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ORIGINAL PAPER

Chia Sprouts Elicitation with Salicylic Acid and Hydrogen Peroxide to Improve their Phenolic Content, Antioxidant Capacities *In Vitro* and the Antioxidant Status in Obese Rats

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Abstract

Elicitation is a biotechnological approach to improve phenolic compounds content and antioxidant properties of ready-to-eat functional foods. This study aimed to evaluate the chemical elicitation effects using salicylic acid (SA) and hydrogen peroxide (H_2O_2) in optimized-germination conditions on seedling vigor, phenolic content, and their antioxidant capacities *in vitro* and serum and urine of Wistar obese rats. Optimized-germination conditions of 26.5 °C and 178 h produced a 64% of germination and a sprout length of 56 mm. Only, the elicitation with H_2O_2 (20 mM) enhanced the germination (75%) and H_2O_2 (10 and 20 mM) the sprout length (69 and 59 mm, respectively). In contrast, both elicitors enhanced phenolic contents, being more significant total phenolic compounds content for SA (1 and 2 mM), up to 65.5–73.5%. SA and H_2O_2 improved total flavonoids content (36.5–64.1%), ABTS (19.3–61.1%), and DPPH capacities (51–86%), depending on SA and H_2O_2 concentration, compared with non-elicited chia sprouts. The QUENCHER antioxidant capacities of elicited chia sprouts increased up to three times more than extracts capacities, principally Q-ABTS, which could be attributed to phenolic bounds to dietary fiber. Rats fed with a high-fat and fructose diet (HFFD) and supplemented with chia sprouts, especially 1-mM SA, improve the obesity-related oxidative stress through an increase of antioxidant capacities, using DPPH and ABTS test, on serum (70–118%) and urine samples (80–116%). These results suggest that chia sprouts elicited with 1-mM SA are a source of antioxidant compounds that can be used to decrease obesity related oxidative stress.

Keywords Chia sprouts · Elicitation · Phenolic compounds · Antioxidant capacities · QUENCHER approach · Serum antioxidant capacities

Abbreviations

ABTS	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
DPPH	2,2-Diphenyl-1-picrylhydrazyl
HFFD	High fat and fructose diet
QUENCHER	(Quick, easy, new, cheap, and reproducible) procedure
RSM	Response surface methodology
SA	Salicylic acid
TFC	Total flavonoids content
TPC	Total phenolics compounds

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Introduction

Chia (*Salvia hispanica* L.) seeds induce health benefits such as reduced body weight, hypertriglyceridemia, and hyperglycemia [1]. These diseases could be improved with phenolic compounds and some studies have shown that germination increases the total phenolic compounds in chia seed [2], the total flavonoid content, and specifically some phenolic acids as gallic and protocatechuic [3].

Likewise, biotechnological tools such as elicitation techniques are used as a strategy to enhance levels and change the profile of different bioactive compounds from sprouts at low-cost [4]. Previous studies have demonstrated that

chemical elicitation with salicylic acid (SA) and hydrogen peroxide (H_2O_2) increases seedling growth and phenolic compounds of common bean sprouts [5], lentil sprouts [6], and buckwheat sprouts [7]. Therefore, the elicitation used during germination could improve the beneficial properties of the seeds. On the other hand, antioxidant capacities are one of the main characteristics of phenolic compounds, and these are commonly determined in extractable compounds from food matrices. However, non-extractable bioactive compounds associated with dietary fiber in the whole sprouts also present these properties. In QUENCHER (**qu**ick, **e**asy, **n**ew, **che**ap, and **r**eproducible) procedure [8], these capacities are quantified in whole food without any solvent extraction, determining the soluble and insoluble antioxidant compounds [9, 10]. Both compounds are important in obesity and its complications, diseases characterized by high oxidative stress [11]. In these alterations, phenolic compounds intake produces a healthy antioxidant status, which has been determined by quantifying these compounds and their antioxidant capacities in serum and urine [12, 13].

The antioxidant capacities of serum and urine are related to the sum of endogenous antioxidants (albumin, creatinine, and uric acid) in synergy to exogenous antioxidants as dietary phenolic compounds, carotenoids, vitamins C and E, among others. Therefore, antioxidant capacities evaluated in body fluids, such as serum and urine, provide more accurate information on the ability of phenolic compounds and other antioxidants considering their bioavailability and metabolism; thus, integrating information of the real effects to decrease oxidative stress produced in the organism by components in the diet [12–14]. The changes in antioxidant capacities of biological fluids have been successfully demonstrated after acute and long-term polyphenols/antioxidants compounds intakes from grape/pomegranate pomace [12], Mediterranean diet [13], and functional pasta enriched with lipophilic or hydrophilic bran extracts [14], increasing serum/urine polyphenols compounds content and their antioxidant capacities, resulting in an improvement of oxidative stress.

To our knowledge, there are no studies about the consumption of rich-polyphenols sprouts on serum and urine antioxidant capacities, since most studies quantify antioxidant capacities *in vitro* from food matrices and recommend consuming these for such benefits. Although these *in vitro* analyses are useful to characterize the antioxidant capacities of food, antioxidant compounds need to be absorbed in order to induce relevant antioxidant biological effects inside the body [14]. Therefore, the present study complemented the *in vitro* assessments with serum and urine antioxidant status to confirm the *in vivo* effect of rich-polyphenols sprouts consumption.

The work aimed to evaluate the effects of chemical elicitation, using salicylic acid and hydrogen peroxide, in chia seeds on phenolic compounds content and their antioxidant capacities *in vitro*, and to assess their antioxidant capacities in serum and urine on induced-obese rats.

Materials and Methods

The materials and methods section is provided as supplementary material (SM1).

Results and Discussion

Predictive Models and Optimization by RSM for Germination

Experimental values of germination percentage and sprout length oscillated from 45–70% and 26–57 mm, respectively. The regression models for germination percentage and sprout length adjusted significantly with p-values = 0.038 and p-values = 0.008, and the R^2 values showed that the model explains 76.2 and 71.4% of the variance, respectively (Table 1). The contours plot and response surface plots show that, for sprout length, higher values are presented at temperatures higher than 22.5 °C and times over 168 h (Fig. 1a-b). The contours plot (Fig. 1c) shows that the germination percentage tends to be higher at 25 °C in the interval of 120–168 h; it also has a paraboloid with a maximum stationary point (Fig. 1d), which can be an optimal point.

Table 1 Central composite rotatable experiment design with two factors and five levels for producing chia sprouts at different combinations of time and temperature

Assays		Germination factors		Response variables *	
Randomized	Order	Time (h)	Temperature (°C)	Germination (%)	Sprout length (mm)
8	1	144	32.0	46 ± 2	26 ± 1
4	2	168	30.0	69 ± 0	56 ± 5
6	3	178	25.0	63 ± 7	57 ± 3
9	4	144	25.0	65 ± 3	41 ± 3
7	5	144	17.9	52 ± 0	26 ± 2
2	6	168	20.0	55 ± 3	26 ± 1
10	7	144	25.0	70 ± 0	36 ± 3
12	8	144	25.0	70 ± 4	39 ± 2
1	9	120	20.0	53 ± 3	17 ± 1
13	10	144	25.0	69 ± 1	42 ± 2
3	11	120	30.0	63 ± 0	42 ± 2
11	12	144	25.0	69 ± 0	35 ± 2
5	13	110	25.0	65 ± 1	25 ± 1

*The results are expressed as mean values ± SEM of three independent replicates

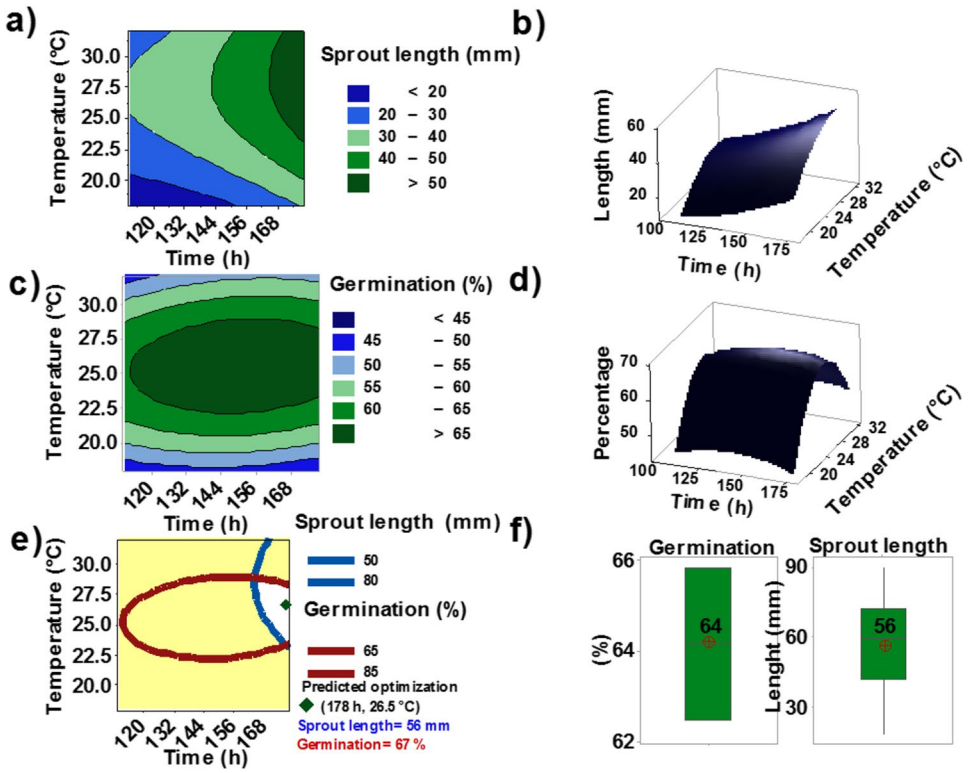

Fig. 1 Contours and response surface plots of sprout length (**a, b**), germination percentage (**c**, **d**) and overlay plot with predicted values on optimal conditions of germination temperature and time (**e**), as well as experimental validation of response variables optimized of chia sprouts (**f**)

The best combinations of response variables were obtained superimposing the contours plots to produce an overlay plot (Fig. 1e), which allowed predicting the conditions to produce optimized chia sprouts. The diamond point represents the region of the optimized response conditions: 26.5 °C and 178 h, for obtaining predicted response solutions; 67% and 56 mm for germination percentage and sprout length, respectively (Fig. 1e). The experimental validation was performed with the optimal conditions for the germination process (Fig. 1f), obtaining a germination percentage of 64 ± 2.3 and chia sprouts of 56 ± 20 mm; indicating that the RSM analysis was appropriated; germination was reproducible yielding experimental values similar to predicted. Our germination percentages results (45–70% without elicitation procedure) are in the range reported by Paiva et al. [15] for chia seeds germination at constant darkness conditions (55–80%).

Chia seed germination showed sensitivity at high temperatures, at which the metabolic rate could be reduced, and the imbibition process of the seed decreased. Temperatures, above 30 °C, modify some seed structures that affect the semi-permeability of the seed membrane, which makes the imbibition process difficult, causing thermal stress that likely affects enzymatic activity and results in germination reduction [15, 16]. Sprout length results are similar to reported by Paiva et al. [15], who demonstrated that the chia sprout growth depends on temperature; high temperatures reduced the length of chia sprouts due to the thermal stress, which affects the proteins synthesis and decreases the supply of free amino acids.

Effects of Chemical Elicitation on Germination Percentage and Sprout Length

Elicitation in seed germination might cause a reduction in germination percentage and sprout length [15]. This effect is well-documented when high concentrations of chemical elicitors are used, or with application of overloads; however, it could depend on plant species (genetics), and on the elicitor´s nature [4]. For this reason, the effects of chemical elicitation on sprout length and germination percentage of chia at optimized temperature (26.5 °C) and different times (72–178 h) were evaluated.

The germination percentages were from 55 to 74.5%, showing the highest values for 178 h with 20 mM H_2O_2 compared with non-elicited chia sprouts ($p < 0.05$) (Fig. 2a). At the same time, SA elicitation had no statistical differences compared with non-elicited chia sprouts. In general, seedling growth tends to increase through germination time (Fig. 2b); in almost all cases, the significant ($p < 0.05$, capital letters) highest values occurred at 178 h; specifically, elicitation with H_2O_2 produced sprouts statistically equal or longer than non-elicited and SA elicited sprouts, throughout time. Thus, at 178 h, the sprout length oscillated from 39 to 69 mm, and the elicitation with H_2O_2 was more effective, showing the significantly highest values ($p < 0.05$, lowercase

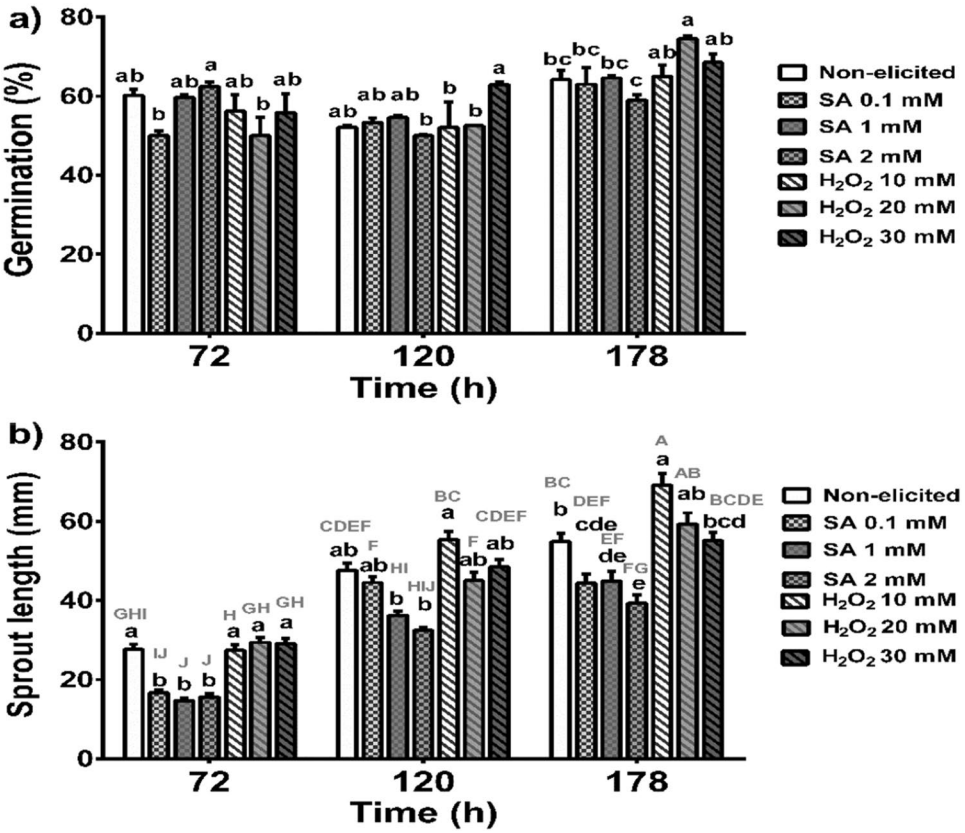

Fig. 2 Effects of chemical stress on germination percentage (**a**) and sprout length (**b**) at optimal germination temperature (26.5 °C) and different times. Mean values ± SEM. Different letters indicate significant difference ($p < 0.05$) by Tukey test (capital letters throughout time, lower cases within the same time). SA, salicylic acid

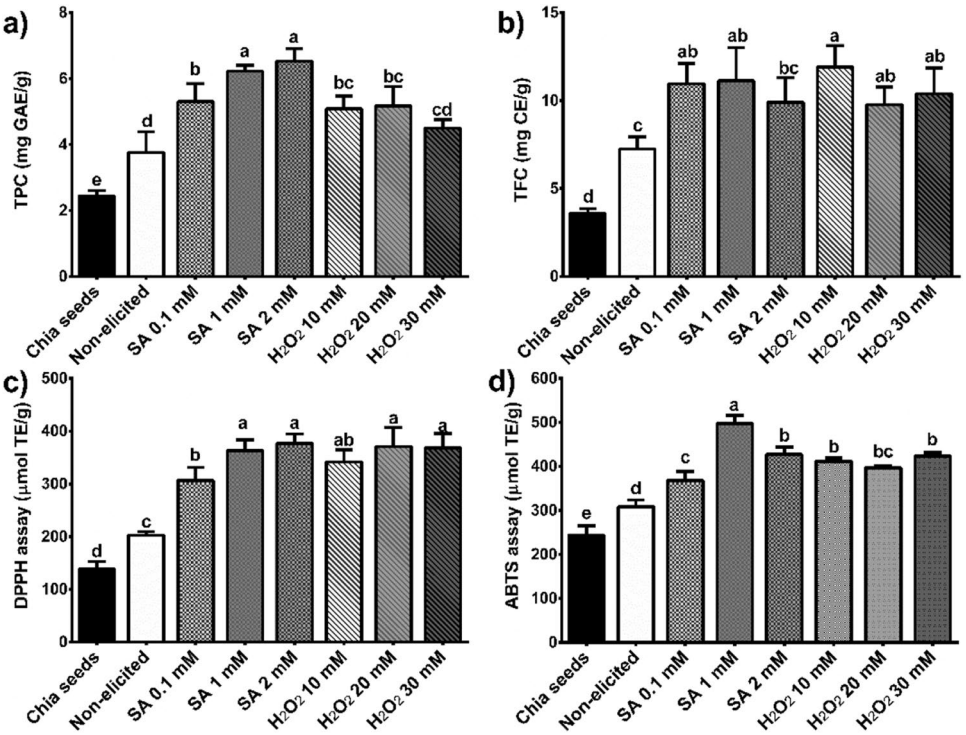

Fig. 3 Effects of induced chemical stress in chia sprouts on the content of **a**) total phenolic compounds, **b**) total flavonoids content; and the antioxidant capacities by **c**) DPPH and **d**) ABTS radical scavenging at the optimal germination temperature (26.5 °C) and time (178 h). Results are expressed on dry basis as mean value ± SD. Different letters indicate significant difference ($p < 0.05$) by Tukey test. SA, salicylic acid

letters) at the lower concentration (10 mM), which decreased when concentration increased. On the other hand, elicitation with SA decreased the sprout length with respect to non-elicited sprouts (Fig. 2b). The observed decrease in sprout length could be related to high oxidative stress induced by both elicitors used at high concentrations. This effect has been reported for H_2O_2, which was applied to lentil sprouts resulting in smaller sprout length [6].

Effects of Elicitation on Phenolic Compounds and Antioxidant Capacities of Chia Sprouts

The best agronomic characteristics were shown at 178 h, and this condition was selected for the following determinations. Non-elicited chia sprouts presented an increase of TPC (1.5-fold), TFC (2-fold), DPPH inhibition (1.5-fold), and ABTS inhibition (1.2-fold) compared with ungerminated chia seed (Fig. 3), analyzed in extracts. The highest concentrations of SA (1 and 2 mM) presented the highest TPC values increasing significantly until 73.5%. While SA 0.1 and 1 mM and H_2O_2 treatments (10 and 20 mM) only increased 37–40%, compared with non-elicited chia sprouts (Fig. 3a). For TFC, the highest content was shown for 10 mM H_2O_2, followed by 0.1 and 1 mM SA, as well as 20 and 30 mM H_2O_2; showing a significant increase, up to 65%, compared to non-elicited chia sprouts ($p < 0.05$) (Fig. 3b). Elicitation increased the antioxidant capacities of all chia sprouts. The treatment with high concentration of SA (1 and 2 mM) and with all concentrations of H_2O_2, produced the chia sprouts with the highest values for DPPH assay, which significantly (82–86%) increased compared to non-elicited chia sprouts (Fig. 3c). For ABTS assay, 1 mM SA showed the highest ($p < 0.05$) values (61% increased), followed by 2 mM SA (38%), and all concentrations of H_2O_2 (29–37%) compared to non-elicited chia sprouts (Fig. 3d).

Our findings are in accordance with earlier studies, which reported the increase in TPC and antioxidant capacities through germination in chia sprouts without elicitation. For example, Gómez-Favela et al. [2] reported an increase in TPC from 1.9 to 3.38 mg GAE/g (1.7-fold), and ABTS antioxidant capacity from 24 to 105 µmol TE/g (4.3-fold). In another study, Pajak et al. [3] reported an increase in TPC from 0.92 to 4.40 mg GAE (4.8-fold) and in the antioxidant capacities measured by ABTS assay from 4.3 mg to 32.9 mg TE/g (6.8-fold), and DPPH assay from 0.8 to 8.3 mg TE/g. Therefore, in agreement with those authors, the differences might be dependent on various factors, such as germination conditions (time, temperature, day to harvest), morphology, plant variety, and methodology applied (solvents and phenolic fraction extractions).

Although further investigation is required to explain this finding, a possible explanation might be that elicitors in chia sprouts could activate enzymes and genes of defense to increase the accumulation of bioactive compounds [2, 3]. It has been reported that elicitation with H_2O_2 overexpressed antioxidant enzymes such as catalase and peroxidase and key enzymes, such as phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), to produce phenolic compounds on lentil sprouts [17]. In addition, SA is a well-known inducer of plant systematic acquired resistance indeed induces gene expression related to biosynthesis of secondary metabolites in plants [4, 18–20].

The elicited chia sprouts showed higher inhibition of ABTS compared to DPPH radicals. Although both tests have the electron-transfer principle, both radicals solve differently (DPPH/methanol and ABTS/aqueous conditions), and molecular sizes are different; they might have different affinities towards other compounds that are in the samples and their solubility. Therefore, the results suggest that in the extracts could be present components with different antioxidant properties because of their distinct affinity and solubility; thus, they could contribute to the reaction rate in a different way, when reacting with radicals. In addition, it has been reported that non-elicited chia sprouts have more accumulation of phenolic acids than seeds [3], and respect to elicited chia sprouts results suggest that could change their profiles characterized by different molecular weights and polarities. In this sense, 1 mM SA chia sprouts and all concentrations of H_2O_2 might have higher content of polar and low weight molecular phenolic compounds (data not shown).

Effects of Elicitation on QUENCHER Antioxidant Capacities of Chia Sprouts

Non-elicited chia sprouts increased the Q-TPC (41%), and Q-DPPH (100%) antioxidant capacities compared with ungerminated chia seed, while Q-ABTS did not present significant changes (Fig. 4). On the other hand, SA (1 and 2 mM) and H_2O_2 (30 mM) concentrations presented the highest values for Q-TPC compared to non-elicited chia sprouts, with an increase until 45% (Fig. 4a). Regarding Q-DPPH, elicitation with H_2O_2 enhanced the response in this test compared to non-elicited chia sprouts, up to 28% ($p < 0.05$) (Fig. 4b). Finally, for Q-ABTS, 1 mM SA chia sprouts presented the highest values, with an increase up to 260%, followed by the 10 mM H_2O_2 chia sprouts with an increase of 230%, then 20 mM and 30 mM H_2O_2 with 178% and 198%, respectively, compared to non-elicited chia sprouts (Fig. 4c). Total antioxidant capacities index was increased, and the highest values were obtained using SA (1 and 2 mM) followed by all concentrations of H_2O_2 (Fig. 4d).

The results showed a significant increase in the QUENCHER measurements of chia sprouts, especially for TPC (438%) and ABTS assay (72%), compared to the values quantified in the extracts, which suggests an underestimation

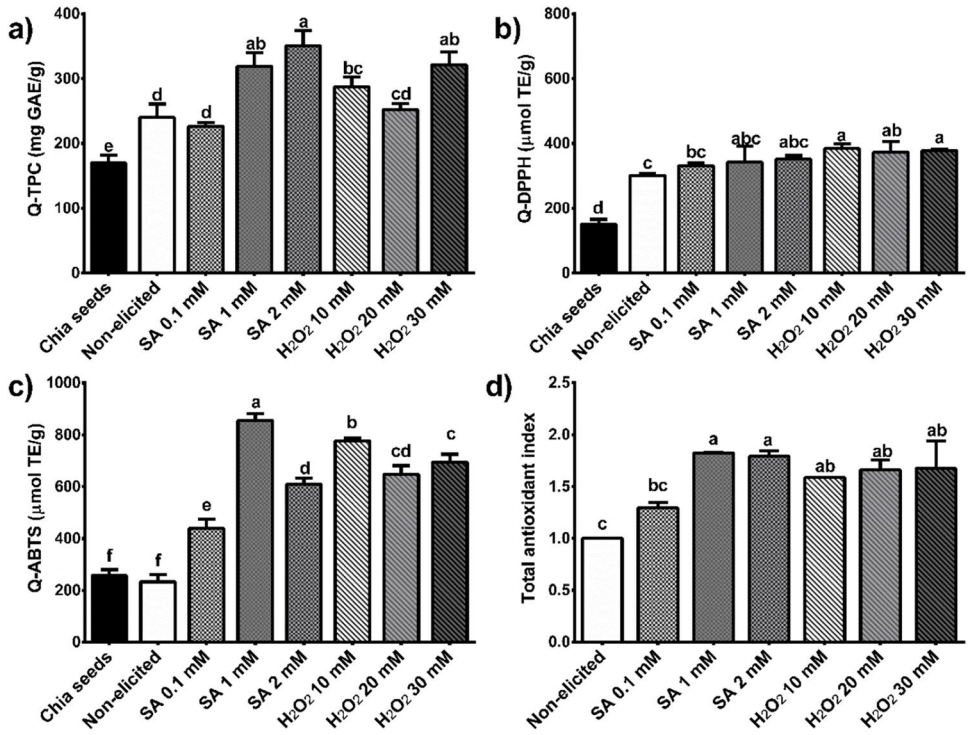

Fig. 4 QUENCHER (Q-) antioxidant capacities of solid matrices of chia seeds and chia sprouts produced at optimal germination conditions (178 h and 26.5 °C). **a**) Q-TPC, **b**) Q-DPPH assay, **c**) Q-ABTS assay, and **d**) Total antioxidant capacities index. Results are expressed on dry basis as mean value ± SD. Different letters indicate significant differences ($p < 0.05$) by Tukey test. SA, salicylic acid

of the conventional techniques based on liquid–liquid reactions. This behavior could be explained by the presence of phenolic compounds bound to insoluble polysaccharides in chia sprouts insoluble matrix, which react with the radicals in the QUENCHER tests. It could also be attributed to the QUENCHER capacities by antioxidants in chia sprouts did not measured in the extraction-solvent methods, such as vitamins (vitamin C and E), free amino acids, chlorophylls, melanoidins and melanoproteins, and lipophilic compounds: carotenoids, among others [9, 10].

Effects of Elicited Chia Sprouts on the Antioxidant Status of Serum and Urine in Obese Rats

Chia sprouts elicited with SA (1 mM), and H_2O_2 (20 mM) were selected to evaluate their antioxidant effects on obese rats due to agronomic characteristics and *in vitro* antioxidant capacities (Table 2). The obese (HFFD) group showed minor values of phenolic compounds and antioxidant capacities on serum and urine samples. These effects were more significant in urine samples (expressed as mmol TE/g creatinine) for TPC (35%), DPPH (38%), and ABTS (23%) compared to the group fed with the standard diet.

The consumption of elicited chia sprouts improved the antioxidant status of HFFD-induced obese rats. Regarding to the *in vivo* antioxidant capacities in serum, non-elicited chia sprouts and 1 mM SA chia sprouts supplementation increased, significantly ($p < 0.05$), the DPPH antioxidant capacity; whereas, ABTS measured antioxidant capacity increased ($p < 0.05$) only with 1 mM SA chia sprouts supplementation, when were compared to the HFFD group (Table 2). On the other hand, respecting the antioxidant capacities in urine, all supplemented diets increased the antioxidant values measurement by both assays compared to the HFFD group ($p < 0.05$).

Considering that the fasting serum samples were deproteinized before antioxidant capacities measurement, the DPPH and ABTS radicals uptake detected in the serum samples of treated animals could not be associated with proteins. Therefore, these measurements might be related to the intake of a high amount of antioxidants in the diet of the animals, due to the consumption of the elicited chia sprouts (with a high TPC content and antioxidant capacities), which contributes to improving the antioxidant status in obese rats, especially measured by the ABTS test, suggesting that extractable polar phenolic compounds could be partially bioavailable [21], and non-extractable phenolic compounds associated with the dietary fiber could be biotransformed by the gut microbiota and absorbed [22]. Overall, a remarkable serum and urine antioxidant effects is attributed to consuming chia sprouts, especially for 1 mM SA chia sprouts. In this sense, 1 mM SA chia sprouts presented the highest *in vitro* ABTS antioxidant capacities and a high TPC content, suggesting that these sprouts could present an important content of bioactive compounds (*i.e.*, polyphenols, dietary fiber, lipids) that could be associated with improving the oxidative state of obese rats.

Although further investigation is required to explain these findings, in the sense that chia sprouts present different classes

Table 2 Antioxidant status in serum and urine of induced-obese rats, fed with high fat and fructose diet and chia sprouts dietary supplementation

Parameters			Groups					
			Standard diet	HFFD	Chia seed	Non-elicited	SA 1 mM	H_2O_2 20 mM
Serum$^+$	DPPH	(mmol TE/L)	1.8 ± 0.3 ab	1.1 ± 0.1 b	1.5 ± 0.1 ab	2.0 ± 0.1 a	2.4 ± 0.3 a	1.9 ± 0.2 ab
	ABTS	(mmol TE/L)	9.7 ± 0.3 ab	7.4 ± 0.9 b	9.6 ± 1.1 ab	8.7 ± 0.4 ab	12.6 ± 1.3 a	9.0 ± 0.8 ab
Urine*	TPC	(mg GAE/g creatinine)	52.2 ± 1.5 a	25.2 ± 1.8 c	37.5 ± 2.0 ab	45.4 ± 4.7 a	38.9 ± 3.2 ab	40.8 ± 2.7 ab
	DPPH	(mmol TE/g creatinine)	1.2 ± 0.1 a	0.6 ± 0.0 b	1.2 ± 0.0 a	1.3 ± 0.1 a	1.3 ± 0.1 a	1.2 ± 0.1 a
	ABTS	(mmol TE/g creatinine)	0.14 ± 0.01 a	0.05 ± 0.01 c	0.09 ± 0.01 b	0.11 ± 0.01 ab	0.09 ± 0.01 b	0.10 ± 0.0 ab

Mean values ± SEM ($n = 8$). Different letters in the same row indicate statistical differences by Tukey test ($p < 0.05$). $^+$ Deproteinized fasting serum samples; * Urine polyphenols extracts adjusted with urine creatinine content; HFFD, high fat and fructose diet; SA, salicylic acid

of antioxidants that may contribute to the *in vivo* antioxidant capacities, a possible explanation might be the metabolism fate of polyphenols [21]. Due to the short half-life of polyphenols in the bloodstream, most phenolic compounds would not be present in serum, in amounts able to increase the *in vivo* antioxidant capacities after an overnight fast [12, 14, 21]. Nevertheless, since antioxidant capacities were measured in the urine of 24-h samples, the period during which the animals consumed the administered diets, these parameters could be directly related to phenolic intake from chia sprouts, as absorbed polyphenols are rapidly metabolized and excreted in urine [12, 12, 13]. In this sense, diets supplemented with chia seed and sprouts resulted in higher urine TPC contents compared to the HFFD group, suggesting that the phenolic compounds intakes were partially bioavailable. And that polyphenols metabolites from microbial metabolism could also exert effects in obese rats, inducing an increase of polyphenol circulation and, perhaps, exerting their antioxidant action on the target tissues, and subsequently be excreted [21, 21, 21]. Summarizing, polyphenols, *in vivo*, act stabilizing radicals and reactive oxygen species, as a first line defense; secondly, activating antioxidant enzymes. Recently they have been studied in their role as cellular signaling messengers to regulate the antioxidant compounds and enzymes [22, 22]. Although this work aimed to establish whether the intake of elicited chia sprouts rich in phenolic compounds (measured by TPC and Q-TPC) affected overall biological antioxidant capacities, further studies would be necessary to elucidate the nature of the compounds responsible for the effects observed.

Conclusions

Chia sprouts elicited with SA 1 mM decreased sprout length but maintained the germination percentage, increased the content of phenolic compounds, the *in vitro* antioxidant capacities (in extracts and whole solid food matrices), and improved the obesity-related oxidative stress in serum and urine compared to non-elicited sprouts. Although H_2O_2-elicited chia sprouts improved antioxidant capacities *in vitro* compared to non-elicited sprouts, the latter had slightly better *in vivo* effects suggesting that induction could generate compounds with less absorption capacity. Therefore, chia sprouts, mainly those elicited with SA 1 mM, decreased obesity related oxidative stress.

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Availability of Data and Material The materials and methods section is provided as SM1.

Declarations

Conflict of Interest The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

Ethics Approval All experiments produced with the rats were approved by Animal Experimentation Ethics Committee of the Autonomous University of Querétaro, Mexico (UAQ; Case N°. 15/4462).

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