxidant and pro-oxidant properties which has effects on the regulation of insulin sensitivity and insulin secretion. ALA is widely prescribed in patients with diabetic polyneuropathy due to its positive effects on nerve conduction and alleviation of symptoms. It is, moreover, also prescribed in other insulin resistance conditions such as metabolic syndrome (SM), polycystic ovary syndrome (PCOS) and obesity. However, several cases of Insulin Autoimmune Syndrome (IAS) have been reported in subjects taking ALA. The aim of the present review is to describe the main chemical and biological functions of ALA in glucose metabolism, focusing on its antioxidant activity, its role in modulating insulin sensitivity and secretion and in symptomatic peripheral diabetic polyneuropathy. We also provide a potential explanation for increased risk for the development of IAS.

Keywords: alpha-lipoic; glucose metabolism; diabetes prevention; complications

1. Introduction

Alpha-lipoic Acid (ALA) is a natural compound with diverse biochemical functions, especially in its reduced form dihydro-lipoic acid (DHLA). It acts as a metal chelator, regenerates endogenous antioxidants such as vitamins C and E and is a modulator of the signalling transduction of several pathways [1]. Many studies have indicated its potential role in the regulation of glucose metabolism, highlighting its effects on insulin sensitivity [2], insulin secretion [3], the reduction of circulating lipid levels [4] and the increase of nitric oxygen [5]. Further, ALA also seems to play a role in improving peripheral diabetic polyneuropathy [6]. It is, therefore, widely prescribed in both type 1 and type 2 diabetes (T1D; T2D) [7,8] diabetic neuropathy and in other insulin resistance conditions such as metabolic syndrome (MS), polycystic ovary syndrome (PCOS) and obesity [9–11].

However, numerous reports have been published in the last 15 years describing cases of Insulin Autoimmune Syndrome (IAS) in genetically predisposed subjects taking ALA. The first case was reported in Japan [12] and followed by reports from other countries, including Italy [13,14]. Our group has also described two new cases of IAS triggered by ALA and associated with HLA-DRB1*04:03, in two women [15].

Here we aim to review the main chemical and biological functions of ALA in glucose metabolism, focusing on its antioxidant activity, its role in modulating insulin sensitivity and secretion and in improving symptomatic peripheral diabetic polyneuropathy.

2. Chemistry and Metabolism of ALA

Alpha-lipoic acid, or 1,2 dithiolane-3-pentanoic acid or thioctic acid, is an endogenous cofactor of important enzyme complexes necessary to produce energy. Initially described

as a vitamin used for bacterial growth, it was isolated for the first time in 1951 by the biochemist Lester Reed from a liver sample [16].

The term alpha-lipoic acid derives from the fact that it is soluble in fats. Its molecule consists of two atoms of sulphur and eight of carbon. It is amphiphilic and due to this property is readily taken up in tissues, including the nervous system, as it can cross the blood-brain central barrier [17,18]. It has an asymmetric carbon atom with two optical isomers, the dextrorotatory (R-ALA or +ALA) and the levo-rotatory (S-ALA or −ALA). R-ALA form is naturally available and can be found free or conjugated to lysine residues, making it an essential cofactor, while synthetic ALA is a racemic mixture of +ALA and −ALA (+/− ALA). The oxidized and reduced form (ALA/DHLA) works as a powerful antioxidant inactivating free radicals and reactive oxygen species (ROS) and enhancing the activity of other endogenous antioxidants (vitamin C, E, glutathione) [18,19].

Although its biosynthesis pathway is not yet entirely clear, there is evidence of ALA synthesis in the mitochondria of mammalian cells [20]. ALA synthesis starts from a fatty acid of eight carbon atoms and cysteine [21,22] probably in the liver, which also seems to be responsible for its catabolism [22].

ALA is found in high concentrations in both animal and plant sources, although the highest content is found in animal tissues with a high metabolic activity, such as the heart, while muscle tissue has lower concentrations [23]. Good animal sources of ALA are pork and calf meat, with the highest amounts found in heart, liver and kidneys. Good plant sources are spinach, broccoli, tomatoes, peas, brussels sprouts and rice bran (in descending order of content) [17]. However, in foods, the R enantiomer of alpha-lipoic acid is often covalently linked to the lysine residues of proteins, which reduces its systemic bioavailability [23].

3. ALA: Cellular Energy and Oxidative Stress

ALA is both an anti-oxidant and a pro-oxidant agent [24,25], inactivating free radicals and reactive oxygen species and modulating several signalling cascades involved in aging. ALA has diverse functions which can vary in relation to other compounds present in the cell, e.g., ALA can stimulate glucose uptake especially in muscles and adipocytes.

In describing the properties of this molecule, we will start by analysing the basic biochemical ones and then move on to those involved in glucose metabolism.

ALA is a cofactor of the pyruvate-dehydrogenase complex, which converts pyruvate obtained from glycolysis to acetyl-CoA. Lipoic acid in its oxidized form (ALA/lipoate) is first reduced (DHLA) and then oxidized again [26]. Through this biochemical reaction the pyruvate obtained from glycolysis can, by transforming into acetyl-CoA, start the Krebs cycle, which promotes the production of ATP and therefore the energy necessary for the cell to perform its functions. It has therefore been hypothesized that ALA could reduce the Warburg effect in cancer cells by stimulating the transformation of pyruvate into acetyl-CoA, rather than lactate, through the cofactor activity of the enzyme pyruvate-dehydrogenase (PDH) [27].

ALA acts as both a direct and indirect antioxidant: it is a scavenger of hydroxyl radicals, hypochlorous acid, singlet oxygen and peroxyl radicals [19,27]. It also increases the intracellular amount of glutathione and ascorbate [25,28], thus indirectly increasing the antioxidant ability of the cell and chelates iron and copper [24]. There is also some evidence that ALA can reduce the advanced glycation end products in neuronal cells with promising results for the treatment of Alzheimer's disease [27].

ALA's effects on cellular metabolism and oxidative stress probably have several systemic implications for both glucose and lipid metabolism. In the following sections we will discuss in depth how these properties can interact with glucose metabolism. We will first analyse ALA's effects on insulin sensitivity and secretion, then discuss related diseases such as insulin resistance and diabetes. With regard to lipids, ALA seems to reduce both total cholesterol and triglycerides in animal models [4], suggesting a possible role also in the treatment of dyslipidaemias [29].

4. ALA and Glucose Metabolism

4.1. Effect of ALA on Glucose Uptake

Several studies over the past years have demonstrated that both ALA and DHLA can increase glucose uptake [2,30,31]. In particular, Eason et al. demonstrated that glucose uptake increased by 300% when muscles of ob/ob mice, a model of severe insulin resistance, were incubated with ALA alone, while no changes occurred when muscles were incubated only with insulin, thus demonstrating the effect of ALA on insulin resistance [30].

Further studies have confirmed this effect and several molecular pathways have been explored. One of these pathways includes the activation of the insulin receptor cascade. Treatment with R (+) alpha-lipoic acid stimulates the activity of PI3K and the phosphorylation of insulin receptor substrate-1 (IRS-1) in 3T3-L1 adipocytes [31]. The phosphorylation of the substrate of the insulin receptor involves the activation of further intracellular mediators and subsequently the translocation of GLUT4 [31]. Diesel et al. revealed a direct binding site for alpha-lipoic acid at the tyrosine kinase domain of the insulin receptor in hepatocytes, suggesting a stabilizing function in loop A that is involved in ATP binding [32]. Thus, ALA can be considered an insulin-mimetic agent [1], since the bond of insulin receptor (IR) and IRS-1 phosphorylation could subsequently lead to GLUT4 translocation and increased glucose uptake. However, there are some controversies over ALA's action on insulin signalling. In a preadipocyte cell culture exposed to ALA [33], Akt is phosphorylated within 30 min while the insulin receptor IR and the IRS-1 are not phosphorylated. Other research findings in adipocyte cell cultures demonstrate that IR is indeed phosphorylated but this is specific to ALA, the reduced form of the compound and not to DHLA [34].

Konrad et al. described two different mechanisms through which ALA induces glucose uptake: ALA induces PI3K and AKT phosphorylation, which determine GLUT4 translocation and it also increases p38 MAPK activity, which determines GLUT4 activation [2]. Therefore, GLUT4 activity seems to be a common element to several hypotheses since the activation of the insulin receptor cascade would also secondarily determine an increase in glucose uptake by GLUT4.

Furthermore, ALA has different effects on AMPK expression in peripheral tissues such as skeletal muscles and hypothalamus. A three-day ALA treatment increases glucose uptake, evaluated by euglycemic hyper-insulinemic clamp, reduces plasma lactate and increases α -2 AMPK activity in the skeletal muscle [35] while decreasing AMPK activity in the hypothalamus [36].

AMPK is a cellular fuel sensor whose activation is associated with ATP production through fatty acid oxidation. Moreover, it can induce the translocation of GLUT4 to the cell membrane and plays an important role in mitochondrial biogenesis [37,38]. ALA seems to share the same mechanisms as metformin, which can also activate AMPK in hepatocytes and skeletal muscles [39].

Moreover, within the cell ALA could carry out additional functions associated with glucose metabolism; in particular, it could influence mitochondrial biogenesis and endoplasmic reticulum (ER) activity. In fact, ALA stimulates the expression of mitochondrial markers such as TFAM, PPAR γ and PGC1 α in C2C12 cells (a myoblast cell line) and the expression of gene encoding for representative antioxidant enzymes such as glutathione peroxidase (GPX1) and superoxide dismutase 1 (SOD1) [40]. In glucose-treated rats, the decrease in PPAR γ protein levels caused by oxidative stress is prevented or attenuated if they are fed with ALA [41]. However, to date, the contribution of ALA-induced PPAR γ expression to insulin sensitivity cannot be clearly quantified. In the endoplasmic reticulum, ALA increases the endogenous expression of DNAJB3 cochaperone, heat shock proteins (HSP25 and HSP27) in C2C12 cells [40,42]. DNAJB3 cochaperone has been associated with reduced metabolic stress, improved insulin signalling and glucose uptake in previous *in vitro* and *in vivo* studies (3T3-L1 adipocytes and human obese subjects) [43,44]. However, it is unclear whether DNAJB3 exerts a direct effect on ER stress or acts indirectly through other pathways. Similarly, low HSP expression could play a critical role in the

induction of insulin resistance and diabetes [45]. ALA's pro-oxidant properties also include the induction of HSP and the activation of PI3K.

Thus, the effect of ALA on GLUT4 is certainly the major determinant of the increase in glucose uptake, making ALA an insulin mimetic agent. However, it is still unclear how ALA interacts with the insulin receptor and insulin receptor cascade. It is possible that ancillary effects of ALA on mitochondria and the endoplasmic reticulum further improve insulin sensitivity. The effects of ALA on glucose metabolism seem not to be limited to a peripheral insulin mimetic effect, as some evidence suggests that it also acts on beta-cells.

4.2. Effect of ALA on Pancreatic Beta-Cells

Several studies over the last two decades have explored changes in insulin secretion during ALA treatment. Most of these studies investigated the effect of ALA *in vitro* in the presence of substances toxic for beta-cell function, such as oleic acid (OA), which can interfere with mitochondria and insulin secretion [46] and 2-deoxy-D-ribose (dRib), a strongly reducing sugar which induces apoptosis and oxidative stress in beta-cell lines [47]. Pre-treatment with ALA (10 mM) prevented OA-induced decrease in insulin secretion in MIN6 cells [48] and reversed low mRNA insulin levels in a dose-dependent manner in dRib [49] stimulated HIT-T15 cells. These studies suggest that in beta-cells ALA acts as an antidote to toxic substances which reduce insulin secretion.

This hypothesis has also been confirmed in studies *in vivo*. In T2D rats fed with fructose, ALA did not affect insulin sensitivity but induced greater insulin excursions after glucose administration [50]. In a more recent study, the co-administration of lipopolysaccharide S (LPS) (an inducer of chronic subacute hepatic inflammation, which can also impair pancreatic insulin secretion) [51] and ALA produced no changes in insulin sensitivity [52], assessed by euglycemic hyper-insulinemic clamp [53], but determined a restoration of first-phase and second-phase insulin secretion [54] (assessed by hyperglycaemic clamp), compared to rats treated with LPS alone. In aged rat islets, ALA significantly increased insulin secretion and decreased reactive oxygen species [55]. We can consider the latter as a model of oxidative stress induced cell damage, even though it differs from the other studies due to the absence of exogenous toxic substances.

In relation to the structure of pancreatic beta-cells, further studies suggest that ALA could exert a protective effect against pancreatic injury induced by Cyclosporin A [56] and Valproic acid [57]. Cyclosporin A is a potent immunosuppressive drug, listed among the possible etiologic causes of post-transplant diabetes mellitus (PTDM). In this specific context, ALA seems to protect islets from atrophy and increase insulin-immunoreactive granules.

IL-1 β is produced by activated macrophages and could be the responsible for early beta-cell injury in type 1 diabetes. When ALA was administered to isolated islets from NOD mice treated with IL-1 β , insulin secretion returned to normal. However, this effect was dose-dependent since although a 10^{-9} M lipoic acid concentration was effective, 10^{-6} M and 10^{-12} M concentrations produced no effects. Interestingly, when the 10^{-9} mM lipoic acid concentration was tested alone without the addition of IL-1 β , insulin secretion was not restored, but, in fact, decreased significantly (68%) compared to the control condition [58]. The latter observation suggests that the effect of ALA on insulin secretion can change in relation to more complex intracellular dynamics [59].

The administration of ALA alone leads to similar results *in vitro*, as shown in two different clonal beta-cell types (HIT-T15 cells [60] and in MIN6 cells [3]), as ALA inhibited glucose-induced insulin secretion in both. While in HIT-T15 cells this finding was also accompanied by a reduction in beta-cell apoptosis, in MIN6 cells this effect was associated with AMPK α -subunit phosphorylation and activation and was compared to other well-known AMPK activators, such as AICAR and metformin. In high glucose conditions all these compounds, administered chronically, decreased insulin secretion and, moreover, ALA treatment produced an acute decrease in glucose-stimulated insulin secretion (GSIS) while AICAR and metformin did not. Thus, ALA's effects on beta-cell AMPK parallels its effects in skeletal muscle, even though with opposite effects, as it increases insulin

sensitivity while decreasing insulin secretion. The administration of ALA alone, therefore, seems to worsen insulin secretion, at least in beta-cell lines. However, we could consider the overall effect as protective, because improved insulin sensitivity accompanied by a smaller demand for insulin production may protect beta-cells from exhaustion [7,61,62].

In addition, a recent study by Azzam et al. suggests that lipoic acid and ascorbic acid—among other antioxidant compounds—have a significant inhibitory activity on the fibril aggregation of amylin [63], which is physiologically co-localized in granules with insulin in beta-cells. Thus, both ALA and ascorbic acid could play a protective role in β -cell overload, which has been correlated with plasma amylin levels in T2D [64]. Furthermore, ALA successfully counteracts other mechanisms of amyloidosis such as the formation of β -amyloid fibrils [65].

The effects of ALA on insulin secretion, therefore, vary according to the oxidative balance of the cell or the presence of other substances. In vitro and in vivo models suggest a role in the prevention or attenuation of the pathogenetic mechanisms that lead to type 1 and type 2 diabetes, diabetes associated with liver diseases and post-transplant diabetes.

4.3. ALA for the Treatment of Diabetes

Diabetes is a chronic disease characterized by chronic hyperglycaemia. While Type 1 diabetes is the result of a reduced or absent insulin secretion, Type 2 diabetes is characterized by both insulin resistance and reduced insulin secretion in different proportions [66].

Given the positive effects of ALA on insulin sensitivity and the protective effects on damaged beta-cells, we would expect some therapeutic efficacy in the management of diabetes.

A metanalysis [67] of six studies including uncomplicated T2D patients, showed no differences in terms of HbA1c between the control group and the group treated with ALA. Most of the patients enrolled were taking other antidiabetic drugs like metformin, sulphonyl-ureas and insulin in different combinations. None of these studies specifically included drug-naïve patients and only one study showed an improvement in HbA1c and other metabolic parameters in patients with complicated T2D treated with ALA [68]. The latter study evaluated 90 elderly type 2 diabetic patients with acute cerebral infarction randomized to receive ALA or vitamin C for 3 weeks plus insulin therapy. With the same insulin doses, FPG, 2HPG and HbA1c decreased after treatment in both groups, but the changes in the ALA group were greater compared with the control group.

Another metanalysis [69] reported a significant decrease in FPG, HbA1c, insulin concentrations, HOMA-IR, triglycerides and LDL cholesterol in individuals with metabolic diseases treated with ALA. In this case, the overall population analysed included subjects who were only overweight or had diabetes, metabolic syndrome, or polycystic ovarian syndrome. Half of the total number of eligible studies (i.e., 12 papers) examined the effects of ALA on glucose control and/or lipid profiles in T2D patients, only a few evaluated changes in HbA1c and the rest evaluated only FPG or HOMA-IR.

Therefore, despite the promising results in animal and in vitro models, treatment with ALA has had controversial and mostly disappointing results in studies on the treatment of diabetes in humans.

A recent study [70], not included in the previous metanalysis, confirms these data in glibenclamide/metformin-treated patients as no statistically significant differences were found in terms of HbA1c and glutathione peroxidase (Gpx) between ALA group and placebo group. The authors, however, argue that the negative results are due to low doses of ALA. A dose dependent effect could also be assumed in the study by Porasuphatana [71], which included metformin and sulphonyl-ureas-treated patients randomly divided into five groups to receive either placebo or four different doses of ALA. The changes between pre- and post-treatment in each group failed to reach a statistical significance due to the small sample size. Only when all patients in the ALA groups were pooled and compared to the placebo group was there a significant difference in terms of HbA1c and a significant

correlation between ALA doses and HbA1c. Studies evaluating the effect of ALA on HbA1c in humans are listed in Table 1.

Thus, further studies with high doses of ALA are needed and the drug should be tested as add-on therapy to newer and safer antidiabetic drugs such as SGLT2 inhibitors and GLP-1 receptor agonists.

Although the effects on HbA1c are limited, the studies carried out with hyperinsulinemic euglycemic clamp in T2D showed increased insulin sensitivity both after acute parenteral administration [72] and after one-month oral treatment [73,74]. In particular, in the study by Kamenova [74], glucose disposal rate after treatment did not differ statistically from that of a control group of normal glucose-tolerant subjects. This discrepancy between ALA's effect on insulin sensitivity and glycaemic control is not surprising. Insulin resistance and glucose control are different parameters and insulin resistance counts only partially towards the pathogenesis of diabetes and its manifestations. Thus, ALA could be effective in those phases in which the insulin sensitivity defect is predominant but not when beta-cell failure has already occurred. This would also explain the difference in results between the two previously mentioned meta-analyses, as the latter probably included a higher number of insulin resistant patients with normal insulin secretion. This ambivalent effect has also been observed in prediabetes, which is also characterized by a beta-cell defect [75]. In this setting, ALA had no effect on body weight, percentage body fat, blood pressure and glucose level compared to placebo, but decreased plasma insulin and HOMA-IR [76]. In women with gestational diabetes, however, the administration of ALA 100–300 mg per day led to a reduction in fasting glucose levels, although no insulin data were available [77,78]. Studies evaluating the effect of ALA on insulin sensitivity and secretion in human are listed respectively in Tables 2 and 3.

Table 1. Studies on ALA's effects on HbA1c *.

	Country/Population	Intervention	Duration	Other Antidiabetic Drugs	Results
[6] Ziegler et al., 1997	Germany/T2D	ALA 800 mg or placebo (oral administration) per day	4 months	14 patients on oral antidiabetics while 47 patients on insulin therapy	No HbA1c differences between ALA and placebo
[79] Heinisch et al., 2010	Austria/T2D	ALA 600 mg or placebo (oral administration) per day	3 weeks	23 patients on oral antidiabetics (metformin, glitazones and sulphonylureas), 5 patients on insulin and one patient no therapy	No HbA1c differences between ALA and placebo
[80] Hegazy et al., 2013	Egypt/T1D	ALA 600 mg or placebo (oral administration) per day	4 months	Insulin therapy	No HbA1c differences between ALA and placebo
[71] Porasuphatana et al., 2011	Thailand/T2D	ALA 300 mg, 600 mg, 900 mg, 1200 mg or placebo (oral administration) per day	6 months	Metformin and/or sulphonylureas	No differences between placebo and other groups, HbA1c significantly decreased in all treatment groups compared to placebo
[81] Udupa et al., 2012	India/T2D	ALA 300 mg or Vitamin E or Omega-3 or placebo (oral administration) per day	3 months	Metformin plus Glimepiride	No HbA1c differences between ALA and placebo
[82] Huang et al., 2013	China/T2D	ALA 600 mg or placebo (intravenous infusion) per day	3 months	Short term continuous subcutaneous insulin infusion	No HbA1c differences between ALA and placebo
[68] Zhao et al., 2014	China/T2D complicated by acute cerebral infarction	ALA 600 mg or placebo (intravenous infusion) per day	1 month	Insulin therapy	ALA significantly decreased HbA1c compared to placebo
[70] Mendoza-Núñez et al., 2019	Mexico/T2D	ALA 600 mg or placebo (oral administration) per day	6 months	Metformin/glibenclamide	No HbA1c differences between ALA and placebo

* Glycated Haemoglobin (HbA1c) estimates blood glucose levels of an individual over the last 3 months.

Table 2. Studies on ALA's effect on insulin sensitivity (M and insulin sensitivity indexes) **.

[72] Jacob et al., 1996	Germany/T2D	ALA 100 mg/500 NaCl (intravenous infusion) or placebo	During glucose clamp study	Oral antihyperglycemic therapy	Mean insulin-mediated glucose disposal increased by about 50% in ALA group (from 3.76 mL/kg/min to 5.82 mL/kg/min, $p < 0.05$), no significant change in control group (from 3.57 mL/kg/min to 3.91 mL/kg/min)
[73] Jacob et al., 1999	Germany/T2D (About 20% of the patients exhibited diabetic complications)	ALA 600 mg, 1200 mg, 1800 mg or placebo (oral administration) per day	1 month	Oral antihyperglycemic therapy	No insulin-mediated glucose disposal differences between placebo and other groups. Insulin sensitivity significantly increased in all the active groups pooled together (+27%, $p < 0.01$) compared to placebo
[74] Kamenova et al., 2006	Greece/T2D	ALA 1200 mg (oral administration) per day. A group of normo-tolerant subjects served as control	One month	Metformin (850 mg once daily to three times daily)	Mean insulin-mediated glucose disposal significantly increased in ALA group (from 3.202 to 5.951 mg/kg/min, $p < 0.01$), and after treatment was not statistically different from control group.
[83] Masharani et al., 2010	USA/PCOS with oligomenorrhoea without hyperandrogenism	Controlled-release ALA 1200 mg per day (No control groups)	4 months	Oral contraceptives and/or spironolactone	Mean insulin-mediated glucose disposal significantly increased (from 9.7 ± 1.3 mg/min/kg/mU to 11.1 ± 1.7 mg/min/kg/mU after treatment). Two subjects not on oral contraceptives experienced a doubling of periods respect to four months prior to study entry.
[84] Xiao et al., 2011	Canada/Overweight and obese patients without history of diabetes	Patients were treated in random order with oral placebo followed by NaCl infusion (SAL), oral placebo followed by intralipid infusion (IH), oral ALA 1800 mg per day followed by NaCl infusion (ALA) and oral ALA 1800 mg per day followed by intralipid infusion (ALA + IH)	2 weeks each phase	No medications	Insulin sensitivity index and disposition were 19 and 25% lower in IH and IH + ALA, respectively, vs. SAL ($p < 0.05$), indicating lipid-induced insulin resistance. Insulin sensitivity in ALA was similar to SAL.

** Insulin-mediated glucose disposal (M) (mg/kg/min), insulin sensitivity index ($I^2 \times \text{kg}^{-1} \times \text{min}^{-1} \times \text{pmol}^{-1}$) and disposition index ($I^2 \times \text{kg}^{-1} \times \text{min}^{-2}$) are obtained from height, weight and glucose infusion rate (GIR) during the last 30–60 min of euglycemic hyper-insulinemic clamp.

Table 3. Studies on ALA's effect on insulin secretion (ISR) ***.

[84] Xiao et al., 2011	Canada/Overweight and obese patients without history of diabetes	Patients were treated in random order with: oral placebo followed by NaCl infusion (SAL), oral placebo followed by intralipid infusion (IH), oral ALA 1800 mg per day followed by NaCl infusion (ALA) and oral ALA 1800 mg per day followed by intralipid infusion (ALA + IH)	2 weeks each phase	No medications	Insulin secretion rate was similar between treatment groups
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*** Insulin secretion rate (ISR) (pmol/min) is derived from deconvolution of C-peptide concentrations measured during hyperglycaemic clamp.

Therefore, alpha-lipoic acid could be considered in the prevention of glucose metabolism alterations rather than in their treatment. In the next section, we will analyse the effects of ALA on syndromes characterized by alteration of insulin levels and signalling without impaired glucose levels.

4.4. ALA for Treatment of PCOS and Conditions of Insulin Resistance

Polycystic ovary syndrome is characterized by hyperandrogenism and ovarian dysfunction [85]. It is also well known that these pathological findings often overlap with obesity (32% of PCO patients in USA) [86] and insulin resistance (up to 75% according to some authors) [87]. Moreover, insulin resistance is an important contributor to the development of T2D [88] and its prevalence is also increased in PCOS [10,89]. A number of studies have performed a detailed analysis of the metabolic profile of obese PCO patients, showing that these subjects have reduced insulin sensitivity, but increased insulin secretion compared to obese adolescents without PCOS, as both first-phase and second-phase insulin secretion were higher compared to controls [90]. Therefore, the prevalence of a beta-cell defect appears to be low in these patients.

Of all the studies exploring the effect of ALA treatment in PCOS, only one performed a euglycemic hyper-insulinemic clamp, which is the gold standard for the evaluation of insulin sensitivity [91], showing a significant improvement in insulin resistance, without changes in body weight, after ALA treatment [83].

Other studies report an improvement in insulin sensitivity expressed by HOMA-I or maximal insulin response during OGTT. In a group of obese PCOS patients, ALA administration significantly decreased insulin, glucose, BMI and HOMA index after 12 weeks compared to baseline values. The degree of improvement was higher especially in those with a family history for diabetes [9]. This could suggest a protection against diabetes onset in subjects at risk of diabetes. In another study by the same group [92], overweight patients were treated with myo-inositol 1 g/die, or alpha-lipoic-acid 400 mg/die, or myo-inositol 1 gr/die + alpha-lipoic acid 400 mg/die. All treatments determined a reduction in HOMA-IR and maximal insulin response. No significant differences were found in the ALA groups in subgroups with and without T2D familiar history. ALA treatment, however, does not affect sexual hormone parameters while myo-inositol does.

The combination of alpha-lipoic acid and inositol has been evaluated in several studies and beneficial effects have been reported despite different dosages and length of observation [93–95]. HOMA-IR decreased especially in the studies that documented a condition of greater insulin resistance at baseline. For example, in the study by Fruzzetti et al. [93], HOMA-I significantly decreased only in insulin resistant subjects, while no changes were observed in the group of subjects without insulin resistance.

In addition, the combination of ALA and inositol also determined a decrease in menstrual abnormalities [94,95]. Cycle length was progressively reduced in oligomenorrheic women after 24 months of treatment [94] and there was also a significant decrease in BMI [93,94]. Hormonal parameters also improved in the study by De Cicco et al. [96] in which treatment was administered at a double dose (ALA 800 mg and myo-inositol 2000 mg). They found that androstenedione and DHEA-S significantly decreased and mean SHBG increased and AMH serum levels decreased significantly compared to baseline. Promising results were also obtained in patients undergoing in vitro fertilization [97,98]. Moreover, the association of alpha-lipoic acid, myo-inositol and metformin (1.7 g) determined a greater improvement in hyperandrogenism, BMI and HOMA index than metformin alone (3 g) [99].

Few studies have been conducted on the effects of this molecule in other states of insulin resistance. In a study by Xiao et al. [84], there were no beneficial effects on lipid-induced impairment in insulin sensitivity and secretion, evaluated with clamp techniques (hyperglycaemic clamp followed by euglycemic hyper-insulinemic clamp the same day) in overweight and obese patients without T2D.

By contrast, other studies [100–102] show better metabolic profiles following ALA treatment. In a cross-over, double-blind placebo-controlled study, ALA 1200 mg positively affected blood pressure, BMI, waist circumference and triglyceride levels compared to placebo [100]. Moreover, two metaanalyses [101,102] have confirmed a significant—even though minimal—reduction of BMI and weight with ALA treatment. In the metaanalysis by Kucukgoncu et al., overall weight loss was 1.27 kg greater with ALA treatment than in the placebo group and BMI difference was 0.40 kg/m² between the two groups. In the metaanalysis by Namazi et al., there was a slight effect on body weight and BMI: respectively 0.69 kg and 0.39 kg/m² compared to placebo. However, the reduction in waist circumference was not significant and there was no correlation between weight loss and ALA dose. Interestingly, both metaanalyses indicate that shorter treatment duration achieves greater BMI reduction than longer intervention. Therefore, we could conclude that ALA is effective for weight loss but with no certain durability of the results.

5. ALA and Diabetic Neuropathy

Diabetic neuropathy is a known microvascular complication of diabetes. Data on the benefits of alpha-lipoic acid for both diabetic polyneuropathy (DPN) and diabetic autonomic neuropathy (DAN) started emerging in the 1990s [103,104]. DPN is one of the main clinical presentations [105] and is characterized by “negative” symptoms such as hypoesthesia and “positive” symptoms such as paraesthesia, dysesthesia, allodynia, or pain. The latter has been identified as the major determinant of depressive symptoms in people with diabetes, as it interferes with the ability to enjoy life, with daily activities and with sleep [106,107]. Electroneurography is the main diagnostic tool, confirming the presence of DPN in symptomatic patients and highlighting alterations in peripheral nerve conduction [105]. Systematic reviews and metaanalyses [108,109] have evidenced the beneficial effects of ALA in the treatment of diabetic polyneuropathy (DPN). ALA, administered at a dosage of 600 mg or more either intravenously or orally, improves the Total Symptoms Score (TSS) and Neuropathy Impairment Score (NIS) in people with DPN [108,109]. ALA was shown to improve night pain, paraesthesia, muscle atrophy and difficulty in walking in a cohort of 20 patients. Better results were obtained in patients with lower HbA1c <7% than in those with higher HbA1c >7% [110].

The effects of ALA appear not to be limited solely to the symptoms of diabetic neuropathy. Indeed, ALA can be considered as the only treatment that acts on the pathogenesis of the disease while the others— α 2 δ ligands, tricyclic antidepressants and opioids—are only symptomatic treatments for pain. The mechanism may be related to an improvement in nerve blood flow and distal nerve conduction mediated by the antioxidant action [111,112]. A systematic review including 15 articles, examined the effect of ALA 300–600 mg i.v. per day for two to four weeks, on motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV). Both these parameters increased after ALA administration even though most of the studies included in this review were of poor methodological quality [113]. Despite these positive premises, to date, no pharmacological therapies (ALA included) have been able to modify the history of the disease or reverse it [114] and, according to the American Academy of Neurology, there are no conclusive data for the use of ALA or B vitamins in the treatment of pain [115].

The manifestations of autonomic diabetic neuropathy are very disparate since different systems can be involved. Cardiovascular autonomic neuropathy (CAN), for example, can manifest with tachycardia, reduced heart rate variability, QT lengthening, orthostatic hypotension, reverse dipping and reduced sympathetic-adrenergic response to hypoglycaemia. CAN is a risk factor for cardiovascular morbidity and all-cause mortality [116]. In the Deutsche Kardiologie Autonome Neuropathie Studie [117], diabetic patients with CAN—on oral anti-hyperglycaemic therapy—treated with ALA, experienced an improvement in heart rate variability, while QTc decreased but not significantly.

As regards the use of ALA in the management of other microvascular complications of diabetes, the results are rather disappointing. In the REPITON trial [118], a daily dose of 600 mg ALA did not prevent the occurrence of clinically significant macular oedema in diabetic patients. Similarly, a recent metanalysis concluded that ALA supplementation does not improve biological indices that reflect diabetic nephropathy in humans, even though with limited evidence [119].

The possibilities for use of this molecule for glyco-metabolic pathologies are therefore ample, but attention must be paid to possible side effects.

6. ALA and Insulin Autoimmune Syndrome

Insulin Autoimmune Syndrome is a rare cause of endogenous hyper-insulinemic hypoglycaemia [120]. It was first described in 1970 in Japan by Hirata and colleagues. Since then, several cases have been identified and nowadays IAS is reported to be the third most common cause of spontaneous hypoglycaemia in Japan [121].

IAS is characterized by the presence of a high titre of insulin autoantibodies (IAA) which bind to insulin and proinsulin. They thus interfere with insulin action, determining a transient phase of hyperglycaemia and a prolonged pancreatic secretion of insulin and C-peptide. When IAA dissociate from insulin, hypoglycaemia occurs [122]. It can be lasting and even more dangerous than that seen in patients with insulinoma.

In 2007, Uchigata et al. published a report on the first case of ALA-induced Insulin Autoimmune Syndrome [123]. To date, 49 cases can be retrieved by a comprehensive literature search [124]. The majority of cases have been reported in Japan, while in Europe most cases have been described in Italy, two by our group in 2018 [15]. The pathogenesis of ALA-induced IAS has not been fully elucidated. It seems that certain individuals could be more susceptible to the development of the disease after the introduction of substances containing sulfhydryl groups, such as ALA. The presence of the Human Leukocyte Antigen HLA-DR4 [125] and, in particular, the DRB1*04:06 and DRB1*04:03 alleles (most of the European cases) are associated with an increased risk of IAS. In Japan, 97% of affected patients are HLA-DR4-positive and 43% are also DRB1*04:06-positive [12]. In Europe, the predominant allele is *04:03. This difference is also linked to the different prevalence of these two alleles: in Japan the frequency of the allele*04:06 is reported to range between 5.3% and 13.2% while in Europe it is between 0.1 and 1%. Conversely the frequency of allele*04:03 is 1.6–12.3% in Japan while its prevalence is 0.4% to 3.9% in Europe [126]. Before ALA-induced IAS was discovered, other sulfhydryl compounds, such as methimazole, had been associated with IAS [127]. These compounds, due to the reducing properties conferred by sulfhydryl groups, could linearize insulin α chain by cleaving its disulphide bond. In this way hidden fragments of human insulin would be exposed and recognised by T lymphocytes, triggering an immune response and eventually antibody production. Matsushita et al. [128] detected the insulin fragment (TSICSLYQLE) with the highest affinity to DRB1*04:06. Conversely the allele DRB1*04:05, which differs from the latter for some amino acid residues, exhibits an IC_{50} value 44 times higher [128]. HLA-DRB1*04:03 is closely related to HLA-DRB1*04:06 and it has been suggested that HLA-DRB1*04:03 is an evolutionary predecessor of HLA-DRB1*04:06 [129]. Interestingly, allele DRB1*04:03 is protective against type 1 diabetes [130] and primary adrenal insufficiency [131] while both DRB1*04:03 and DRB1*04:06 could predispose to pemphigus [132]. To date, the exact interactions of ALA and methimazole with insulin in IAS are unclear, but it can be asserted that both a reducing ambient and a genetic predisposition seem to be necessary for the development of the disease.

In case reports, the time to onset of IAS after taking ALA ranges from 1 week to 4 months (7–120 days) with no obvious association between dose and time to onset. Hypoglycaemic episodes are generally postprandial, but they have also been reported in the fasting state. Insulin levels are very high and C-peptide levels are normal or high. After PEG precipitation, recovery of insulin is low (5–10%) compared to control samples (70%) [13]. The reason for high C-peptide levels is still controversial. C-peptide may cross-react with the same IAA and may also be “incorrectly” reported as “free” C-peptide by some immunoassays [122]. Furthermore, the initial transient hyperglycaemia—due to insulin binding to antibodies—in a usually euglycemic subject could stimulate beta-cells and determine C-peptide release. The severity of hypoglycaemia is linked to antibody titres and their affinity: low affinity antibodies are more likely to cause hypoglycaemia [122,133]. However, in some clinical situations IAA are not pathogenetic. In a study on T2D patients, insulin antibodies were detected in 48 of 118 patients (40.7%) on insulin therapy and were unexpectedly found in seven of 263 insulin naïve patients (2.7%) [134].

IAS usually resolves once the trigger is removed. There is no agreement as to whether to introduce a therapy or even which therapy to use. In case reports, diazoxide, prednisone and other immunosuppressants were administered after ALA suspension. In all cases, hypoglycaemic events disappeared after a comparable period of time (one week to three months) [124]. Thus, the introduction of drugs is not mandatory, but based on the severity of symptoms and the patient’s general clinical condition. However, the positioning of a flash glucose monitoring (FGM) device could be a reasonable option to monitor the possible persistence of hypoglycaemic episodes over time [135]. In only one case report ALA was wrongly re-administered after disease remission in a South Korean 67-year-old woman with type 2 diabetes and neuropathy. She underwent two ALA re-challenges after the first administration and in both cases developed Insulin Autoimmune Syndrome [136].

Recently the European Commission has asked the European Food Safety Authority (EFSA) to review existing data on the possible link between ALA administration and IAS and provide advice on the dietary intake of foods supplemented with alpha-lipoic acid. A panel published by EFSA in April 2021 [124] concluded that the addition of ALA to foods is likely to lead to an increased risk of development of IAS in individuals with certain genetic polymorphisms. On the other hand, no link has been found between the intake of foods naturally containing ALA with IAS. Furthermore, an ALA dose below which IAS is not expected to occur cannot be derived based on the available data. This is probably due to the autoimmune pathogenesis of the disease.

Therefore, we can conclude that the safe daily administration of ALA would require the analysis of HLA, although this is rarely feasible in clinical practice. An accurate evaluation of the medical history of the patient could help identify any pathologies sharing the same HLA predisposing alleles of ALA-induced IAS, such as pemphigus, thus avoiding the introduction of the drug.

This Figure 1 summarizes the main effects of ALA on glucose metabolism: it can activate the insulin receptor by extracellular binding, but it can also cross the cell membrane and activate AMPK. The latter interaction is responsible for GLUT4 expression on cell surface and increased glucose uptake. This promotes glucose entry into the cell and glycolysis. ALA then contributes to the beginning of Krebs cycle through its interaction with pyruvate dehydrogenase. On the other hand, ALA could modify insulin structure through the cleavage of disulphide bond and the subsequent exposition of hidden fragments to immune system. In this way lymphatic organs produce IAA which bind insulin and proinsulin and determine IAS.

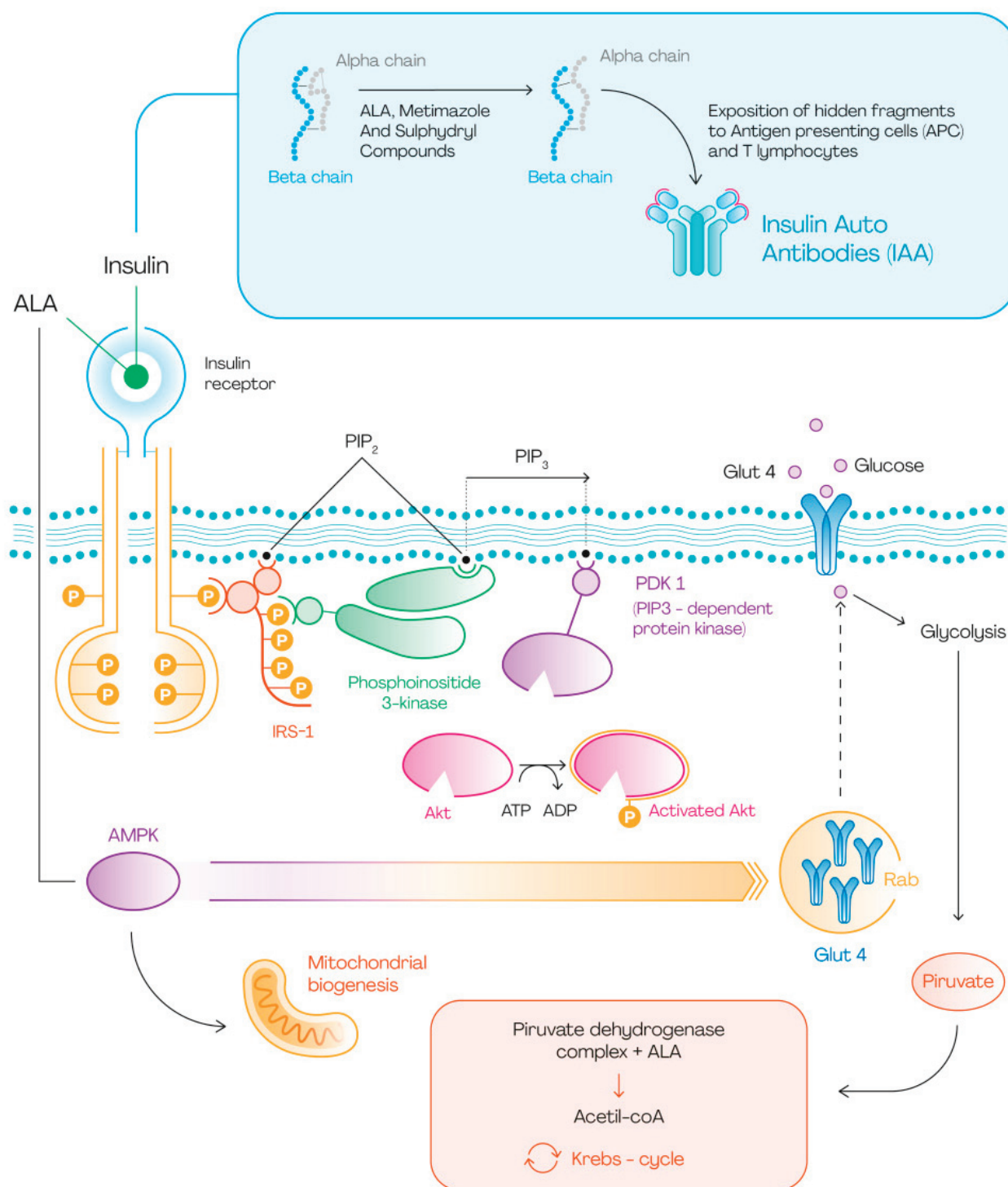


Figure 1. ALA and glucose metabolism.

7. Conclusions

Alpha-lipoic acid has pleiotropic effects on glucose metabolism (Figure 1), many of which are still under investigation. This compound is widely prescribed for the treatment of insulin resistant states, such as polycystic ovary syndrome and for diabetic neuropathy, due to the amount of supporting evidence.

Blood glucose is the result of many sophisticated intra- and extra-cellular processes on which ALA seems to have only a partial impact. We have seen how the effect on insulin secretion primarily depends on the degree of cellular oxidative balance [55], but that this

molecule can have only a limited effect on beta-cell metabolism tout court [67]. To date, the evidence on ALA's validity as an antihyperglycemic drug is rather disappointing, but there are few human studies on the addition of alpha-lipoic acid to more recent therapies such as SGLT2i and GLP1-RA. Further studies are needed to determine its possible role in the treatment of diabetes, identifying any sub-categories of diabetic patients who could benefit from this type of therapy [137].

However, it cannot be excluded that alpha-lipoic acid could be used to prevent rather than treat the alteration in insulin sensitivity that may precede the development of diabetes by years. The studies on ALA in PCOS seem to confirm this hypothesis. For example, overweight and obese PCO patients are characterized by increased insulin secretion and reduced insulin sensitivity [90] but have an increased risk of developing diabetes [88]. Family history is known to be a risk factor for its development and ALA has been shown to improve insulin sensitivity and reduce blood glycaemia particularly in those patients with diabetic relatives [9]. Another area where ALA treatment has become consolidated is diabetic neuropathy, due to its beneficial effects both on the pathogenesis of the disease and on pain control [113]. However, curative treatments for this pathology are still lacking and the response rates to this and other treatments are often incomplete [115].

Considering the effects of this molecule on glucose metabolism, we cannot disregard its possible interference with the immune system leading to severe hypoglycaemia. In recent years there have been many reports of ALA-induced Insulin Autoimmune Syndrome, which, however, seems to occur only in genetically predisposed individuals [124]. The exact interactions of ALA with insulin in this disease are still unclear. It probably reduces disulphide bonds of insulin α chain displaying usually hidden amino acid residues to the immune system, leading to the production of autoantibodies [128]. Great attention must be paid to the possible onset of insulin autoimmune syndrome. The analysis of HLA would make the introduction of the drug safe, but this is rarely feasible in clinical practice. When this test is not available, we believe that this molecule should be prescribed with great care particularly in pregnancy and in fragile subjects in which a hypoglycaemic crisis could have particularly serious consequences. We consider it reasonable to discourage its use in patients with a history of pemphigus. It is also important that physicians become familiar with the early symptoms and signs of this pathology in order to avoid the onset of the most severe forms. Any hypoglycaemic event in a person taking alpha-lipoic acid must lead to suspicion of IAS and to immediate discontinuation of therapy.

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Abbreviations

ALA	alpha-lipoic acid
DHLA	dihydro-lipoic acid
T1D	type 1 diabetes
T2D	type 2 diabetes
MS	metabolic syndrome
PCOS	polycystic ovary syndrome
IAS	insulin autoimmune syndrome
IAA	insulin autoantibodies
IRS-1	insulin receptor substrate—1
IR	insulin receptor
HSP	heat shock proteins
PTDM	post-transplant diabetes mellitus
DPN	diabetic polyneuropathy
DAN	diabetic autonomic neuropathy
NIS	neuropathy impairment score
TSS	total symptoms score
CAN	cardiovascular autonomic neuropathy
FGM	flash glucose monitoring

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Review

A Comprehensive Review on Nutraceuticals: Therapy Support and Formulation Challenges

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Abstract: Nutraceuticals are the nourishing components (hybrid of nutrition and pharmaceuticals) that are biologically active and possess capability for maintaining optimal health and benefits. These products play a significant role in human health care and its endurance, most importantly for the future therapeutic development. Nutraceuticals have received recognition due to their nutritional benefits along with therapeutic effects and safety profile. Nutraceuticals are globally growing in the field of services such as health care promotion, disease reduction, etc. Various drug nutraceutical interactions have also been elaborated with various examples in this review. Several patents on nutraceuticals in agricultural applications and in various diseases have been stated in the last section of review, which confirms the exponential growth of nutraceuticals' market value. Nutraceuticals have been used not only for nutrition but also as a support therapy for the prevention and treatment of various diseases, such as to reduce side effects of cancer chemotherapy and radiotherapy. Diverse novel nanoformulation approaches tend to overcome challenges involved in formulation development of nutraceuticals. Prior information on various interactions with drugs may help in preventing any deleterious effects of nutraceuticals products. Nanotechnology also leads to the generation of micronized dietary products and other nutraceutical supplements with improved health benefits. In this review article, the latest key findings (clinical studies) on nutraceuticals that show the therapeutic action of nutraceutical's bioactive molecules on various diseases have also been discussed.

Keywords: cardiovascular diseases; interactions; nutrition; prevention; therapeutics

1. Introduction

Nutraceuticals are characterized as 'specially designed preparations', formulated with the aim of fulfilling specific dietary requirements and/or offer preventive health care. Nutraceuticals are the formulation of nutrient/nutrients which helps in prevention and treatment of some diseases, in addition to a supplement diet. Nutraceutical is a term given by Dr. Stephen De Felice in 1989 and came from two words "nutrition" and "pharmaceutical". These are foods or a part of foods that are beneficial in providing various health benefits including the treatment and/or prevention of the disease. Science of nutrition has increasingly achieved new horizons, starting from the anticipation of deficiencies in nutrients to prominence on human health and prevention and treatment of chronic ailments. Terms 'nutraceuticals', 'food supplements', 'dietary supplements' have evolved after the concept was originated by Dr. De Felice. There is no sharp demarcation between food supplements and nutraceuticals given by regulatory authorities. Literature of recent years emphasizes on redefining the concept of nutraceuticals, taking into consideration the efficacy, safety and toxicity of these products. Food products are nourishing substances that

are eaten, drunk or otherwise taken to sustain life, provide energy and promote growth. Currently, isolation of nutrients from these food products are well recognized and used. The starting point to differentiate food/dietary supplements and nutraceuticals is the identification of an epidemiological target, followed by safety and efficacy studies that understand the mechanism of action. One approach to differentiate these two types of formulations is describing 'food supplements' as agents to compensate deficiencies in micro- or macronutrients; in addition, the use of a "nutraceutical" in the treatment of a pathological disease must be supported by strong scientific evidence [1]. With adequate clinical evidence, nutritional supplements should have a strong safety profile with few undesirable side effects and better bioavailability. There is a very fine line of demarcation between two type of formulations: the same ingredients may work as a nutraceutical or food supplement, but may be demarcated on the basis of claims. Nutraceuticals include single or combinations of pro- and pre-biotic foodstuff and food for special medical uses; and food supplements includes single or combinations of mineral, vitamins, protein supplements, functional foods and herbal products. By prolonging or eliminating the need for pharmaceuticals in subjects to fit for an alternative nonpharmacological treatment to a pathological condition, the incorporation of nutraceuticals into daily diet may aid in the prevention of pathological disorders. There are claims that foods including spices and herbs possess the tendency to decrease the risk of many diseases and can be highly beneficial in improving the quality of life [2]. There is a plethora of benefits that nutraceuticals have provided, including their promising results in the prevention and treatment of complicated diseases. However, there is a need of administration and prescription of nutraceuticals and they should be strictly regulated in order to prevent their uncontrollable use and side effects [3]. Several researchers have studied drug compound-based nutraceuticals to improve the efficacy as well as bioavailability. The safety and efficacy of various statins have been used in the prevention of cardiovascular diseases even in pregnant women. Nutraceuticals with an effective safety profile and well-established impact on pregnancy might be a suitable therapeutic option for preventing diabetes mellitus and hypertensive disorders, or as an adjuvant to therapy with standard medications. Calcium, omega-3 polyunsaturated fatty acids, vitamin D, folic acid, resveratrol, alpha-lipoic acid, zinc, inositol, and probiotic supplements are potentially proven candidates as novel nutraceuticals [4]. Researchers have evaluated the nutraceutical associated with the drug compound ezetimibe for patients at risk of elevations of statin level, which further leads to cardiovascular diseases [5]. The use of a novel nutraceutical in blend with non-steroidal anti-inflammatory drugs (NSAIDs) has been proven a potential candidate for osteoarthritis, thus improving its efficacy and safety for commercial use [6].

The market remains robust and ever-growing for nutraceuticals such as antioxidants; omega-3 fatty acids; plants such as algae, aloe vera, seaweed, and wheatgrass; teas and herbs such as ginseng and Echinacea. A detailed findings including clinical data on nutraceuticals are shown in Table 1 [7,8]. A recent survey suggested that the nutraceutical market is expanding globally and the probability states that it may reach up to \$340 billion by the year 2024. The compound annual growth rate (CAGR) of nutraceuticals is estimated to be 7.2% in the year 2016 to 2024. This increment in the growth of the nutraceuticals-based industry is associated with various factors such as a rise in demand for nutraceuticals, an awareness among people for the benefits of nutrition and an incremental rate observed in the healthcare graph [9,10]. Currently, Europe, USA and Japan account for >90% of the total global nutraceutical market and the global market is supposed to reach \$336 billion by 2023 from \$247 billion in 2019 at a CAGR of 8%. With this attainment of maturity of global markets, now the focus of nutraceutical players has been shifted towards developing economies, especially those across Asia Pacific, including India. The Indian market had only 2% market share of total global nutraceutical market in 2017. It is estimated to reach \$11 billion by 2023, increasing at a CAGR of 21%. By 2023, India is also expected to hold at least 3.5% market share of the global market [11,12].

Table 1. Clinical data findings on nutraceuticals.

Nutraceuticals	Bioactive Molecule	Dosage	Formulation	Duration of Study	Action	Population Size (Volunteers)	References
Omega-3	Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA)	60 mg/kg/day	Capsule	12 months	Cystic fibrosis	15	[13]
Omega-3	Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA)	900 mg in the omega-3 rich group and 250 mg in the omega-3 poor group	Capsule	15 days	Reduction of chemotherapy-related toxicities	61	[14]
Omega-3	Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA)	2000 mg/day	Capsule	21 days	Appetite	72	[15]
Aloe vera	aloe-emodin, aloin, aloesin, emodin, and acemannan	-	Gel	2 months	Acute dermatitis	120	[16]
Aloe vera	aloe-emodin, aloin, aloesin, emodin, and acemannan	60 mL	Juice	5 months	Irritable bowel syndrome	110	[17]
Seaweed	polysaccharides, proteins, lipids and polyphenols	2000 mg/d	Extract	12 weeks	High-density lipoprotein (hdl) cholesterol	34	[18]
Wheatgrass	vitamins, (A, B, C and E), minerals such as iron, calcium, magnesium, benzo(a)pyrene, ferulic, gallic, caffeic, syringic and p-coumaric acid	-	Cream	12 weeks	Plantar fasciitis	134	[19]
Wheatgrass	vitamins, (A, B, C and E), minerals such as iron, calcium, magnesium, benzo(a)pyrene, ferulic, gallic, caffeic, syringic and p-coumaric acid	100 cc/day	Juice	1 month	Active distal ulcerative colitis	23	[20]
Ginseng	ginsenosides	100 mg twice a day	Capsule	12 weeks	Psychomotor functions	16	[21]
Ginseng	ginsenosides	-	Hydrogel	2 weeks	Skin homeostasis	20	[22]
Echinacea	polysaccharides, glycoproteins, alkamides, cichoric acid, caftaric acid and chlorogenic acids	20 mg or 40 mg twice a day	Powder	6 weeks	Anti-anxiety and anti-depressant	104	[23]
Echinacea	polysaccharides, glycoproteins, alkamides, cichoric acid, caftaric acid and chlorogenic acids	5 mL	Oral Suspension	6 months	Tonsillitis	300	[24]

The COVID-19 virus is having a knock-on effect for every industry. The long-term repercussions are hard to predict as of yet. In a post-COVID-19 pandemic situation, the global economy is still struggling and its impact on the global trade and nutraceuticals market is rising with rapid deterioration in the supply of products irrespective of high demands. Nutraceutical industry is not able to bridge the gap between high demand and low supply as 75–80% of raw material used in nutraceuticals was sourced from China in North America, Europe and Asia Pacific. Due to China's manufacturing shutdown, and the sudden recessive conditions in native regions, the nutraceutical production has reduced. The sharp surge in demand was observed for dietary supplements from consumers, as they provide a strong immune function and decrease possible health threats [25].

However, an officially shared and accepted definition of nutraceuticals is missing; these are also referred as 'pharma food'—a powerful toolbox that is beyond the diet but before the drugs. Various formulations containing macronutrients (required in large

amounts, e.g., omega-3 fatty acids, magnesium, potassium and calcium) [26], micronutrients (required in lesser amounts, e.g., minerals or vitamins) [27] and phytochemicals that are present in the food source [28] and are available at drug stores. Further, probiotics [29], minerals [30], polyunsaturated fatty acids [31], carotenoids [32], amino acids and proteins [33], vitamins [34], dietary fibers [35], spices and phytochemicals [36] have also become part of these formulations. Nutraceuticals exist as various types and may range from herbal products to isolated nutrient diets and may go up to existing genetically modified foods [37–39]. Plant foods such as vegetables, whole grains and vitamins are rich in dietary phytochemicals. Dietary supplements are consumed as such, or as isolated active ingredients. These phytochemicals are immensely diverse and these include carotenoids [40], phenolics [41], alkaloids [42], organosulfur [43] and nitrogen containing compounds [44]. However, these products may not be substantiated by scientific data on their safety, efficacy and effect on health and/or pathological conditions.

In the pharmaceutical industry, it is mandatory to do clinical tests on animals or in vitro for the verification of a compound's effects. On the contrary, in nutrition, there was no such method in the past for the verification of effects of foods in preventing or treating diseases. In recent years, however, the food composition has been scientifically tested and verified as people are becoming more and more aware of health-related issues and how food can directly or indirectly be responsible for maintaining proper health and preventing diseases (Figure 1) [45,46].

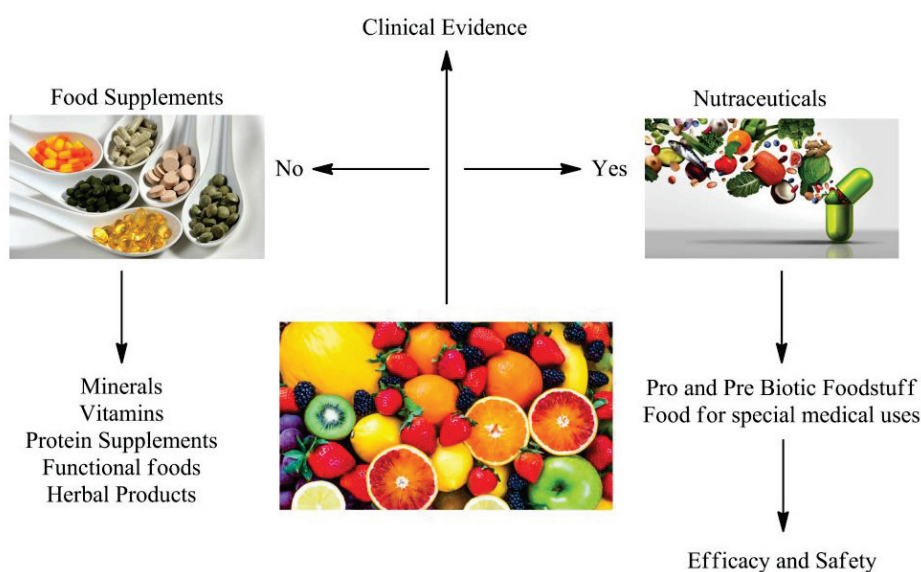


Figure 1. Potential roles of nutraceuticals.

Nutraceuticals provide their benefits in a wide range of therapeutic areas such as cough and cold [47], anti-arthritis [48], digestion [49], sleeping disorders [50] and treatment of cancers [51], depression [52], diabetes [53], cholesterol [54], blood pressure [55] and pain killers [56]. The research and development sectors for nutraceuticals are working at their peaks to discover how various nutraceuticals can prove to be of significance in the pharmaceutical industry. Scientific needs for nutraceuticals demand standardization of the constituents and cautious development of protocols and implement clinical studies which will form the foundation for consumer health and impact on nutraceutical companies [57,58].

In the last 10 years, a huge growth has been observed in the awareness of nutraceuticals and their use as powerful therapeutic supplements. Nutraceutical medicine has now been accepted as a part of Complementary and Alternative Medicine (CAM) and, thus, it has been incorporated as a new branch of CAM [59,60].

Due to dynamic action of nutraceuticals (nutritional and medicinal action), their popularity amongst general public and healthcare providers has increased over medicines.

A current review comprehensively discusses the use of nutraceuticals in preventive and support therapy, followed by compiled literature on patents published on said topic.

2. Nutraceuticals in Various Diseases

Nutraceuticals help enhance health, wellbeing and modulating immunity, thus preventing and treating various diseases and health issues (Figure 2) [61]. There are a variety of diseases that can be treated with the help of nutraceuticals which are discussed below:

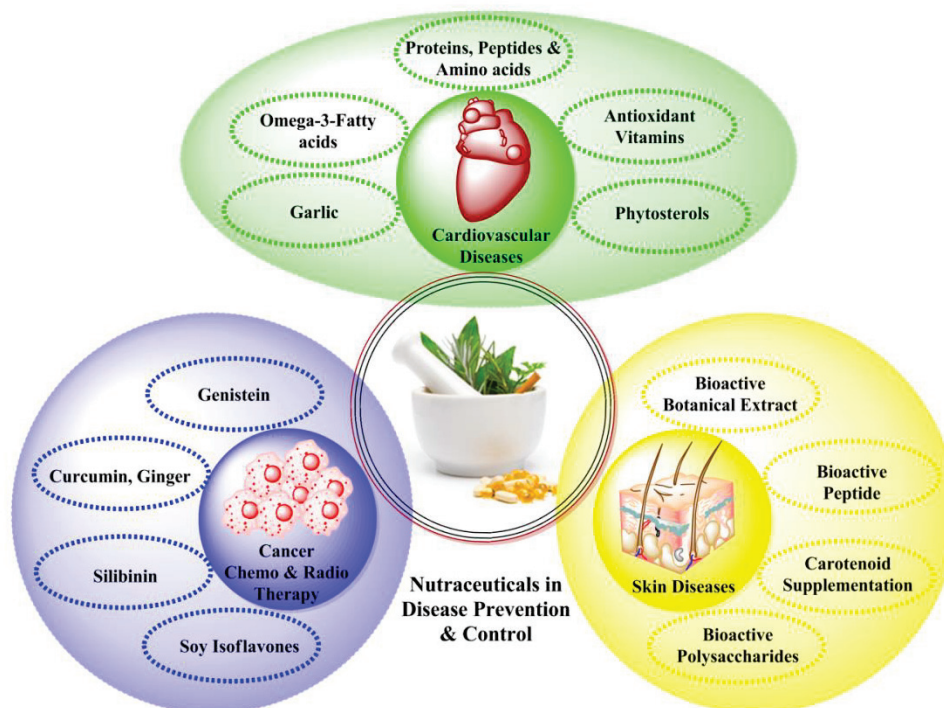


Figure 2. Nutraceuticals and Dietary Supplements in various diseases.

2.1. Nutraceuticals in Cardiovascular Diseases

Among all other diseases, cardiovascular diseases reveal significant risk-related factors acquiescent to nutraceutical intervention [62]. There is significant evidence indicating that nutraceuticals can be used in cardiovascular diseases [63,64].

Cardiovascular diseases (CVDs) mainly affect the blood vessels and the functioning of the heart. CVDs are one of the biggest causes of mortality, as they account for about 30% of deaths all over the world annually [65]. Dietary supplements have been proven to be beneficial in risk management and prevention of cardiovascular diseases and can be classified broadly into the ones used in the treatment of arrhythmias [66], Congestive heart failure [67], angina [68], hypertension [69] and hyperlipidemias [70,71]. Some of the nutraceuticals and dietary supplements that are used for the treatment and prevention of CVDs are discussed below.

2.1.1. Allicin and Alliin

Ischemic heart disease and atherosclerosis are associated with elevated levels of plasma triglycerides and blood-cholesterol are associated with. *Allium sativum* is antihyperlipidemic in nature and it exerts its effects by the elimination of cholesterol and its end-products in high amounts in the feces and by decreasing the cholesterol synthesis endogenously [72]. This helps in producing more favorable ratio of HDL and LDL. Allicin and alliin can effect cholesterol levels, if these can be protected by gastric acids. Garlic supplementation on serum cholesterol was assessed using thirteen placebo-controlled trials concerning 781 patients. Garlic also has some inherent antihypertensive effect, apart from being antihyperlipidemic [73,74].

2.1.2. Omega-3 Fatty Acids

Omega-3 fatty acids are derived from marine sources and are called polyunsaturated fatty acids (PUFAs). Docosahexaenoic acid (DHA) and Marine omega-3 eicosapentaenoic acid (EPA) plays a critical role in the treatment and prevention of cardiovascular diseases. In one study, it has been reported that intake of fish oil supplements reduced mortality rate by 29% for over a period of 2 years in the diet and reinfarction trial (DART), which was a randomized trial involving 2033 men post-myocardial infarction. Consumption of fish oil led to a noteworthy reduction in unexpected demises by 45%, cardiovascular diseases deceased by 30% and a 20% decrease in overall mortality. According to the recent clinical trial studies, it has been found that the risk of cardiac arrhythmias is decreased with the help of omega-3 fatty acids, and they also improve the health of the patients suffering from plaque formation caused by atherosclerosis. Omega-3 fatty acids enhance the electrical-stability of heart cells, thereby extending its relative refractory period and helping treat arrhythmias [75,76].

2.1.3. Soy Isoflavones

Soy proteins and soy isoflavones are important nutrients with potentially medicinal benefits such as antihyperlipidemic, antihypertensive, anti-hyperglycemic, antioxidant, anticancer, anti-inflammatory, anti-obesity and neuroprotective activities that support the biological plausibility for observational associations. It is evident from clinical study reports that consumption of soy protein reduces serum cholesterol levels in humans [77]. In addition, USFDA has evidenced that 25g of soy proteins or isoflavone per day intake showed a lowering of blood pressure in postmenopausal women. Moreover, soy proteins exert favorable effects on the serum lipid concentrations, especially in hypercholesterolemic patients. In a study, subjects fed with a diet low in saturated fat leads to the decrease in risk of coronary heart disease. Soy isoflavone had no effects on the lipid profiles [78]. Further a study reported significant reduction of the ratio of LDL to HDL after intake of new soy products, having high levels of isoflavones, cotyledon soy fiber and soy phospholipids (Abaco and Abalon) [79].

2.1.4. Proteins, Peptides and Amino Acids

Hypertension is associated with cardiovascular diseases. ACE (angiotensin converting enzyme) inhibitors have been a chief line of therapy to treat the condition, but these drugs lead to side effects such as hypotension, elevated levels of potassium, impaired renal function, coughing and skin rashes [80]. Natural, ACE inhibitors are found in casein and whey protein derived from milk. It is also evidenced from animal studies that these milk-derived proteins exert antihypertensive effects. The same has been reported in clinical studies where a statistically significant hypotensive effect has been observed [81].

2.1.5. Antioxidant Vitamins

Antioxidants have been used as potential supplements in chronic diseases such as cardiovascular diseases and cancer. They reduce LDL-cholesterol oxidation by counter-acting the damaging effects of free radicals. Vegetables, fruits, fish and fixed oils contain antioxidant vitamins in large volumes, which works by preventing the formation of oxygen free radicals or by entrapping them. It has been evidenced in some epidemiologic studies of CHD patients on a diet of large quantities of antioxidants that they result in fewer incidences of morbidity and mortality. Supplements having antioxidant vitamins C and E help in preventing CHD. However, supplementation with β -carotene can produce adverse effects and, thus, is not recommended. The National Health and Nutrition Examination Survey-I cohort study observed that risk of CHDs decreases in vitamin C intake, which was found in the observation of over 10 years in American men and women of age group of 25–74 years, in which subjects were randomized with diverse combinations of 10 nutritional supplements for over five years [82].

2.1.6. Phytosterols

Phytosterols are structurally similar to cholesterol. They tend to compete for absorption through the small intestine. These are found naturally in vegetable oils, seeds, nuts, grains, wood pulp, etc. [83]. Intake of phytosterols led to increased hepatic uptake of LDL, reduced blood LDL levels, and reduced absorption of cholesterol. Studies have indicated up to 15% reduction in LDL levels by intake of plant sterols [84]. Plant sterols are derived from natural grains such as soy, sunflower and corns. Various studies evidenced that the consumption of 2–3 g/day of plant sterols/stanols tend to reduce LDL cholesterol levels up to 20%; although, there is substantial variation amongst individuals [85].

2.2. Nutraceuticals in Cancer Chemo- and Radiotherapy

Radiotherapy and chemotherapy are conventional therapies for cancer therapy but have serious side effects and various complications (e.g., pain, fatigue, diarrhea, vomiting, nausea and hair loss) [86,87]. There are some cancers that are highly resistant to chemo- and radiotherapy and, because of this, systemic cytotoxic chemotherapy and radiotherapy are not very operative at cultivating patient subsistence [87,88]. In this situation, various combination therapies overlay an efficient means to treat cancer. Likewise, there are a variety of plants and natural supplements that are observed to reduce side effects of radiotherapy and chemotherapy. Thus, these should be used in the combination with radio- or chemotherapy for the reduction of side-effects and to augment treatment effectiveness. Proliferation of cells leading to cancer results in the need to treat, and the nutraceutical industry is evolving to treat the needs of the consumers. The evolution of the nutraceutical industry can be recognized as it has now reached disease prevention after it started from health promotion. A plethora of currently used herbs and phytochemicals are safe pharmacologically and have been proven to be potent nutraceuticals in suppressing tumor progression, alleviating the disadvantages of radio- and chemotherapy and increasing the sensitivity of these therapies [89,90]. Caponio and his team evaluated the effects of the phenolic compounds found in Aglianico Grape pomace (GP) on colorectal cancer cell lines at varying stages of development after subjecting them to an *in vitro* digesting model. Aglianico GP extract was found to have strong effects on cell proliferation and apoptosis, as well as on other cellular processes. A substantial upregulation of Bax, as well as the Bax/Bcl-2 ratio and caspase-3, was observed in both HT29 and SW480 cells. UHPLC-DAD analysis revealed that anthocyanins, phenolic acids, and flavonoids were the primary components responsible for the elevated (total phenolic content) TPC and antioxidant activity in the Aglianico GP digested extract [91]. In 2021, Zhang and his co-workers studied the combined effect of chrysin and apigenin by suppressing the activity of P38-MAPK/AKT pathway in colorectal cancer. Apigenin and chrysin, both at 25 μ M, substantially inhibited clone number, migration and invasion, while increasing apoptosis in both colorectal cancer (CRC) cell lines. Additionally, chrysin and apigenin significantly suppressed p-P38 and p-AKT. Anisomycin, a P38 agonist, effectively mitigated the tumor-inhibiting action of apigenin and chrysin. Together, apigenin (25 μ M) and chrysin (25 μ M) had a synergistic impact in limiting the proliferation and metastasis of CRC cells by suppressing the P38-MAPK/AKT pathway [92].

2.2.1. Curcumin (Diferuloyl-Methane) from Turmeric (*Curcuma Longa*)

Curcumin has been classified as a commanding nutraceutical for cancer treatment. Pre-clinical studies with curcumin suggest that it inhibits carcinogenesis in different types of cancers, such as pancreatic, colorectal, prostate, gastric and hepatic cancer; in addition, it has been able to suppress it at every step, that is, angiogenesis, metastasis and proliferation. It is much more effective when it is in combination with the chemo- and radiotherapies for cancer treatment [93,94].

2.2.2. Ginger

Ginger is an antimutagenic, antioxidant and anti-inflammatory nutraceutical and is known to diminish the side effects of radio and chemotherapy. It is these properties

of ginger that provide helpful radio-protector activity. A reported loss in the doses of morphine in cancer patients is seen with the help of Ginsenoside Rf and Ginseng and its polysaccharides are helpful in reducing the side effects of cancer treatment therapies, which has reported to cause a 50% less risk in the recurrence of cancer [95].

2.2.3. Genistein

Genistein is a potent isoflavone and has promising anti-carcinogenic properties. In vitro studies have shown that there are a few components that exert their antitumor effects only at higher concentrations that are not possible to achieve at normal dietary consumption [96]. Thus, it is difficult to achieve the desired effect at the tumor site, which leads us into thinking that the mode of delivery is a very important factor that needs to be considered in in-vivo studies and clinical trials. For a therapy to be formulated, the non-toxicity of the natural components is a very important factor. Nevertheless, it has been found that some compounds are more potent if administered early in life and Genistein is one of them [97].

2.3. Nutraceuticals in the Treatment of Prostate Cancer (PCa)

Prostate cancer is the most common type of cancer and has been recorded as the second leading cause of mortality by cancer in American males. It has been noticed that men descendent from United States and Africa have the highest prostate cancer mortality rates compared to those of European descent. Although current strategies of treatment are quite potent and effective, there are always opportunities of resistance towards disease and progression to metastasis and many more that may develop over time. Therefore, more effective and non-toxic therapeutic approaches are required to overcome these major hurdles and provide proper management and treatment of this disease. Thus, in this regard, various potential safe nutraceuticals are available as effective anti-PCa agents. Evidently, execution of nutraceuticals might help in the development of precision in the design of the medicines and reduce the toxicities associated with chemotherapy and decrease the resistance of disease and have the potential of treating the disease in both localized and advanced stages. Some of the nutraceuticals that can potentially be used as treatment are discussed below [98,99].

2.3.1. Silibinin

Silibinin that is, flavanolignan from milk thistle “*Silybum marianum*” seeds have potent anti-carcinogenic effects for a plethora of tumors including PCa. A pre-clinical animal model shows significant anticancer activity of silibinin in the treatment of PCa, and phase II clinical trials bioavailability studies have also been evaluated. There is still a need of larger clinical trials to be performed to confirm the biological efficacy and effectiveness of silibinin as a nutraceutical for effective clinical management of advanced or localized form of PCa [100,101].

2.3.2. Soy Isoflavones

Soy Isoflavones are members of the polyphenolic flavonoid family, mainly found in soybeans, red clover, kudzu root, etc., and are widely used in cuisines from Asia and Africa. Clinical studies have proven some benefits against the disease PCa with the help of Soya Isoflavones as it has a marked effect on inflammatory signaling and insulin. Among others there have also been reports in which Isoflavones have shown immuno-modulatory properties in the plasma of asymptomatic bio-chemically-recurrent PCa patients. It was found out that the high concentration of genistein in plasma was linked to a 69% reduction in the risk of future development of PCa in Chinese patients. If isoflavones are administered short term, such as for a period of 6 weeks, they show an inhibitory effect in the cell cycle of prostate tumor and also shows apoptotic-associated signaling; however, it does not have any influence on the levels of testosterone, PSA, free testosterone and total cholesterol in patients suffering from PCa. A clinical trial performed for 6 months showed that the intervention of soy protein had no effect on molecular markers determining proliferation

and apoptosis, i.e., EGFR, Bax:Bcl-2, Bax:PCNA ratios in patients with high-risk and low-grade PCa. On the contrary, soy protein (alcohol-washed) intake decreased the tissue levels of Bax and PCNA in comparison with patients receiving milk protein treatment [102,103]. Recently, Zhu and his team reported that the potential cholesterol-reducing effects of soy protein isolate were enhanced by glycation with soy soluble polysaccharide at higher degrees of glycation (DG) [104].

2.4. Nutraceuticals for Skin Treatment

The skin is known to be the body's largest organ and it offers protection against all sorts of microorganisms, ultraviolet radiations and chemicals also participating in sensitivity. As a result of having a major role in protecting the body, skin may face alterations, such as immune dysfunction, photo-aging and inflammation, which may result in harm on human health [105]. A potential strategy of delaying or diminish pre-mature ageing of the skin and alleviation of skin-related disorders can be found with the help of nutraceuticals. These nutraceuticals can be bioactive peptides, bioactive polysaccharides, botanical extracts, carotenoids, etc. Supplementation with these products in several human trials has evidenced fewer signs of ageing and also protection against UV-radiation ageing [106].

2.4.1. Bio-Active Peptides

Peptide moieties are the combination of two or more amino acids and are short sized with low molecular weight (<3 kDa), and some may perform important biological actions that are termed bioactive peptides. Bioactive peptides have been isolated from a wide variety of dietary proteins, including plant and animal. Eggs, milk (casein and whey), and meat proteins are the most common sources of animal protein. Soy, oat, pulses (chickpea, bean, pea and lentil), canola, wheat, flaxseed and hemp seed are common plant sources for bioactive peptides [107]. Peptides that are used for cosmetic purposes are usually derived from collagen and typically serve as nutraceutical formulations because of their increased bioavailability and solubility [108]. It has typically been observed in a controlled study of VERISOL[®], which contains bioactive collagen peptide (BCP). In this study VERISOL[®] and a placebo were given to subjects for 8 weeks and skin wrinkles were measured before the treatment and after 8 weeks. It was observed that BCP promoted a significant decrease in the eye-wrinkle volume in comparison to placebo after a period of 8 weeks of treatment. Not only this, BCP intake showed an increase in the content of elastin and procollagen type1 along with an increase in the fibrillin content. Thus, this treatment reduced wrinkles and has encouraging effects on skin matrix synthesis [109].

Peptan F and porcine origin Peptan P are some other nutraceuticals of fish origin containing collagen peptides used to slow aging by effectively maintaining the moisture content within skin layers. Recently, a study indicated improved skin properties without risk of oxidative damage by use of a nutraceutical product Celergen[®], proving it a safe and effective supplement. This nutraceutical is based on a marine collagen peptide derived from deep sea fish, grape skin, coenzyme Q10 and leutinin [110,111].

2.4.2. Bio-Active Polysaccharides

These are sugar-based polymers that have the energy storage and structural functions. They are present in life forms such as plants, fungi, animals and prokaryote organisms having diverse monosaccharide combinations, physicochemical properties and structures. The most useful of them for the nutraceutical formulations is the Glycosaminoglycans from the marine origin. The basic unit of these are- an un-branched disaccharide (repeating) unit of amino sugar called N-acetylglucosamine or N-acetylgalactosamine and an uronic acid called glucuronic or iduronic acid [112,113]. A human trial of the formulations containing these was conducted using Imedeen[®] DermOne[®], these contain some protein fractions as well along with the glycosaminoglycans and served as dietary supplements for skin care. In addition to the protein the supplement contained zinc gluconate and vitamin C which are relevant for skin care. In the trial conducted, 10 women were treated with an amount of 500

mg of Imedeen® for a period of 90 days. Parameters evaluated were, dryness, brittleness of hair and nails, wrinkles and mottles. It was seen that after 90 days, all these signs were improved, and observations established skin thickness and elasticity [105].

2.4.3. Bio-Active Botanical Extracts

These extracts are multifaceted mixtures of various compounds having diverse structures and origin. Since long times they are being used and reviewed. Polyphenols are one of the key natural compounds with cosmetic applications with a plethora of families and structures. These are plant-based micronutrients available from diet. These are beneficial as support therapy in the prevention of diseases and also improved the outcome of diseases. Various polyphenols have significantly different bioavailability and the most abundant polyphenols in our diet possess the maximum concentration of active metabolites in target tissues. Their composition and proportion vary depending on the procedure of extraction and families [114]. Pycnogenol® is a formulation being made with the help of these and is rich in catechins, flavonoids and procyanidins (B1, B2, B3, B7 C1 and C2), also, they contain phenolic acids such as ferulic acid and caffeic acids. It is also confirmed to have several effects such as cholesterol lowering and cardiovascular benefits because of its antidiabetic, anti-inflammatory and antioxidant properties [115–117].

2.4.4. Carotenoids

These are naturally occurring pigments found in algae, photo-synthetic bacteria and various plants. These have linear tetra terpenoid structure. These are found in natural sources such as fruits, vegetables etc. α -carotene, β -carotene, β -cryptoxanthin, lutein, zeaxanthin, and lycopene are the most commonly used dietary carotenoids [118]. These carotenoids are used for skin health such as anti-ageing and photo protection of skin. The probiotics and carotenoids are reported for decreasing the skin damage due to UV-exposure and also in modulating early skin biomarkers of UV effects. A Carotenoid mixture supplement of α -carotene, β -carotene and lutein are proved effective in photo protection. Similarly, a mixture of beta-carotene, lutein and lycopene carotenoids is reported for protection against erythema. The vitamin C and E is studied for the photoprotective effect and found to be effective in skin health care [119]. Vitamin C is a hydrophilic vitamin, commonly taken in large doses via consuming various food products with the intent of inhibiting the formation of carcinogenic nitrous metabolites. It acts as a cofactor for the synthesis of collagen fibers and inhibits the biosynthesis of elastin in fibroblasts thereby preventing its accumulation, which is highly present in photo-damaged skin. In combination with vitamin E, it acts synergistically working with its mechanism of transformation. Vitamin E is the main lipophilic antioxidant and is found in the form of tocopherols. It binds with peroxy radicals, thereby preventing lipid peroxidation of polyunsaturated fatty acids. In addition, its use for preventing photodamage, sunburn, atopic dermatitis, etc., is clearly evidenced [120,121].

2.5. Nutraceuticals as Specialized Medical Products

According to the legal basis, dietary foods and enhancements for distinct medical purposes are specialized medical products. These dietary supplements should be regulated according to the regulatory agencies such as 'European Food Safety Authority' and the 'U.S. Food and Drug Administration', in addition to numerous national protocols issued most often by the 'Ministry of Agriculture' and/or 'Ministry of Health' of various countries around the world [122].

Nutraceuticals are non-specific biological therapies used to promote wellness, prevent malignant processes and control symptoms. Figure 3 shows a flow chart indicating role of nutraceuticals in health promotion and disease prevention. Various nutraceuticals in health promotion are summarized in Table 2 [123–125].

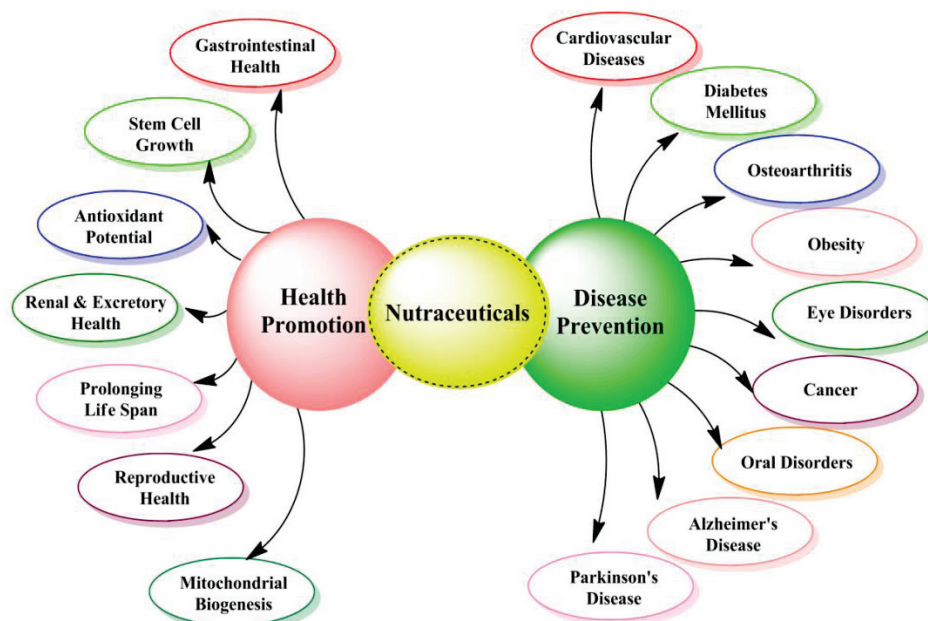


Figure 3. Role of nutraceuticals in disease prevention and health promotion.

Table 2. List of nutraceuticals with health benefits.

Nutraceuticals/ Dietary Supplements	Nutrients	Health Benefits
Water Soluble Vitamins	Vitamin C	Wound healing, Antioxidant
	Vitamin B1	Carbohydrate metabolism, Neurological function
	Vitamin B2	Energy metabolism, Nerve function
	Vitamin B3	Brain function
	Vitamin B6	Convert proteins to energy
	Vitamin B12	Formation of RBC's, Synthesis of amino acids
	Folic acid	Metabolism of fat, protein and carbohydrate
	Pantothenic acid	Formation of RBC's, Formation of genetic material of cells Intraneuronal synthesis of acetylcholine Synthesis of cholesterol, steroids, and fatty acids
Fat Soluble Vitamins	Vitamin A	Cancer, Skin disorder, Healthy vision
	Vitamin D	Antioxidant
	Vitamin E	Absorption of calcium, Formation of bones and teeth
	Vitamin K	Boost immune system, Antioxidant Blood clotting
Minerals	Calcium	Maintaining bone strength, blood clotting
	Iron	Oxygen transport, Energy production
	Magnesium	Healthy nerve and muscle function and bone function
	Phosphorus	Phosphorylation process, Genetic material
	Copper	Heart functioning, Iron absorption
	Iodine	Functioning of thyroid gland
	Chromium	Diabetes
	Selenium	Antioxidant
	Zinc	Sperm production, wound healing
Herbals	Aloe vera	Anti-inflammatory, Wound healing
	Evening primrose oil	Treatment of atopic eczema
	Garlic	Anti-bacterial, Anti-fungal
	Ginger	Carminative, Anti-emetic
	Ginseng	Adaptogen
	Green tea	Cell mediated immunity, Antioxidant

3. Formulations and Challenges Involved

A quality nutraceutical formulation with physical and chemical stability, adequate safety, technological feasibility and still cost effective entails many challenges. When compared with drug molecules that are well defined chemical entities, botanicals are complex ingredients containing multiple chemical constituents and usually several classes of compounds are present in a single product. Most of these botanicals are susceptible to heat, light, oxygen, alkaline pH and elevated humidity. These are usually having poor flow, bulk density and variable particle size distribution. Thus, successful development of nutraceutical formulation requires knowledge of the fundamental aspects of the physicochemical properties of the different types of ingredients, the use of adequate techniques of manufacturing, selection of the right excipients and the addition of suitable manufacturing overages based on critical stability studies [126,127]. Here, emphasis is given on:

- Challenges with various dosage forms;
- Approaches to deal with formulation challenges;
- Excipients selection.

3.1. Challenges in the Formulation of Nutraceuticals and Dietary Supplement

While formulating nutraceuticals, the poor aqueous solubility, high melting point of nutraceuticals and chemical instability of active constituents pose difficulties. For example, omega-3 fatty acids, carotenoids, oil soluble vitamins, curcumin possess high nutritional value but are poorly soluble. Therefore, the possible approach is to formulate these as novel delivery systems. These novel delivery systems make them costly. Thus, efforts are needed to make these formulations cost effective [128].

Another challenge in formulating nutraceuticals is their high melting point. For example, phytosterols, fatty alcohols and carotenoids all have high melting points that may cause instability to formulation. Therefore, the possible approach is to prepare solid dispersion/dissolve in suitable grade solvent and introduce in food as suspended nanocrystals. However, the challenge is again that it leads to deteriorated stability and shelf life, disagreeable appearance, and obnoxious odor and mouthfeel, which affect market value and customer demand. Therefore, there is need to develop cost-effective technologies [129–131].

Chemical instability is another challenge. For example, omega-3 fatty acid rich oils, such as fish oils, flaxseed oil, cod liver oils; carotenoids; lycopene or curcumin all have stability issues. The extent of the chemical degradation is completely dependent on the composition of the bioactive product, the environmental conditions such as temperature, pH, pressure, etc., or the presence of metals or other such oxidation-promoting agents. For such compounds, the development of nanoscale products is essential to protect them from degradation [132–138]. Further, in the case of the development of probiotics, special, selective bacterial strains are necessary. A current challenge is the selection of the proper strain followed by their incorporation in foods. Application of any mismatching bacterial or toxic cultures, in addition to their negligent handling, can cause disastrous consequences. Apart from these challenges, other considerations are solid dosage formulation and process design for drug products and nutrition products that are similar, but the purpose and regulatory requirements may differ [139,140].

Finally, there is a challenge in the formulation of nutraceuticals and dietary supplement dosage forms that are suitable for different groups of the aging population, especially older adults and children. This is because this group of people has limitations in solid dosage form (tablets or capsules) swallowing (dysphagia). Therefore, advanced dosage forms such as orodispersible tablets, fast dissolving films and easy-swallowing gels, which are normally used in pharmaceutical applications, have to be considered in nutraceutical and dietary supplement administration [141–143].

3.2. Approaches to Deal with Formulation Challenges

One of the most widely used approaches is isolation or preparation of concentrates of nutraceuticals from natural sources. It is advantageous as most of herbal nutraceuticals are

to be administered in a large dose per daily serving. Further, multiple “active ingredients” are present in different sources. There is significant variation in active ingredient compression and flow characteristics within one dosage form. There are large variations in heat and moisture sensitivity of ingredients within one formula. Significant stability challenges are there with multiple opportunities for interaction. Different extraction processes involve microwave-assisted extraction, counter current extraction, maceration, percolation and Soxhlet extraction. Natural bioactive compounds are plant extracts; herbal concentrates; fruit, vegetable and specialty concentrates; and fungal and microbial materials, which are included in feedstock to produce concentrates (bio-fermentation process). Concentrates may include excipients used in production (such as spray dried carriers) [144,145].

Another promising approach is modification in delivery systems. Novel Drug Delivery Systems have been used to modify the properties of various compounds to produce new generations of drug compounds. They also play a role in the food industry and in various kinds of nutritional supplements. Various formulations of supplements prepared by nanotechnology, called nano formulations, that have increased bioavailability, reduced side effects and protect the active ingredients against the process of degradation, have been reported [146,147]. The polyphenols obtained as nutraceuticals in the form of dietary sources have proved greatly advantageous in improving diseased symptoms as observed in preclinical and clinical studies. One of the most commonly encountered disadvantages is its lower bioavailability due to the lesser gastrointestinal absorption in the upper tract attributable to hydrophobicity, presence in the polymeric or glycosylated form in foods, and they bound tightly with the food matrices. All these factors lead to the minimum bio-accessibility of polyphenols inside the body. Therefore, food processing-based research and development techniques along with nanoformulations, enzymatic treatment, probiotics combination therapies, among others, have proved advantageous to overcome this challenge [148,149].

3.2.1. Liposomes and Nanoemulsions

Liposomes and Nanoemulsions are also termed as bilayer phospholipid vesicles and possess greater potential for the industry of nutraceuticals as they can simultaneously encapsulate both hydrophilic and lipophilic materials. This ensures a synergistic effect and is also helpful in the protection of bioactive compounds that are highly sensitive, ensuring enhanced bioavailability, sustainable release and storage stability. As the nanoliposomes have unique properties, they can be effectively used in operative prevention against diseases and also for health promotion. One of the latest examples is lipid-based nanocarriers, nanophytosomes, which help in enabling the delivery of botanical nutraceuticals. They have the potential to be used in various food products for the design of novel functional beverages and food products. A study indicates increased chemical and physical stability of rutin when rutin complexes were formed in the form of phytosomes. These phytosome complexes of rutin were formed with the help of phosphatidylcholine (PC) with the molar ratio of (rutin: PC) being (1:3) with a particle size of less than one hundred nm and 99% encapsulation efficiency, as it was able to mask the undesirable properties of rutin. These were termed as phytosomes, phosphatidylcholine (PC)—rutin complexes [150,151].

3.2.2. Lipid-Based Carriers

Lipid formulations in the form of nanocapsules, micronized carries are potential candidates to be used to effectively enhance the controlled release, solubility and bioavailability of phenolic compounds. As an example, β -Car nanocapsules (>300 nm), due to their physical stability showed only negligible variations during storage, which suggested that they can be widely used as functional foods and beverages along with being nutraceutical products [150,152].

3.2.3. Polysaccharide Matrices

These are the kind of matrices that have numerous enzymatic exposures that guarantee degradation at specific points in the large and small intestine. When being used as a nanoparticle coating, they can effectively retard the nonspecific release of bioactive components encapsulated within until the coating is exposed in the intended environment where it was intended to be released. These coated nanoparticles can potentially be used to target various diverse organs of the GI tract to help improve the oral bioavailability [150,153].

3.3. Excipient Selection

Next, the approach to deal with formulation challenges of nutraceuticals is modification in formulation parameters by appropriate selection of excipients. For nutrition, the final formula must be robust to accommodate the variable physical characteristics of natural ingredients in a complex formula. Materials and manufacturer must meet internal quality and safety, specifications and performance requirements. One formulation's functionality can be another formulation's dysfunctionality. Excipient functionality can only be properly assessed in the context of a particular formulation and manufacturing process. For natural product formulation, excipient functionality in a particular formula is heavily influenced by the complex combination of multiple active ingredient characteristics. Occasionally, seemingly equivalent excipients are not equivalent in functionality [154].

4. Safety and Quality Control of Nutraceuticals

Nutraceuticals are taken as supplements by users, which are available as over-the-counter products. Therefore, their safety is of prime concern, else it may lead to lethal effects. The most commonly observed issues are contamination, adulteration (inadvertent or intentional) or misleading labels. To demonstrate adulteration, three different detection strategies may be adopted as (1) the presence of an undeclared substance, (2) that a component is deviated from its normal level (content) and (3) that a profile is unlikely to occur [155].

Adulteration may be inadvertent adulteration or intentional adulteration. Inadvertent adulteration may exist due to different conditions. For example, during the different stages of plant growth, the formulation and manufacturing of nutraceuticals, or during storage, contamination with fertilizers, heavy metals, fertilizers or microbial agents may take place. Adulteration may also take place with synthetic drugs, substitute species, dust, pollens, insects, rodents, parasites, microbes, fungi, mold, toxins and heavy metals. Any of this type of contamination may lead to infections or even serious illnesses such as gastritis and associated complications, liver injury and even life-threatening conditions. Therefore, raw-material and finished-product quality control is required and may be determined by specifications outlined in certain monographs, along with stability of active compound(s) and microbiological control [156].

Serious harmful effects may result from the intentional adulteration in supplements or herbal remedies. It usually occurs with synthetic compounds, mostly undeclared. It usually occurs with the intentions of altering pharmacological response and earns economic benefits. The sourcing of nutraceuticals from plants is often very limited, and extract preparation is time- and cost-consuming [157,158]. Therefore, these adulterations are not allowed from regulatory authorities.

Some examples of adulterations are quoted in below sections.

- (a) Ibuprofen hydrochloride monohydrate is a drug molecule that works by inhibiting serotonergic and noradrenergic reuptake and shows effects as an anti-obesity drug, and is a common adulterant. In a study conducted with twenty-two samples of dietary supplements in China, eleven were found to be contaminated with phenolphthalein, *N*-mono-desmethylsibutramine, and sibutramine. In another similar study performed on fifteen samples in China, four of them contained sibutramine and *N*-di-desmethylsibutramine [159]. Further, it has also been reported that two pregnant women in Turkey lost their wombs due to consumption of adulterated Chinese herbal medicine "meizitanc" [160]. Sibutramine has also been reported as a

solvent in slimming preparations. It has led to mania-like psychosis in two women in Hong Kong [161].

- (b) Fenfluramine is another drug that was used as an adulterant in Chinese traditional medicines and found in many slimming preparations. It caused primary pulmonary hypertension and valvular heart disease. This drug was withdrawn from market in 1997 [162].
- (c) In some weight control programs using anorexigens, diuretics, stimulants and laxative agents, it has been demonstrated that these products contain adulterants as ephedrine, norephedrine, caffeine and furosemide [163].
- (d) Morphological substitute usage is another common example of adulteration that may cause serious health issues. For example, *Panax ginseng* (Araliaceae), also known as “Asian or Korean ginseng”, is used as traditional medicine. It has been found to be adulterated with roots of *Panax quinquefolius* L. (American ginseng) and *Eleutherococcus senticosus* Maxim (Siberian ginseng), which may cause health problem [164,165]. Another similar example is *Panax ginseng* being used as adulterant in roots of *Mandragora officinarum* L. (Solanaceae) because they are morphologically similar, but have completely different pharmacological effects and phytochemistry [166]. Further, roots of *Pfaffia paniculata* (Mart.) Kuntze (Amaranthaceae), also known as “Brazilian ginseng or suma root”, morphologically resembles *P. ginseng* roots, but the phytochemical content is different and, hence, pharmacological action is also different [167].
- (e) Some other examples of physical similarity between species include the flower *Anthemis nobilis* L. and the chamomile, *Matricaria chamomilla* L. (Asteraceae), both of which are listed in the European Pharmacopoeia as therapeutic plants, as well as several other species in the family Asteraceae, such as *Tanacetum parthenium* (L.) Sch. Bip., *Tanacetum cinerariifolium* (Trevir.) Schultz Bip., *Tripleurospermum callosum* (Boiss. et Heldr.) E. Hossain, *Bellis perennis* L. and *Leucanthemum vulgare* L. Furthermore, the pharmacological activity differs depending on the phytoconstituents [168,169].
- (f) One of the serious intentional adulterations is use of peanut skin extract in different grape products. Grape seed-containing drugs are said to have very high bioactive polyphenols content and used in prevention of cardiovascular and neurodegenerative disorders. The peanut skin is used as it is widely available, is a high-volume byproduct and is very cheap compared to grape products. However, it is a potential allergen, which may cause serious concerns when used as adulterant, which has been reported in a study where tested products contained no detectable quantities of grape seed extract, but only peanut skin as adulterant [170].
- (g) One of the most important categories affected by adulteration is species containing essential oils (spices). As these spices are of high economic value and are export-oriented commodities, these are widely adulterated with natural and synthetic adulterants. One of the most commonly reported examples is adulteration of pure *Lavandula angustifolia* Mill. (Lamiaceae) oil with other species of same genus, which are almost six times cheaper. Essential oils from citrus are usually adulterated with sweet orange essential oil. Another quoted case is use of citronella oil (*Cymbopogon winterianus*), which is quite cheaper, as an adulterant in very high economic value essential oil from *Melissa officinalis* herb (balm oil). *Melissa officinalis* herb contains citronellal as the main constituent whereas citronella oil contains enantiomeric mixtures of citronellal [171,172].
- (h) Another intention of adulteration is to add to the industrial value to the product. For example, synthetic α -irone and β -irone is added to iris (*Iris* sp., Iridaceae) oil to enhance the commercial value of the product, linalyl acetate or linalool, as the olfactory quality of bergamot or lavender oil becomes improved [171,172]. Other similar cases are when vegetable oils are added to increase the weight of other products—for example, lemongrass oil is diluted with coconut oil and sandalwood oil with polyethylene glycol. Leaf cinnamon essential oil contains lesser quantity of cinnamaldehyde but has the same olfactory notes as those of cinnamon bark essential oil, and, therefore, it has been reported as adulterant. Cheaper petit-grain oil made from leaves is used as adulterant in neroli oil made from the flowers of *Citrus aurantium* L. spp. *amara* L.

var. *pumilia* (Rutaceae) [171]. Another case study reports that samples of the dietary supplements containing the leaf extract of *G. biloba*, used for cerebrovascular diseases, tinnitus and Alzheimer's type of dementia, were demonstrated to be adulterated with free flavonols and glycones (such as quercetin and kaempferol, respectively) as well as genistein, the isoflavone derivative [173]. In another case, abietic acid has been reported as an adulterant in a herbal preparation used for the treatment of psoriasis [174].

- (i) One potential hazardous adulteration that has harmful effects on human health is adulteration with allopathic drugs. The commonly used molecules in such types of adulterations are non-steroid anti-inflammatory drugs (NSAIDs), steroids, anti-diabetics and analgesics. For example, glibenclamide and metformin are reported in anti-diabetich herbal/botanical supplements [175–177]. In addition, it has been reported that herbal anti-diabetic remedies contain chlorpropamide, gliclazide, glimepiride, glipizide, pioglitazone, tolazamide and tolbutamide. Many analgesic compounds, including codeine, indomethacin, ketoprofen, morphine, oxyphenbutazone, paracetamol, phenylbutazone, diclofenac, dipyron, ibuprofen, mefenamic acid, salicylamide and salicylic acid, are found in adulterated dietary supplements. It has been reported that herbal preparations contain anabolic steroids and hormones, such as rostenidione, betamethasone valerate, betamethasone, clenbuterol, dexamethasone, flumethasone, hydrocortisone, prednisolone, prednisone, testosterone propionate, testosterone isocaproate, testosterone phenylpropionate and testosterone decanoate [178]. Another significant case that has been reported is adulteration with phosphodiesterase type-5 (PDE-5) inhibitor analogues, such as sildenafil citrate (Viagra[®], Pfizer, New York, NY, USA), vardenafil hydrochloride (Levitra[®], Bayer, Leverkusen, Germany), and tadalafil (Cialis[®], Eli Lilly, Indianapolis, IN, USA), have been found in dietary supplements containing well-known natural constituents such as *Panax ginseng* L., *Astragalus membranaceus* (Fisch.) Bunge, *Schizandra chinensis* (Turcz.) Baill., *Ginkgo biloba* L., and many others. One of the possible consequences of such adulteration with prescription-only drugs such as phosphodiesterase type-5 (PDE-5) inhibitor analogues, which are contraindicated in men taking nitrates, may lead to unsafe drop in blood pressure. Such reported adulterations are homosildenafil in a food beverage; acetildenafil and hydroxyhomosildenafil in some other herbal products; vardenafil, sildenafil, tadalafil, and vardenafil in an herbal product sold in Hong Kong; benzamidenafil in herbal products; and thiosildenafil, a thioketone analogue of sildenafil, in herbal aphrodisiac supplements; nitroso-prodenafil, a prodrug of aildenafil, is also a reported adulterant, which is as carcinogenic as nitroso derivatives. Researchers examined 91 herbal items for the presence of PDE-5 inhibitors and found that 74 of them actually contained the PDE-5 inhibitor analogs, despite the fact that none of the labels mentioned the presence of a synthetic inhibitor. Eighteen of twenty-three herbal dietary supplements on the Dutch market tested positive for sildenafil or a comparable PDE-5 inhibitor in a similar investigation. [179,180].

Misleading labels are also one of the sources that lead to harmful effects on human health. In one study conducted on five samples of soy-based dietary supplements, the isoflavone content was analyzed and compared with claimed label checked. It has been reported that three of five supplements failed to contain the claimed amount of the isoflavones, i.e., genistein and daidzein. It has also been mentioned that one of the preparations that claimed to contain 60 mg of isoflavone based on genistein per tablet and was found to contain 1.538 mg of genistein *per* tablet after HPLC analysis [181].

5. Formulation Challenges

Drug interactions may be described as the situation when the activity of one active constituent is affected due to presence of other constituents. It may be food–drug interaction or drug–drug interaction. The pharmacological response may alleviate, lessen or induce side effects [182,183].

- (a) Garlic (allicin) exhibits a hypotensive property and a hypocholesterolemic effect, acts as an anti-inflammatory agent and possess anti-bacterial as well as anti-fungal properties. When it is administered with anticoagulants (such as warfarin), it may lead to increased bleeding. With hypoglycemic drugs, such as insulin or glipizide, it may cause hypoglycemia. With protease inhibitors (such as indinavir or saquinavir), garlic decreases their blood levels and effectiveness [184,185].
- (b) Ginger is commonly used to treat various types of stomach problems—such as, to expel gas, motion sickness, diarrhea, nausea (anti-emetic) and loss of appetite. It is also used in pain relief from arthritis, menstrual pain, upper respiratory tract infections—coughs and bronchitis. Ginger taken with anticoagulants may lead to risk of bleeding. If ingested with hypoglycemic drugs, such as insulin or glipizide, it may cause hypoglycemia. When co-administered with calcium channel blockers, ginger might reduce further or cause an irregular heartbeat [186,187].
- (c) Green tea (polyphenols) improves mental alertness and thinking. It is also used to treat a plethora of other medical conditions, including Crohn's disease, Parkinson's disease, cardiovascular disease, diabetes, hypotension, chronic fatigue syndrome (CFS), tooth decay, kidney stones and skin conditions. Consuming green tea with stimulant medications could have dangerous consequences, such as elevated heart rate and blood pressure. Bortezomib (Velcade) may not be as effective against some cancers if used with green tea. Consuming green tea may reduce the effectiveness of warfarin [188,189].
- (d) The leaf extract of *Ginkgo biloba* is effective in the treatment of Alzheimer's disease and other forms of dementia, Raynaud's syndrome, peripheral vascular disease, vertigo and dizziness, premenstrual syndrome (PMS) and improving color vision in people with diabetes. Ginkgo, when administered with anticoagulants/with NSAIDs, it may increase the risk of bleeding. When administered with anticonvulsants, it may reduce the effectiveness in preventing seizures [190,191].
- (e) Licorice has been used for various digestive system complaints such as stomach ulcers, heartburn, colic and chronic gastritis. It is also used for sore throat, bronchitis and in treating infections caused by bacteria or viruses [192]. Licorice is also used in an herbal form called Shakuyaku-kanzo to increase fertility in women. It is also used to treat prostate cancer and the skin disorders such as eczema, in combination with other herbs. It may make antihypertensive drugs less effective, as it may increase salt and water retention. When taken with anti-arrhythmic drugs, it may decrease their efficacy as it may increase the risk of an abnormal heart rhythm. In some cases, it decreases the levels of potassium as it increases urine formation. In such cases, the risk of digoxin toxicity also increases, if patient is on digoxin [193–195].
- (f) Kava root (kava-lactones) medicine, native to South Pacific, is used to calm anxiety, stress and to treat insomnia. It is also used in the treatment of attention deficit hyperactivity disorder (ADHD), depression, migraines and other headaches, chronic fatigue syndrome (CFS), epilepsy, psychosis, common cold and other respiratory tract infections, muscle pain, tuberculosis and cancer prevention. Kava is applied to the skin for some skin disorders such as leprosy, to promote wound healing. It is also used in urinary tract infections (UTIs), pain and swelling of the uterus, menstrual discomfort and hot flushes in women with menopause. It is also used as pain reliever in toothaches. When co-administered with barbiturates and benzodiazepines, it may prolong or intensify their effects [196,197].
- (g) Chamomile (tea extract) is used as tea or dietary supplement for stomach cramps, to treat irritation from chest colds. It is also used for slow healing wounds, abscesses, gum inflammation, and skin conditions such as eczema, chickenpox and diaper-rash. The risk of bleeding increases when it is co-administered with anti-coagulants. Iron absorption also reduces in the presence of tea extract. The pollen is allergic in some cases. If one is allergic to ragweed pollen, chamomile use must be prohibited [198,199].

6. Patent Literature

Several published patents on nutraceutical applications in various areas, such as the agriculture and healthcare sector (such as promotion of health and disease prevention), and as nanocarriers for the improved delivery of nutraceuticals as a support therapy for many diseases, have been summarized in Table 3. Many nutritional products are commercially available and used widely.

Table 3. Patent Literature on nutraceuticals.

Patent Number	Patent Title	Year	Reference
CN104193540B	Fertilizer and insecticide use nutraceuticals disease prevention, disease prevention nutraceutical insecticidal fertilizer slow-release agent and the use thereof	2016	[200]
CN104262041A	Nutritional disease-preventing pesticide fertilizer and application thereof, nutritional disease-preventing pesticide fertilizer slow release agent and application thereof	2015	[201]
CN101371628A	Disease-preventing nutrient bag and manufacture method thereof	2009	[202]
US9669199B2	Bio-synchronous transdermal drug delivery for longevity, anti-ageing, fatigue management, obesity, weight loss, weight management, delivery of nutraceuticals and the treatment of hyperglycemia, Alzheimer's disease, sleep disorders, Parkinson's disease, aids, epilepsy, attention deficit disorder, nicotine addiction, cancer, headache and pain control, asthma, angina, hypertension, depression, cold, flu and the like	2017	[203]
WO2008054788A3	Transdermal delivery techniques for drugs, nutraceuticals and other active substances	2008	[204]
EP1835818B1	Product and method for producing a vehicle for oral administration of nutraceuticals	2011	[205]
US5955269A	Methods of screening foods for nutraceuticals	1999	[206]
EP2349302A4	Cardio-protective effects of nutraceuticals isolated from nigella sativa seeds	2012	[207]
US10517316B2	Combination of 25-hydroxyvitamin D and antioxidants/anti-inflammatories for bovine health	2016	[208]
US20080118583A1	Phyto-nutraceutical synergistic composition for Parkinson's disease	2008	[209]
AU2005200614A1	Nutraceuticals for the treatment, protection and restoration of connective tissues	2005	[210]
US7494674B2	Nutraceutical with tart cherries and method of treatment therewith	2009	[211]
CN101534807A	Pharmaceutical and nutraceutical products comprising vitamin k2	2009	[212]
US20100063153A1	Anti-cholesterolemic compounds and methods of use	2010	[213]
US6630160B1	Process to modulate disease risk with doses of a nutraceuticals	2003	[214]
US6080788A	Composition for improvement of cellular nutrition and mitochondrial energetics	2000	[215]
US20160089411A1	Method and composition for treating symptoms of sickle cell disease	2016	[216]
CN102088995B	The use of angiogenin and angiogenin agonist for treating diseases and disorders	2016	[217]
US20080193590A1	Highly refined cellulose nutraceutical compositions and methods of use	2008	[218]
US20040009244A1	Composition comprising melissa leaf extract for anti-angiogenic and matrix metalloproteinase inhibitory activity	2004	[219]
US20110009360A1	Nutraceutical Composition and Methods for Preventing or Treating Multiple Sclerosis	2011	[220]

Table 3. Cont.

Patent Number	Patent Title	Year	Reference
CN1822768A	Nutraceutical for the prevention and treatment of cancers and diseases affecting the liver	2006	[221]
US20210292265A1	Chalcones and derivatives for use in medicaments and nutraceuticals	2021	[222]
US20210113554A1	Nutraceuticals Having Sustained Release for Improved Bioavailability and Method of Production	2021	[223]
US20210290722A1	Nutraceuticals supplement composition for regulating metabolism and anti-aging	2021	[224]
US10981083B2	Process for fractionation and extraction of herbal plant material to isolate extractives for pharmaceuticals and nutraceuticals	2021	[225]
US20220040248A1	Prevention of Neuroinflammation associated Memory Loss Using Nutraceutical Compositions	2022	[226]
US11270791B2	In silico methods for obtaining nutraceutical compositions	2022	[227]
US20220160744A1	Multilayer pharmaceutical or nutraceutical solid dosage forms comprising pyrimidine and/or purine derivatives and b vitamins, preparation and uses thereof	2022	[228]
US20220184100A1	Enhanced d vitamin nutraceutical compositions and methods for making and used same	2022	[229]

7. Conclusions and Future Prospects

To conclude, nutraceuticals area potentially growing sector and are engaged in both the fields, either medical treatment or nutrition so as to assure integrated medical assistance. These act as potential dietary supplements, prevention of diseases such as CVD, the support and treatment of various types of cancer, and other healthcare benefits. Therefore, nutraceutical industries now understand and perceive extensively about the potential success of nutrients that affect people in healthcare. At present, medical care is assessed to be the domain of drugs. On the contrary, nutrition is only appraised to be a product for healthy living. In the forthcoming years, it is anticipated that work will be performed, as they both interact and complement each other. The implementation of newer technologies such as the application of genetically modified technology in the food industry, nanotechnology-based nutraceuticals, etc., leads to better medical treatment and health care benefits, which further extended the increase in the nutraceuticals revenue market. The scientific research ratifies that the improved safety and potential effects of newly developed nutraceutical products will further stimulate the investments in newer technologies, such as nutrigenomics, converging techniques, varied imaging technologies and its applications in nutrition development and healthcare.

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A Systematic Review of the Effect of Polyphenols on Alterations of the Intestinal Microbiota and Shared Bacterial Profiles Between Metabolic Syndrome and Acne

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Abstract: Introduction: Microbiota, composed of micro-organisms like bacteria, viruses, and non-pathogenic fungi, plays a crucial role in digestion, vitamin production, and protection against dangerous microbes. Several factors, including age, diet, alcohol consumption, stress, environmental microorganisms, and therapies (particularly antibiotics), as well as birth and nursing, could modify the microbiota. Recent research has highlighted its alteration and involvement in a various disease, including metabolic syndrome and acne. This systematic review aimed to identify common biomarkers and microbiota alterations shared between metabolic syndrome and acne, and to explore how the potential prebiotic activities of polyphenols may promote intestinal eubiosis. **Materials and methods:** A comprehensive search in PubMed and EMBASE resulted in 4142 articles, from which nine studies were selected based on specific criteria after removing duplicates and reviewing abstracts and full texts. All studies correlated the microbiota alteration in both pathologies and the activity of polyphenols in metabolic syndrome. **Results:** This review suggests that acne may be influenced by some of the same microorganisms involved in metabolic syndrome. While the literature highlights the effectiveness of polyphenols in treating metabolic syndrome, no studies have yet demonstrated their specific impact on acne. **Conclusions:** The research points to the potential benefits of polyphenols in modulating the microbiota, which could be relevant for individuals with metabolic syndrome. However, due to the limited data available, it was not possible to establish a direct correlation between metabolic syndrome and acne.

Keywords: nutraceuticals; microbiota; bacteria; metabolism; systematic review

1. Introduction

1.1. Gut Microbiota

The gut microbiota, also known as the gut flora, is a community of microorganisms that reside in the human gastrointestinal tract. This community consist of a large number of cells, estimated at up to 10^{13} , that belong to all three domains of life (*Bacteria*, *Archaea*, and

Eukaryotes) [1]. The gut can host thousands of bacterial species belonging to different phyla, including *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Fusobacteria*, *Tenericutes*, *Spirochaetes*, *Cyanobacteria*, and *Saccharibacteria* [2,3]. In contrast, the diversity of fungi in the human gut is limited. From samples of 98 human adults, a pyrosequencing study identified about 66 fungal genera, with *Saccharomyces*, *Candida*, and *Cladosporium* being the most prevalent [1,4]. The gut microbiota plays a pivotal role in maintaining host health, influencing various physiological functions and contributing to digestion, nutrient absorption, immune system maturation, and protection against harmful pathogens [5]. A healthy balance of the gut microbiota is often associated with good health, while an imbalance, known as “dysbiosis”, can be linked to various pathological conditions [6]. The gut microbiota plays a crucial role in the fermentation of non-digestible substrates such as dietary fiber and endogenous intestinal mucosa. This activity favors the development of specialized microorganisms that generate short-chain fatty acids (SCFAs) such as acetate, propionate, and butyric acid, and gas [7]. Butyric acid, which represents the main energy source for colon cells, positively impacts human health. This substance can stimulate the intestine’s production of glucose and encourage the colon’s natural death of cancer cells, which helps control blood sugar levels and preserve a healthy energy balance [8]. In addition, butyrate is essential in facilitating the consumption of high oxygen amounts by epithelial cells through β -oxidation. This activity generates a state of hypoxia, which helps to preserve the oxygen intestinal balance and prevent any imbalances in the microbiota. Propionate is transported to the liver, regulating gluconeogenesis and satiety through interaction with intestinal fatty acid receptors [9]. Acetate, the most present among SCFAs, represents an essential metabolite for bacteria proliferation, spreading into peripheral tissues and participating in cholesterol metabolism and lipogenesis [10]. Foods contribute significantly to the intestinal microbiota composition, suggesting that nutrition can prevent or alleviate diseases related to an altered microbial composition. As previously mentioned, the intestinal microbiota is involved in the food substrates fermentation, promoting or inhibiting the growth of specific types of bacteria [11]. Beyond nutrition, probiotics currently represent the most effective and safe method to specifically modify the intestinal microbiota and promote the improvement of the host’s health (Figure 1).

Microbiota composition

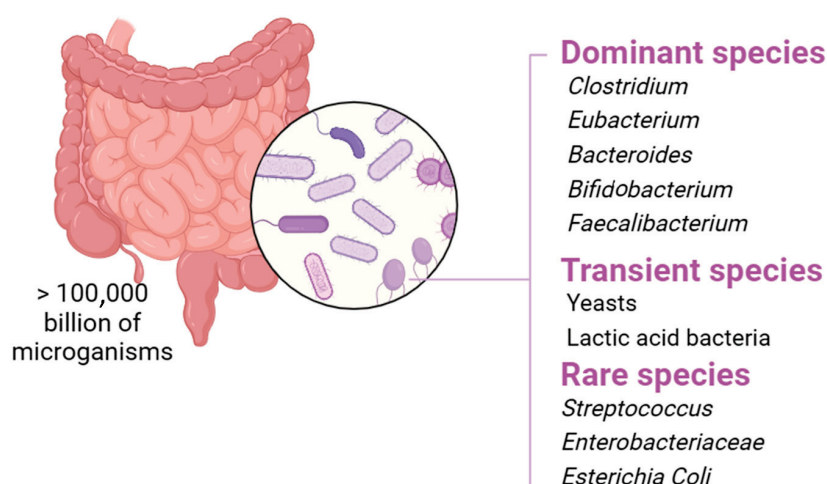


Figure 1. Different microbial species inhabit the host’s gut microbiota.

1.2. Intestinal Microbiome in Adults with *Acne Vulgaris*

In the 1930s, Stokes and Pillsbury used experimental data to establish a connection between inflammation of the skin and microbial flora. They noted that up to 40% of people with acne showed hypochlorhydria, suggesting that hydrochloric acid deficiency

could induce migration of bacteria from the colon tract to the small intestine, disturbing the balance of normal intestinal flora [12]. In recent years, it has been confirmed that hypochlorhydria represents a significant risk factor for bacterial overgrowth in the small intestine (SIBO), which can cause an increase in intestinal permeability (“leaky gut”), resulting in the development of systemic inflammation [13,14].

Bifidobacteria and *Lactobacilli*, normally present in the intestine as lactic-acid-producing bacteria, could play a beneficial role in the treatment of inflammatory skin diseases, including acne [15].

The gut microbiome plays a key role in the management of both systemic and local inflammation through interaction with the immune system. Microbial communities support the integrity of the intestinal barrier by converting complex, indigestible polysaccharides into short-chain fatty acids and vitamins, especially K and B12. The integrity of the intestinal barrier, supported by mucus, immune cells, IgA, and antimicrobial peptides (AMPs) produced by epithelial cells, is fundamental in preventing the access of intestinal bacteria to the bloodstream, thus contributing to the maintenance of skin homeostasis [1].

A study highlighted a significant decrease in the presence of *Actinobacteria*, *Bifidobacterium*, *Butyricoccus*, *Coprobacillus*, and some *Lactobacillus* species in people suffering from acne vulgaris, accompanied by a significant increase in *Proteobacteria* [16,17].

Furthermore, studies have observed that acne patients have a low presence of *Firmicutes* and an increase of *Bacteroidetes*. According to one hypothesis, the stimulation of sterol regulatory element-binding protein-1 (SREBP-1), fatty acids, and sebum triglycerides occurs following an interruption of the nutritional signal, favoring the proliferation of *Propionibacterium acnes* [17]. Several metabolic pathways are involved in the onset of acne vulgaris, such as the mTOR pathway which is activated by high blood sugar levels [18]. This event contributes to increasing insulin-like growth factor signaling, improving the cytoplasmic expression of FoxO1, ultimately leading to the development of acne [19].

1.3. Intestinal Microbiota in Adults with Metabolic Syndrome

In recent years, several studies have suggested that gut microbiota dysbiosis is closely associated with many metabolic diseases, including obesity, diabetes, and non-alcoholic fatty liver disease (NAFLD). A reduction in the abundance of *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Bacteroides* is observed, contrasted with a significant increase in the *Phylum Firmicutes* [20]. A metagenome analysis of a cohort of lean and obese Chinese adolescents showed a significant decrease in *Bacteroides thetaiotaomicron*, with a negative correlation in serum glutamate concentration [21]. Animal studies confirmed that the administration of *B. thetaiotaomicron* reduced serum concentrations in branched-chain amino acids and mitigated diet-induced weight gain and obesity in mice [22,23]. The gut microbiota can ferment indigestible carbohydrates, producing important metabolites such as SCFAs and succinate. These metabolites play a significant role in obesity and its comorbidities. SCFAs regulate energy balance and prevent obesity by suppressing appetite and increasing energy expenditure [24]. Several studies found significant differences in the composition of the gut microbiota between patients with type 1 diabetes (T1DM) and healthy people [25]. A study performed on Swedish participants highlighted a significant alteration of the intestinal microbiota in patients with type 2 diabetes (T2DM) [26], compared to healthy people. In fact, in the intestines of T2DM patients, a decrease in the amount of *Bifidobacteria* and *Akkermansia* and an increase in *Dallella* were observed [27]. Hypertension and atherosclerosis contribute to the cardiovascular risk associated with metabolic syndrome. In people with hypertension, a significant decrease in the richness, diversity, and uniformity of the intestinal microbiota is observed [28], together with a crucial increase in the *Firmicutes*/*Bacteroidetes* ratio. Other research has indicated a possible connection between intestinal microbiota (GM) and cardiovascular diseases (CVD), underlining bacterial translocation from the intestine to the heart [6,29]. The presence of live bacteria and bacterial DNA detected in atherosclerotic plaques suggests the implication of the microbiome in the development and progression of atherosclerosis. In atherosclerotic

patients, the abundance of *Enterobacteriaceae* and *Enterobacter aerogenes* was significantly higher than in control samples, with an inhibitory effect on the growth of beneficial bacteria [30]. At the same time, several studies, both on animal and human models, have highlighted that intestinal dysbiosis associated with atherosclerosis could increase intestinal permeability, thus favoring the absorption of lipopolysaccharides in circulation [31]. Pathophysiological mechanisms associated with metabolic syndrome, intestinal dysbiosis, and acne vulgaris include chronic inflammation and elevated levels of oxidative stress, which may mediate the connection between the two disorders [32] (Figure 2). These data underline a close correlation between metabolic syndrome and intestinal microbiota, suggesting that the microbiota equilibrium could represent a promising therapeutic strategy to manage this condition.

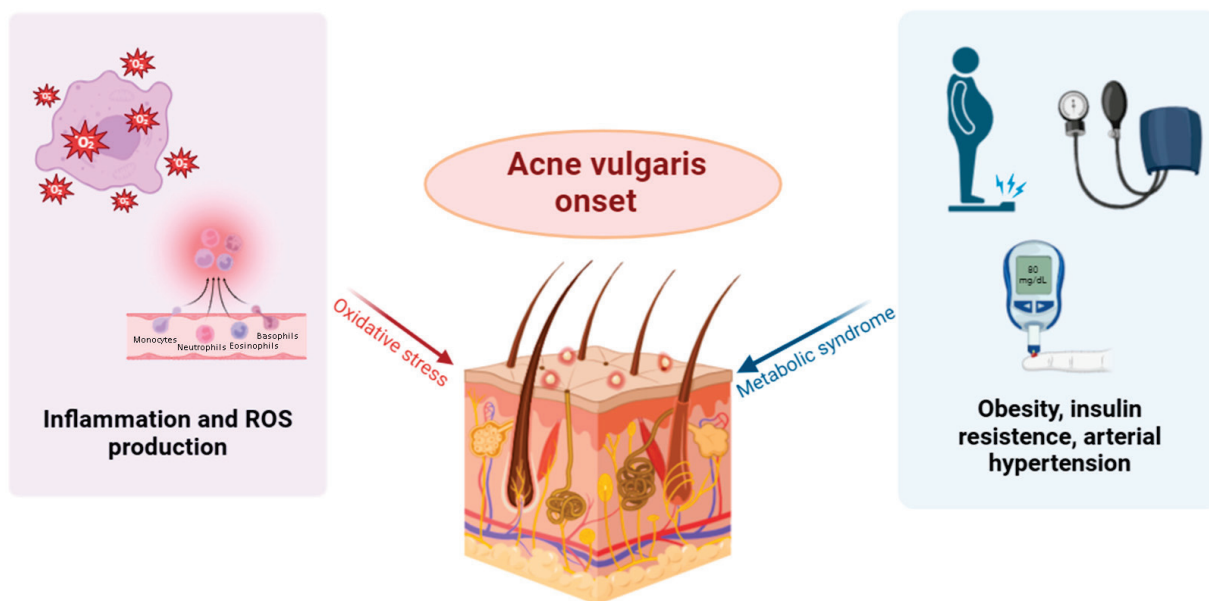


Figure 2. Oxidative stress and metabolic syndrome contribute to the development of acne vulgaris.

1.4. The Role of Polyphenols in Gut Microbiota Modulation

Polyphenols are natural phytochemical compounds present in many plants, fruits, and herbal remedies, including green tea, black tea, apples, coffee, bergamot, and grapes. To date, approximately 8000 different types of polyphenols have been identified [33]. Based on their chemical structure, polyphenols can be roughly divided into several categories, including condensed tannins, hydrolyzable tannins, phlorotannins, polyphenolic caffeoyl ester derivatives, flavones and flavonoids, and other substances. These polyphenolic compounds perform various beneficial activities, including antioxidant action [28,34], anti-inflammatory properties [35], and antibacterial activity [36]. Polyphenols show remarkable potential in improving several health conditions, including cancer, Alzheimer's disease [37], diabetes, NAFLD, insulin resistance, inflammatory bowel disease, and arthritis [38–44]. Polyphenols offer different opportunities for interaction with the intestinal microbiota, mainly due to their wide presence in foods, herbal medicines, and plants, and thus in our diet [43,45,46]. Polyphenols are characterized by an important range of effects, thanks to different molecular structures which create multiple possible interaction with the intestinal microbiota. The low bioavailability of many monomeric polyphenols, as demonstrated by Wan and colleagues in 2021, means that a significant proportion of polyphenol can reach the gastrointestinal tract without being fully absorbed. Finally, they are used directly by microorganisms [47]. The interaction between intestinal microbiota and polyphenols occurs mainly in three distinct ways:

(1) Polyphenols influence the composition of the intestinal microbiota; (2) they alter the metabolic processes within the microbial system; (3) the intestinal microbiota itself is

involved in the processing of polyphenols, influencing their structure and activity. This complex interaction plays a critical role in modulating the biological effect of polyphenols within the intestinal environment, offering greater insight into how these compounds influence host health [33].

Polyphenols are present in different sources such as *Smilax china* L. extracts, along with tea polyphenols and those of *Aronia melanocarpa* (Michx.). They have demonstrated the ability to positively influence the diversity of microbial species in the intestine, known as α -diversity. This influence has been observed in contexts such as metabolic disorders caused by a high-fat diet (HFD). Other polyphenols, including litchi pulp phenols, kaempferol, and resveratrol, have been associated with improving the α -diversity of the gut microbiota, contributing to the relief of colitis. Subsequently, polyphenols were detected as agents capable of altering the overall structure of the intestinal microbiota, evidenced by variations in β -diversity. These structural changes have been observed in individuals suffering from various pathologies, including obesity, hepatic steatosis, atherosclerosis, and colitis. Data analysis revealed a difference in the structure of the gut microbiota between sick and healthy people. Such structural changes have also been found in individuals treated with polyphenols. In conclusion, the action of polyphenols in modulating both α -diversity and β -diversity highlights their significant role in promoting intestinal health and contributing to the treatment of microbiota-related disorders [33]. A study conducted by Lu et al. in 2016 highlighted that polyphenols deriving from green tea have an important role in acne management [48]. A significant decrease in inflammatory lesions was noted on specific areas of the adults' skin undergoing treatment, thanks to the anti-inflammatory and antioxidant action of these compounds (Figure 3).

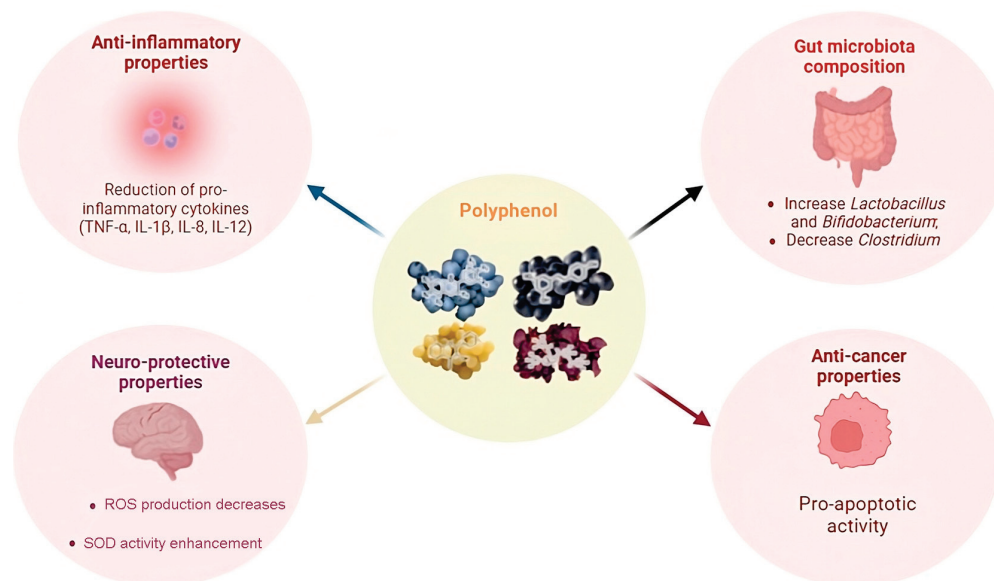


Figure 3. Properties of polyphenols.

2. Objectives

This systematic review aims to identify common biomarkers and microbiota alterations that may indicate significant links between metabolic syndrome and acne. Additionally, it explores how the prebiotic properties of polyphenols could contribute to maintaining intestinal eubiosis, providing a strong scientific rationale for their potential use in therapeutic and preventive approaches.

3. Materials and Methods

3.1. Search Strategy

The systematic review integrates data from experimental studies regarding the changes in the human intestinal microbiome in adults affected by acne and/or metabolic syndrome,

exploring the polyphenols' effect in improving these conditions. The keywords used to search for the articles were: "(acne) AND (gut microbiota)", "(polyphenols) AND (acne)", "(Lactobacillus) AND (acne) AND (Proteobacteria)", "(polyphenols) AND (microbiome) AND (metabolic syndrome)", "(phenols) AND (gut microbiome)", "(gut microbiota) AND (metabolic syndrome)".

3.2. Information Sources

The studies included in the review were retrieved from the PubMed and EMBASE databases, following the PRISMA 2020 Checklist (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement and the PICO (Patient, Intervention, Comparison, Outcome) framework [49]. All articles written in English were evaluated, and the search did not specify a specific time frame. An evaluation of references of all retrieved articles was also conducted to identify additional relevant studies not included in the aforementioned databases.

3.3. Eligibility Criteria

Studies of the following types were included in the analysis:

- studies in women and men with acne;
- studies in women and men with metabolic syndrome or diseases associated with this condition;
- studies reporting oral polyphenol treatments.

Studies were excluded from the analysis for the following reasons:

- use of topical treatments.
- studies conducted on animals;
- studies conducted in vitro.

3.4. Study Results

Two separate qualitative analyses were conducted to account for the heterogeneity of the results. In the first systematic analysis, the aim was to identify the composition variations of the gut microbiota in adults with acne, comparing different clinical studies conducted on men and women. The second systematic analysis compared different human experimental models to better understand variations in gut microbiota composition in people with metabolic syndrome or related conditions. This second analysis examined the effect of oral polyphenol-based treatment on the gut microbiota and showed a qualitative improvement.

3.5. Statistical Analysis

Due to the bacteria and data heterogeneity and the limited number of specific studies on the chosen topic, it was necessary to analyze the data at a qualitative level.

4. Results

4.1. Study Selection

The systematic review identified 4142 documents from the literature search. Of these, 212 articles were excluded as duplicates, and 3565 were excluded as their content did not match the review's objectives. After screening the abstract and full text, 365 articles were assessed for their eligibility, and 88 were excluded because they were reviews or animal studies, involved topical administrations, or had incomplete data. Finally, nine articles describing studies on humans were included in the qualitative analysis (systematic review) and divided into two analyses, one relating to in vivo studies on patients with acne and one relating to in vivo studies on patients with metabolic syndrome. The literature search process and the selection of articles are illustrated in the following Figure (PRISMA flowchart Figure 4).

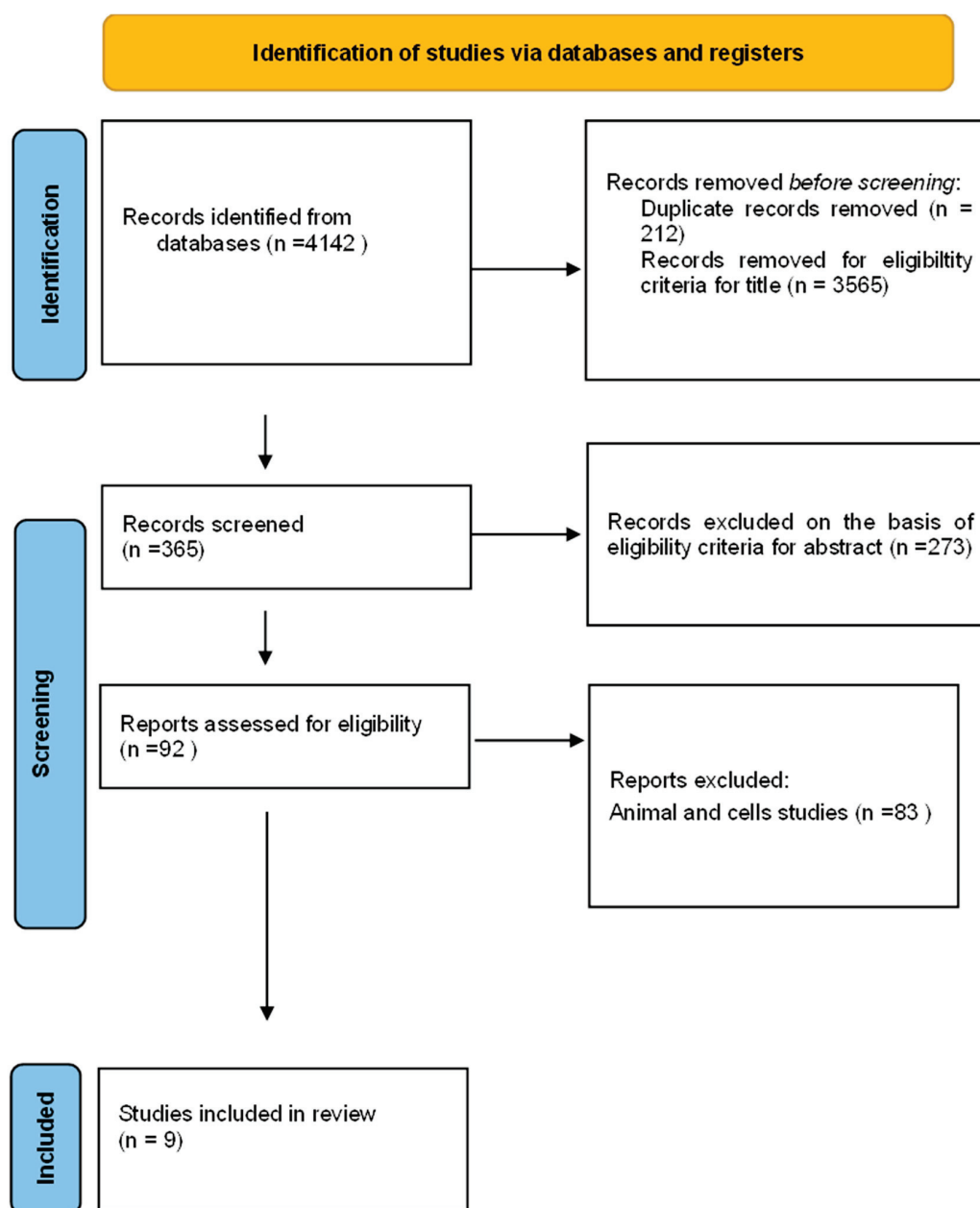


Figure 4. PRISMA flowchart.

4.2. Study Characteristics

4.2.1. Systematic Review on Gut Microbiome Changes in Acne Patients

Table 1 summarizes the main features of the four articles describing the differences in gut microbiome composition between healthy and acne patients. Formerly, it has been observed that gut microbiota plays a crucial role in the pathogenesis of acne. Hui-Min Yan et al. (2018) conducted a study to identify the differences in gut microbiota between acne patients and healthy individuals [28]. The study enrolled 31 healthy individuals (free from any skin disease) and 31 individuals with moderate to severe acne vulgaris. The distribution of the phyla *Proteobacteria* and *Actinobacteria*, the two major populations in the human gut, shows a significant disparity between acne patients and controls [28]. The phylum *Proteobacteria* is more abundant in acne patients, including many pathogenic bacteria such as *E. coli*, *Salmonella*, and *Vibrio cholerae* [28]. In addition, a decreased level of the genera *Bifidobacterium*, *Butyricicoccus*, *Coprobacillus*, *Lactobacillus*, and *Allobaculum* is observed in acne patients [28]. *Bifidobacterium* are Gram-positive bacilli belonging to the

phylum *Actinobacteria*, while *Lactobacillus* are Gram-positive bacilli belonging to the phylum *Firmicutes*. A subsequent analysis conducted by Katherine G. Thompson et al. (2020) was published based on a case-control study investigating the skin and gut microbiota in eight acne patients before and after oral minocycline administration, comparing them with eight controls [50]. Oral antibiotics are the “gold standard” for the treatment of moderate to severe acne. So far, it is not known whether the efficacy of oral antibiotics in acne treatment is due to systemic absorption and, therefore, to the direct action of the antibiotic on the acne lesion, or an indirect action, due to the action of the antibiotic on the gut microbiome. Compared to controls, acne patients showed a reduced gut ratio of *Firmicutes* to *Bacteroidetes*. Following oral antibiotic treatment, acne patients’ microbiome underwent a statistically significant increase in *Bacteroidetes* levels. Furthermore, the gut microbiota in acne patients at baseline, compared to controls, was observed to be poor in Gram-positive probiotic bacilli such as *Lactobacillus iners*, *Lactobacillus zeae*, and *Bifidobacterium animalis* [50]. It has also been observed that increased intestinal permeability may allow the passage of lipopolysaccharides (LPS) by Gram-negative gut bacteria into systemic circulation, promoting inflammation and the development of acne lesions through their action on toll-like receptors TLR2 and TLR4, which are overexpressed in inflammatory acne [50]. Deng and colleagues studied whether the gut microbiota is altered in individuals with acne. It was shown that differences in microbial diversity were found between patients with acne and controls. At the phylum level, the *Firmicutes* abundance was lower in the patient group compared to healthy controls, while that of *Bacteroidetes* was higher [51]. The study conducted by Manzhali et al. examined the effect of *Escherichia coli* Nissle on intestinal inflammation and the intestinal microbiota in patients with intestinal dermatoses. After treatment with *E. coli* Nissle, a significant reduction in pathogenic flora and an increase in *Bifidobacteria* and *Lactobacilli* were observed in the feces of treated patients. This change in the microbiota was associated with a clinical improvement, with normalization of stool consistency, color, and odor. Additionally, an increase in serum IgA levels and suppression of proinflammatory cytokines were found, indicating a beneficial effect of probiotic therapy with *E. coli* Nissle in intestinal dermatoses [52].

Table 1. Studies highlighting changes in the gut microbiota in adults affected by acne.

Study/Year	Population	Age	Sex	Bacteria Phylum	Results
Hui-Min Yan et al., 2019 [28]	31 healthy adults; 31 adults with acne	Healthy adults: 22.87 ± 3.65 Acne: 22.16 ± 4.24	Male 8 Female 26	<i>Actinobacteria</i> <i>Proteobacteria</i>	It has been demonstrated that the phylum <i>Actinobacteria</i> is lower in patients with acne compared to the control group, while <i>Proteobacteria</i> is higher compared to the control group.
Yongqiong Deng et al., 2018 [51]	43 healthy adults; 43 adults with acne	Healthy adults: 20.24 ± 3.06 Acne: 20.19 ± 2.59	Male 17 Female 26	<i>Bacteroidetes</i> <i>Firmicutes</i>	In the study, differences in microbial diversity were found for patients with acne and controls. At the phylum level, the <i>Firmicutes</i> abundance was lower in the patient group compared to healthy controls, but that of <i>Bacteroidetes</i> was higher.
Katherine G. Thompson et al., 2020 [50]	8 healthy adults; 8 adults with acne	Healthy adults: 23~34. Acne: 20~32	NA	<i>Firmicutes</i> <i>Bacteroidetes</i>	It has been observed that at the phylum level, the abundance of <i>Firmicutes</i> was lower in the patient group compared to healthy controls, but that of <i>Bacteroidetes</i> was higher.
Elina Manzhali et al., 2016 [52]	20 healthy adults; 37 adults with acne	Healthy adults: 28 ± 2.5 Acne: 29 ± 3.1		<i>Actinobacteri</i> (Genus <i>Bifidobacteria</i>) <i>Firmicutes</i> (Genus <i>Lactobacteria</i>)	In treatment with <i>E. coli</i> Nissle, a significant reduction in pathogenic flora and an increase in <i>Bifidobacteria</i> and <i>Lactobacilli</i> were observed in the feces of treated patients.

4.2.2. Systematic Review on Changes in the Gut Microbiome in Adults with Metabolic Syndrome or Associated Pathological Conditions

Table 2 presents an overview of the articles considered in the systematic review, which aim to describe the changes in the gut microbiome in adults with metabolic syndrome or pathological conditions closely related to it. In addition, the improvements in the microbiome following the administration of substances/supplements/diets/drinks with a high polyphenolic content are described. In the study carried out by Moreno-Indias et al. in 2016, the possible prebiotic effect deriving from moderate consumption of red wine polyphenols on the modulation of the composition of the gut microbiota and the improvement of risk factors associated with metabolic syndrome in obese patients was evaluated [53]. In the study, 20 patients were involved, 10 of whom were healthy adults. An increased level of *Proteobacteria* and *Firmicutes* has been demonstrated in patients with metabolic syndrome compared to healthy adults. However, after the red wine and de-alcoholized red wine intake, no significant differences at the phylum level emerged between the two study groups [53]. Adults with metabolic syndrome, after the periods of treatment based on red wine polyphenols, presented a higher concentration of *Fusobacteria* and *Bacteroidetes* and a significant decrease in *Firmicutes*, compared to baseline [54]. Within *Firmicutes*, in patients with metabolic syndrome, a lower number of bacteria from the *Clostridium* group and the *Clostridium histolyticum* group was found, accompanied by a significant increase in bacteria belonging to the *Blautia coccoides*–*Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Roseburia*, and *Lactobacillus* groups, after periods of red wine and de-alcoholized red wine intake compared to baseline. Finally, it was observed that the increase in *Actinobacteria* and *Lactobacillus*, together with the decrease in *Clostridium histolyticum* and *E. coli*, predicted the triglycerides' reduction. Instead, the decrease in plasma LPS levels was associated with the *Bifidobacterium* growth and the reduction in *Enterobacter cloacae* number [53]. The study conducted by Vaiserman et al. in 2017 on the Ukrainian population demonstrates the changes in the composition of the gut microbiota between a group of “normal weight” individuals and a group of patients classified as “obese” based on body mass index (BMI). The study found a significant increase in *Firmicutes* and a higher *Firmicutes*/*Bacteroidetes* ratio in overweight and obese people compared to normal-weight adults in the Ukrainian population. The results obtained in the Ukrainian population are consistent with those obtained in other populations [54].

The traditional Chinese herbal formula Yangyin Tiluo Decoction (YTD), rich in polyphenols and used in the study by Ni et al. in 2017, mitigates metabolic syndrome and cardiovascular diseases [55]. In this study, the gut microbiota alterations in elderly people with metabolic syndrome, following natural treatment based on polyphenols, were investigated. In these patients, YTD can improve intestinal microbial composition and reduce the metabolic markers of cerebrovascular disease [55]. Older adults with metabolic syndrome showed an increase in *Lactobacillus* and *Bifidobacterium* species, accompanied by a reduction in butyrate-producing bacteria compared to healthy controls. Although *Lactobacillus* and *Bifidobacterium* are generally considered beneficial bacteria and often used as probiotics, studies have shown an increase in interleukin 1 β (IL-1 β) concentrations and a potential increase in the inflammatory response following their presence. YTD can reduce the lipoprotein A concentration, a known risk factor for cardiovascular disease and atherosclerosis. The decrease in lipoprotein A reflects the clinical efficacy of YTD in mitigating metabolic syndrome in older individuals [55].

A correct diet is essential to maintain the balance of the intestinal bacterial flora. According to Meir et al. (2021), a “green” diet rich in vegetable-derived polyphenols brings benefits to individuals affected by hepatic steatosis, abdominal obesity, and dyslipidemia [56]. The hypothesis has been advanced that the intestinal microbiota plays a crucial role in the pathogenesis of NAFLD, as dysbiosis appears to be linked to the modulation of fat and lipid hepatic metabolism. In this study, nine bacteria at the genus level were initially identified as significantly related to IHF (intrahepatic fat); these represented 5% of bacteria at the genus level, including *Fournierella*, *Anaerospobacter*, *Lachnospiraceae_UCG-003*, and

several genera of the *Ruminococcaceae* family [56]. Out of the nine bacteria identified, eight of them showed an association with the variation in IHF 18 months from the beginning of the study, suggesting that the presence of these bacteria is linked to changes in intrahepatic fat over time. Therefore, adopting a “green” diet enriched in polyphenols combined with physical exercise has been shown to significantly reduce the prevalence of NAFLD and improve intestinal bacterial flora [56]. Finally, in a randomized crossover clinical trial performed by Zhang et al., 2022, it is evaluated whether supplementation with polyphenol-rich red fruits (RRB), alone or in association with a prebiotic (FOS), improves cardiovascular risk biomarkers in adults with prediabetes and insulin resistance, and whether these effects are related to the modulation of the intestinal microbiota [57]. Supplementation with RRB has been shown to reduce hepatic insulin resistance, total cholesterol (TC), and LDL cholesterol (LDL-C) levels in the group of individuals with prediabetes and insulin resistance [57]. Supplementation with RRB increased the presence of *E. eligens* and reduced that of *R. gnavus*, while supplementation with RRB + FOS increased the *Bifidobacterium* spp. presence and decreased that of *B. wexlerae*. Variations in the abundances of *R. gnavus*, *E. eligens*, and *B. catenulatum* were significantly correlated with a reduction in hepatic insulin resistance and a decrease in TC and LDL-C [57]. *R. gnavus* is a fundamental component of the gut microbiota, characterized by the ability to degrade mucus [58]. Evidence suggests an association of *R. gnavus* with several pathologies, including diverticular disease, respiratory allergies in children, and septic arthritis. Furthermore, it is significantly more present in patients with Crohn’s disease, indicating inflammatory dysbiosis of the intestine [59]. The presence of 300% of *R. gnavus* was observed in the PreDM-IR group compared to the control group, which was decreased following supplementation with RRB, a result like that observed in the previously cited study on the Mediterranean “green” diet [60]. Overall, RRB supplementation improved hepatic insulin resistance, total cholesterol, and LDL cholesterol levels, with these effects being correlated with decreased *R. gnavus* and increased *E. eligens* [57]. The addition of FOS to the RRB regimen further enhanced β -cell function and increased the presence of *Bifidobacterium* spp., a gut bacterium known for its beneficial effect [57].

Table 2. Studies highlighting changes in the gut microbiome in adults with metabolic syndrome or related conditions.

Study/Year	Population	Age	Sex	Bacteria Phylum	Polyphenols	Results
Moreno-Indias et al., 2016 [53]	10 healthy adults; 10 adults with metabolic syndrome	48 ± 2 years (range 45–50 years)	NA	<i>Fusobacteriota</i> <i>Bacteroidetes</i> <i>Firmicutes</i>	Red wine polyphenols	Significant increase in <i>Fusobacteria</i> and <i>Bacteroidetes</i> , and significant decrease in the <i>Clostridium</i> and <i>Firmicutes</i> group, in patients with metabolic syndrome, compared to baseline.
Vaiserman et al., 2017 [54]	34 adults with BMI ≤18.5–24; 11 adults with BMI >30	Mean age 44.2 years	Adults with BMI ≤18.5–24: 9 male and 25 female; adults with BMI >30: 4 male and 7 female	<i>Actinobacteria</i> <i>Firmicutes</i> <i>Bacteroidetes</i>	NA	Adults with a high BMI have a higher level of <i>Firmicutes</i> and a lower level of <i>Bacteroidetes</i> than adults with normal weight.
Ni et al., 2018 [55]	11 healthy adults; 12 adults with metabolic syndrome	60–89 years	Healthy people: 6 male and 5 female; metabolic syndrome: 8 male and 4 female	<i>Firmicutes</i> <i>Bacteroidetes</i> <i>Actinobacteria</i> <i>Proteobacteria</i>	Yangyin Tiluo Decoction (YTD)	Adults treated with the Chinese herbal formula (Yangyin Tiluo Decoction) were able to reduce the abundance of potentially pathogenic bacteria and lipoprotein A.

Table 2. Cont.

Study/Year	Population	Age	Sex	Bacteria Phylum	Polyphenols	Results
Meir et al., 2021 [56]	294 adults with NAFLD adulated to a different diet: • 98 MMED • 98HFG • 98 green MED	51 years	260 male and 34 female	<i>Firmicutes</i>	A polyphenol-rich diet (440–800 mg/day)	Adults treated with a diet rich in polyphenols (green MED) demonstrated a reduction in non-alcoholic hepatic steatosis, beneficial changes in cardiometabolic and inflammatory parameters, and improvements in intestinal bacterial flora.
Zhang et al., 2022 [57]	10 healthy adults; 26 with pre-diabetes	35 ± 2 years	Heathy adults: 3 male and 7 female; pre-diabetes adults: 14 male and 12 female	<i>Actinobacteria</i> <i>Firmicutes</i>	Red berry polyphenols	Supplementation with red fruit polyphenols (RRB) reduced total cholesterol and LDL levels in adults with pre-diabetes; RRB increased <i>Eubacterium eligens</i> and reduced <i>Ruminococcus gnavus</i> , while RRB + FOS increased <i>Bifidobacterium</i> spp. and reduced <i>Blautia weylerae</i> compared to baseline.

5. Discussion

Acne, in addition to being a common skin condition, plays an indicative role in detecting alterations in the gut microbiome, similar to what occurs in metabolic syndrome.

The intestinal microbiome plays a fundamental role in maintaining the host's health, exerting a significant impact on several physiological processes, including digestion, nutrient absorption, maturation of the immune system, and defense against harmful pathogens.

An optimal balance of the microbiota is closely related to an optimal state of health, while an imbalance, known as “dysbiosis”, can be associated with various pathological conditions and diseases; systemic and/or local inflammation is a consequence of SIBO which leads to an increase in intestinal permeability and the passage of bacteria into the systemic circulation [14].

Bacterial dysbiosis in adults with acne mainly concerns an increased ratio of *Firmicutes/Bacteroidetes* and *Proteobacteria*; one notable study reported the presence of *Actinobacteria*, *Bifidobacterium*, *Butyricicoccus*, *Coprobacillus*, and some species of *Lactobacillus*, positive bacteria for the balance of the intestinal bacterial flora [16].

Literature studies show a specific imbalance in gut flora composition in people with metabolic syndrome. This alteration is characterized by an increase in the *Firmicutes/Bacteroidetes* ratio and in *Bifidobacterium* reduction; similar conditions can be found in individuals suffering from acne [61,62].

In this systematic review, although it was not possible to obtain a quantitative relevance of intestinal bacteria in adults with acne due to the limited amount of data available, an appreciable qualitative difference emerged. In patients suffering from acne compared to the control group, a significant increase in the presence of bacteria belonging to the *Phylum Proteobacteria* including pathogenic bacteria such as *E. coli*, *Salmonella*, and *Vibrio cholera* and an increased *Firmicutes/Bacteroidetes* ratio have been observed. On the other hand, lower levels of the genera *Bifidobacterium* (*Phylum Actinobacteria*), *Butyricicoccus*, *Coprobacillus*, *Lactobacillus* (*Phylum Firmicutes*), and *Allobaculum* are found in acne patients. It has been highlighted that, at the basal level, the microbiota of acne patients, when compared to controls, showed a significant deficiency of Gram-positive probiotic bacilli such as *Lactobacillus iners*, *Lactobacillus zeae*, and *Bifidobacterium animalis* [63].

Systematic analysis of the bacteria in the gut flora of people with metabolic syndrome shows similarities to changes in the microbiome of people with acne. The microbiomes of adults with metabolic syndrome or closely related pathologies have higher levels of

Proteobacteria and *Firmicutes* than healthy controls. The *Firmicutes/Bacteroides* ratio is also found to be increased in people with obesity.

Treatment with polyphenols leads to an improvement in the balance of the intestinal bacterial flora, which correlates with a significant improvement in several cardiometabolic parameters [56]. These improvements include reductions in total cholesterol, triglycerides, and LDL, as well as a significant incidence of hepatic steatosis.

The bacterial flora restoration induced by polyphenols is characterized by a greater concentration of *Fusobacteria* and *Bacteroidetes*, accompanied by a notable reduction of the *Firmicutes* compared to the controls. Within the *Firmicutes* phylum, a reduced number of bacteria belonging to the *Clostridium* and *Clostridium histolyticum* groups was found, while a significant increase in bacteria was observed in the *Blautia coccoides–Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Roseburia*, and *Lactobacillus* groups. In addition, the polyphenol treatment stimulated the increase of *Bifidobacterium* spp., an intestinal bacterium known for its beneficial effects. This review suggests that acne is caused by some of the same microorganisms that alter in metabolic syndrome. The literature only demonstrates the effectiveness of polyphenols in metabolic syndrome, with no study reporting this effect in acne. This research suggests that the potential benefits of polyphenols at the microbiota level may be relevant for patients suffering from metabolic syndrome. However, due to a lack of data, a direct correlation between metabolic syndrome and acne cannot be established.

Limitations of the Study

Some data observed could influence the results regarding the efficacy of polyphenols in metabolic syndrome or acne. Natural compounds come from different families, increasing heterogeneity. A further critical issue is represented by the bacteria heterogeneity, which prevented conducting the meta-analysis. The limited quantity of items represents a further difficulty. Current information on the potential positive effect of polyphenols on acne is limited; however, it is known that they exert a beneficial effect on the intestinal microbiota. The positive role of polyphenols in reducing cardiovascular risk also emerges from the qualitative analysis carried out on metabolic syndrome. Another important limitation is that anthropometric parameters, such as age, medication use, and the consumption of antibiotics, probiotics, and prebiotics, which can significantly influence the composition of the gut microbiota, were not taken into account.

6. Conclusions

The role of the intestinal microbiome in the development of diseases is unequivocal, even though the results currently available are limited. The gut microbiota acts decisively in the pathological processes that lead to various chronic conditions. In this context, polyphenols derived from plant species seems to hold great potential in the management and prevention of disease. The positive interaction between polyphenols and microbiota opens the way to new strategies for the therapeutic management of chronic diseases and the development of personalized therapies aimed at improving patient recovery during rehabilitation. It is therefore necessary to highlight how implementing a balanced diet, with a high content of polyphenols, can bring important enhancement to the microbiota. Furthermore, considering the beneficial effects of polyphenols on the bacteria involved in metabolic syndrome, this approach could provide new foundations for their introduction in patients with acne, as the bacteria involved are the same. Therefore, a new holistic approach should include both the use of polyphenols and a balanced diet for improved health.

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