The Pharma Innovation Journal 2021; 10(7): 1297-1303

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(7): 1297-1303
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www.thepharmajournal.com
Received: 03-04-2021
Accepted: 09-06-2021

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Effect of elicitation on phenylpropanoid pathway and biological activities of Chickpea sprouts

Chandan Thappa and Sanjay Guleria

Abstract
The present study was carried out to evaluate the effect of different elicitors namely methyl salicylate (MeSA), salicylic acid (SA) and neem leaf extract alone and also in combination with precursor (L-phenylalanine) on the phytochemical composition, antioxidant activity, and enzyme activity of chickpea sprouts. The most effective treatment was found to be the combination of 1% MeSA and precursor treatment which increased the concentration of phenols, flavonoids and tannins in the treated sprouts by 2.3 folds, 3.4 folds and 2.8 folds respectively as compared to control. Also there was an increase in the antioxidant activity of the elicited sprouts as revealed by an increase of 93.54% and 75.96% in the ferric reducing antioxidant power and percent metal ion chelation activity respectively. Elicitation also enhanced DPPH radical scavenging activity and reducing power, IC_{50} value was decreased in elicited sprouted by (IC_{50} 23.98 mg/g FW) DPPH assay and (IC_{50} 92.24 mg/g FW) reducing power assay. Elicitation with optimized concentration of elicitor and precursor also exerted profound effect on phenylpropanoid pathway as revealed by the increase in the activities of the enzymes namely phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL) and peroxidase (POD) by 2.8 folds, 4.9 folds and 2.4 folds respectively. From the above study it may be concluded that, elicitation can be a promising strategy to improve the nutraceutical potential of chickpea sprouts.

Keywords: Elicitor, salicylic acid, methyl salicylate, precursor, phytochemical

Introduction
Many epidemiological studies showed that consumption of plant-based dietary food reduces the effect of various ailments, such as cardiovascular diseases, diabetes, and obesity (Raiola *et al*., 2018) [1]. Edible parts of plant-based food is a source of various bioactive compounds like phenolic compounds, glucosinolates, tocopherols, vitamin C, and carotenoids, with health-promoting properties (Barba *et al*., 2017) [2]. These biologically active compounds possess various biological activities such as anti-inflammatory, anticarcinogenic, antioxidant, and antidiabetic. Several studies have been carried out to search for the strategies for increasing the concentration of bioactive compounds in the edible seeds so as to improve its nutraceutical properties. It has been found that various types of phytochemicals naturally increase during germination of seed leading to enhancement in the biological activities of the sprout. In addition to this, germination process also reduces various anti-nutritional and non-digestible components present in the seed. Several studies have proven that bioactive compounds in cereal, pulses, flower, fruit, vegetable and medicinal plant accumulate at the time of germination (Gan *et al*., 2017) [3]. However, the polyphenol content in the seed can be further enhanced during germination by the method of elicitation. Elicitation is a method of induction of plant defense mechanisms by modification of metabolism by elicitors which results in the enhanced synthesis of phytochemicals. As compared to genetic engineering, elicitation is simple, socially acceptable and cost-effective approach for improving the functionality of plant-based food (Lopez-Martinez *et al*., 2017; Natella *et al*., 2016) [4, 5]. Therefore, to enhance the qualitative value and production of metabolites in the plant-specific concentration of treatment can be used (Baenas *et al*., 2014) [6].
Legumes are good source of bioavailable compounds like proteins, lipids, starch, and minerals. After the various type of processing (e.g., cooking, frying), they are consumed all over the world. Nutritional and nutraceutical value of the legumes are enhanced by sprouting. In addition, various documents support the presence of polyphenols with potent health benefits in germinated seeds (Gawlik-Dziki and Swieca, 2011) [7]. Chickpea or Bengal gram (*Cicer arientum* L) is a rich dietary protein source and the largest legume cultivated in the world. Moreover, chickpeas have also been reported as a rich source of minerals, vitamins, and

phytoestrogens (Pittaway *et al*., 2006) [8]. Chickpea also possesses several medicinal properties. In traditional medicinal system, chickpea seeds were used as a stimulant, tonic, anti-helminthic, appetizer, aphrodisiac, and for relieving burning sensation in the stomach. In the Indian system of medicine, chickpea is used to decrease Kapha as it is considered to be dry (rooksha) and light (laghu). Because of these properties, chickpea has been suggested for treating obesity as well as excessive oily and heavy foods consuming patients (Wu *et al*., 2012; Zia-Ul-Haq *et al*., 2007) [9, 10]. It has been reported that the seeds of chickpea have been employed in the treatment of diabetes mellitus and hypertension over the past 2500 years in the Chinese herbal medicine (Zhang *et al*., 2007a) [11]. Among the medicinal properties that have been assigned to chickpea, antihyperlipidemic activity has attained much focus because of the presence of phytoestrogenic isoflavones formononetin and biochanin-A (Li *et al*., 2008) [12].

The goal of the present study was to evaluate the effects of elicitation on the precursor of the phenylpropanoid pathway on the phenolics content, antioxidant capacity, and nutritional quality of chickpea sprouts.

Material and Methods

Plant material and growth conditions

Chickpea (*Cicer arietinum* var.GNG1581) seeds were procured from the Plant Breeding and Genetics, Division, FoA, SKUAST-J, Chatha. Seed surface sterilization was performed for 10-15 minutes with 1% sodium hypochlorite. After that the seeds were washed (5-7 times) with distilled water to neutralize the effect of sodium hypochlorite and then were immersed in distilled water overnight at 25 ˚C. The seeds were then placed in the petri dishes lined with absorbent paper and dark germinated (12-15 ˚C, 40% relative humidity) for 6 days. 5 mL of distilled water was used for the watering of seedlings every day.

Elictor and treatment

Elicitors used in the study were salicylic acid, methyl salicylate, and neem leaf extract at different concentrations whereas the only precursor used in the study was L-phenylalanine (phenolic precursor). For elicitation of sprouts, salicylic acid (SA) was used at the working concentrations of 10 mM and 20 mM whereas methyl salicylate (MeSA) was used at the working concentrations of 0.5% and 1%. Neem leaf extract was prepared according to the method described by Paul and Sharma (2002) [13]. Using chilled distilled water, 400 g (fresh weight) of mature neem leaves were homogenized in a pre-chilled pestle and mortar using chilled distilled water.

The extract was filtered through four layers of moistened muslin cloth and then distilled water was added to make the final volume up to 1000 mL. The filtrate was then centrifuged at 2000 g for 15 min at 4 ˚C. The supernatant thus obtained was denoted as concentrated neem extract. This extract was then diluted with distilled water to prepare a 1:2 dilution of the concentrated neem extract. The concentration of 0.1 mM L-phenylalanine was prepared in distilled water. All solutions were freshly prepared before spraying. Different concentrations of elicitors and elicitor plus L-phenylalanine (E+P) were sprayed on 3-day-old seedlings for 3 days. Sprouts were then collected after 7 days and stored at -20 °C until further biochemical analysis. All the experiments were performed in triplicates.

Phenolic analysis

Extract preparation

One gram of sprouts were extracted in 3 mL of methanol which was then centrifuged at 10,000 rpm for 20 min. The final volume of the supernatant was raised to 4 mL which was then stored at -20˚C until further biochemical analysis.

Total phenolic content

Folin-Ciocalteu method was used to determine the total phenolic content (Singleton *et al*., 1947) [14]. 100 µL of extract was taken and the volume was raised to 1000 µL by adding 900 µL distilled water. After adding 1 mL of 1 N Folin-Ciocalteu reagent, the reaction mixture was held at room temperature for 5 minutes before adding 3 mL of 20% Na_2CO_3 (sodium carbonate). After 10 min of incubation at room temperature, the absorbance of the reaction mixture was taken at a wavelength of 760 nm by using double beam UV-VIS spectrophotometer. The amount of total phenolics was expressed as milligram gallic acid equivalents (mg GAE) per gram fresh weight.

Flavonoid content

Flavonoid content was determined according to the method reported by Zhishen *et al.* (1999) [15]. 500 µL extract was mixed with 1 mL distilled water and then 75 µL 5% sodium nitrite ($NaNO_2$) solution solution was added. 150 µL of 10% aluminium chloride ($AlCl_3$) solution was added after leaving the reaction mixture undisturbed for approximately 6 minutes at 25°C. After that, there was another 5 minutes of undisturbed incubation at room temperature before addition of 500 µL of 1 M NaOH to the reaction mixture. The absorbance was measured at 510 nm using a double beam UV-VIS spectrophotometer after the volume of the reaction mixture was increased to 2.5 mL using distilled water. Standard curve of catechin was plotted to calculate the flavonoid content. Results were expressed as µg of catechin equivalents (CE)/g fresh weight.

Condensed tannins

Condensed tannins were determined according to the method described by Xu and Chang (2007) [16]. 1 mL of sample extract was mixed with 3 mL of 4% methanol-vanillin solution, followed by 1.5 mL of concentrated HCl. After thoroughly mixing, the mixture was allowed to rest at room temperature for 15 min. Absorbance was measured at a wavelength of 500 nm using double beam UV-VIS spectrophotometer against a reagent blank. The amount of condensed tannins was expressed as µg catechin equivalents (µg of CE) /g fresh weight.

Antioxidant activity

DPPH radical scavenging activity

The method described by Abe *et al.* (1998) [17] was used to determine the DPPH radical scavenging activity. 2 mL of sample was combined with 1 mL of 0.5 mM DPPH solution prepared in methanol, followed by the addition of 2 mL of 0.1 M sodium acetate buffer (pH 5.5). For 30 minutes, the reaction mixture was shaken and incubated at room temperature in the dark. Using a double beam UV-VIS spectrophotome, the absorbance was estimated at a wavelength of 517 nm. Methanol served as the negative control and was used for the base correction of instrument. The radical scavenging activity (RSA) was calculated as a percentage of DPPH radical discoloration, using the equation:

RSA (%) = (1- A_{sample} / A_{blank}) × 100

Where A_{sample} is the absorbance of the reference sample, and A_{blank} is the absorbance of the positive control (which contains all reagents except the test compound). The extract concentration that provided 50% inhibition (IC_{50}) of DPPH radical was calculated by plotting a RSA (%) versus concentration graph. Butylated hydroxyl toluene (BHT) was used as the reference compound.

Reducing power assay

The method defined by Oyaizu (1986) [18] was used to determine the reducing power of extracts. Different concentrations of extract were combined with 2.5 mL of a 0.2 M phosphate buffer (pH 6.6), followed by 2.5 mL of a 1% potassium ferricyanide solution. For 30 minutes, the mixture was incubated at room temperature. After incubation, 2.5 mL of a 10% trichloroacetic acid (TCA) solution was added before centrifuging the reaction mixture at 1036 g for 10 min. The upper layer of the solution (2.5 mL) was taken and combined with 2.5 mL distilled water and 2.5 mL ferric chloride solution (0.1 percent). A double beam UV-VIS spectrophotometer was used to measure the absorbance of the reaction mixture at 700 nm. Absorbance of the reaction mixture is directly proportional to the reducing power of the sample. The extract concentration providing 0.5 of absorbance (IC_{50}) was calculated from the equation obtained by plotting a absorbance versus concentration graph.

Percent metal ion chelation activity

The chelating effect of ferrous ions in sprouts extract was determined by using the method described by Dinis *et al.* (1994) [19] with minor modifications. 50 µL of extract was combined with 2950 µL of methanol and 60 µL of 2 mM $FeCl_2$ (ferrous chloride). The reaction was started by adding 120 µL of ferrozine (5 mM) to the reaction mixture. The reaction mixture was then shaken well and kept at room temperature for 4 min before taking absorbance at 562 nm. Each test sample was repeated thrice. Quercetin was used as the reference compound in this assay. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:

% inhibition = [(absorbance of control – absorbance of sample)/ absorbance of control] ×100. Ferric reducing antioxidant power (FRAP) activity

The method of Benzie and Strain (1996) [20] was used to determine the FRAP activity. For the preparation of FRAP reagent, 10 volumes of 300 mM acetate buffer (pH 3.6) was combined with 1 volume of 10 mM TPTZ (Ferric tripyridyl triazine), prepared in 40 mM HCl and 1 volume of 20 mM $FeCl_3.6H_2O$ (Ferric chloride). 3 mL of FRAP reagent was warmed at 37 °C and then combined with 200 µL of sample (control and elicited) and 800 µL of methanol and the absorbance was taken at a wavelength of 593 nm against reagent blank after incubating for 4 min. FRAP value was calculated from the standard curve of ferrous sulfate and expressed as micromoles of Fe^{2+} equivalent (FE) per gram fresh weight.

Enzymatic activities

Extract preparation

For PAL and TAL assays, sprouts were extracted in borate buffer (pH 8.8). In the pre-chilled mortar and pestle, 4 mL of pre-chilled borate buffer was used to grind one gram of sprouts. The homogenate was then centrifuged for 20 min at 4 ° C at 12,000 rpm. For enzyme assays, the supernatant was collected and stored at -20 ° C. Similarly extraction in phosphate buffer at pH 7.0 was carried out for peroxidase assay. One gram of sprouts were homogenized in 4 mL of pre-chilled phosphate buffer and centrifuged at 12,000 rpm for 20 min at 4˚ C. Supernatant was stored at -20˚and used as enzyme source.

Enzyme assay

Phenylalanine ammonia lyase (PAL) activity

The method described by Assis *et al.* (2001) [21] was used for the determination of PAL activity. 100 µL of extracts (enzyme source) from different samples (control and elicited) were incubated at 37°C for 1 h with 1.9 mL of L-phenylalanine (0.02 M). To stop the reaction, 0.1 mL of 6 N HCl was added after incubation. A UV-VIS spectrophotometer was used to measure the absorbance of the reaction mixtures at 290 nm. Absorbance of the reaction mixtures was taken at 290 nm in a UV-VIS spectrophotometer. Results were expressed as µg of t-cinnamic acid formed/hr/mg protein.

Tyrosine ammonia lyase (TAL) activity

TAL activity was determined according to the method reported by Assis *et al.* (2001) [21]. The reaction was conducted by combining 100 µL of the enzyme extracts from different samples with 1.9 ml of 0.01 M L-tyrosine and incubating the reaction mixtures at 37˚C for 1 h. To stop the reaction, 0.1 mL of 6 N HCl was added after incubation and the absorbance was taken at 333 nm using a UV-VIS spectrophotometer. TAL activity was expressed as µg of *p*-coumaric acid formed/hr/mg protein.

Peroxidase (POD) activity

The method given by Putter (1947) [22] was used to determine the POD activity of extracts. 50 µL of guaiacol solution (20 mM) and 100 µL of the enzyme extract were combined with 3 mL of the phosphate buffer solution (pH 7.0). The reaction was started by adding 30 µL (12.3 mM) hydrogen peroxide solution after taking the reaction mixture in the cuvette. The reaction mixture was vigorously shaken, and the absorbance was measured every 15 seconds for 3 minutes with a UV-VIS spectrophotometer set to 436 nm. POD activity was expressed as units/min/mg protein.

Statistical analysis

All of the tests were repeated three times. The results were calculated as the average of three independent germination experiments. Data were subjected to one way analysis of variance (ANOVA) and significant differences between samples were determined by the least significant differences (LSD) test at $p \leq 0.05$ probability level, using R Studio software (version 3.4.1, 2017).

Results and Discussion

Nowadays, consumers look for those food products which have nutraceutical importance. The food products having potential bioactivity, in addition to nutritional quality, have more food-value and are preferred by the consumers. Food products having bioactive constituents can prevent various diseases and could also promote good health. The digestibility

of the nutrients and vitamin content of foods can be improved in an efficient and economical way by sprouting (Swieca *et al*., 2012) [23]. Various researches have shown the disease preventive and health promoting properties of phytochemical rich foods. Therefore, the nutritional and nutraceutical value of seeds should be improved for diverse human health benefits (Gawlik-Dziki *et al*., 2013) [24]. Elicitation is a promising strategy to enhance the polyphenol content in the seed. Several studies have reported that the nature and dose of the elicitor affect the secondary metabolite composition of sprouts (Baenas *et al*., 2014) [6]. Change in the composition of secondary metabolites is normally are preceded by an increase in the activity of the metabolic enzymes involved (Vasconsuelo and Boland, 2007) [25]. Therefore for the production of optimum amount of desirable metabolites, it is required to optimize the elicitor type, dose, and intervals between the treatments which are different for each plant species. The results obtained from the present study revealed that the optimized concentrations of SA for chickpea sprouts were 10 mM and 20 mM. Enhancement in the production of different types of secondary metabolites such as phenolics, alkaloids, flavonoids, terpenes, and phytoalexins in response to SA treatment has also been reported by Silve *et al*. (2013) [26]. In the present study, 0.5% and 1% concentrations were selected as the optimized concentrations of MeSA for elicitor treatment of chickpea sprouts. Mandal used 0.01% 0f MeSA for elicitor treatment (Mandal, 2010) [27]. The optimized concentrations of Neem leaf extract were NE1 and NE2. {NE1 = Neem extract (1:1 dilution of NE2) and NE2 = Neem extract (100 g fresh leaves of Neem /250 ml D.W.)}. Guleria and Kumar, (2006) [28] used neem leaf extract at 1:2 dilution for the elicitation of sesame seedlings in order to induce resistance against *Alternaria* leaf spot disease. The concentration of the L phenylalanine, the only precursor, was found to be 0.1 mM. Swieca *et al.* (2014) [29] also used phenylalanine as precursor at the concentration of 0.1 mM in which UV light was used as the elicitor.

Effect of different elicitors on phenolic content of chickpea sprouts

The composition of bioactive compounds such as phenols and flavonoids in the elicited sprouts differed significantly as compared to control chickpea sprouts (Table 1). These bioactive compounds possess antioxidant properties and thus are beneficial for health (Lee *et al*., 2017) [30]. Total phenolic content (TPC) of chickpea sprouts was found to vary from 0.96 to 2.05 mg GAE/g FW. Highest total phenolic content was observed in MeSA (1%)+P treated chickpea sprouts (2.05 mg GAE/g FW) followed by the MeSA (0.5%)+P treatment (1.67 mg GAE/g FW). Least TPC was observed in sprouts treated with 10 mM SA (1.12mg GAE/g FW) (Table 1). Similarly, flavonoid content of chickpea sprouts was also elevated in the range of 30.33 to 106.13 µg catechin/g FW. Chickpea sprouts treated with MeSA (1%) + P showed the highest flavonoid content (106.13 µg catechin/g FW) followed by the MeSA (1%) (96.87 µg catechin/g FW). Among all the treatments NE1 showed the least flavonoid content (41.82 µg catechin/g FW) (Table 1). Similarly in the studies conducted by Swieca (2015) [31] on osmotic stress induced lentil sprouts, total flavonoid and condensed tannin contents were also elevated as compared to control. There is much evidence that generation of ROS during stress in plant induces over production of phenolics. Condensed tannins content was also varied from 31.83 to 90.20 µg catechin/g FW. Highest condensed tannins content was observed in the sprouts treated with MeSA (1%) + P (90.20 µg catechin/g FW) followed by the MeSA (1%) (77.89 µg catechin/g FW) (Table 1). In another study, the effect of salicylic acid (SA), chitosan (CH), and hydrogen peroxide (H_2O_2) on the anti-nutritional and nutraceutical content, as well as the antioxidant capacity of bean sprouts (cv Dalia) at different concentrations was observed and it was found that sprouts treated with CH, SA and H_2O_2 have increased content of phenolic compounds (1.8-fold), total flavonoids (3-fold) and antioxidant capacity (37%) as compared to raw seeds (Mendoza-Sanchez *et al.,* 2016) [32]. The increased polyphenolic content may be due to the treatment of sprouts with H_2O_2 which lead to the generation of reactive oxygen species (ROS) that act as secondary messenger in various signal transduction pathways such as phenylpropanoid and shikimate pathways. Activation of these pathways increases the levels and activities of key enzymes (PAL and TAL) of secondary metabolism.

Table 1: Phtyochemical profile of chickpea sprouts

Treatments	Phenols (mg GAE/g FW)	Flavonoids (µg catechin/g FW)	Condensed tannins (µg catechin/g FW)
Control	0.96±0.08^a	30.33±5.92^a	31.83±6.27^a
NE1	1.14±0.02^b	41.82±5.46^a	41.60±1.38^a
NE2	1.25±0.01^b	57.49±0.66^b	47.10±1.56^a
SA (10 mM)	1.12±0.02^b	55.19±3.24^b	47.32±5.23^a
SA (20 mM)	1.34±0.03^b	68.28±10.57^b	60.07±1.00^b
MeSA (0.5%)	1.47±0.02^b	85.77±14.29^b	63.43±2.41^b
MeSA (1%)	1.60±0.09^b	96.87±1.97^b	77.89±12.50^b
P (0.1mM)	1.16±0.11^b	42.35±7.53^a	33.99±2.94^a
NE1+P	1.15±0.09^b	64.32±5.62^b	42.29±0.73^a
NE2+P	1.31±0.07^b	68.49±5.92^b	47.11±4.07^a
SA (10 mM)+P	1.29±0.06^b	65.72±7.04^b	51.21±2.6^a
SA (20 mM)+P	1.53±0.05^b	78.31±11.30^b	76.43±10.6^b
MeSA (0.5%)+P	1.67±0.06^b	95.15±5.28^b	73.22±12.2^b
MeSA (1.0%)+P	2.05±0.015^b	106.13±1.21^b	90.20±2.15^b

*The superscript alphabet b shows the values significantly different from control (($p \leq 0.05$).

NE1; Neem extract (1:1 dilution of NE2), NE2; Neem extract (100 g fresh leaves of Neem /250 mL Distilled water), SA; Salicylic acid, MeSA; Methyl salicylate, P; Precursor (L-phenylalanine), FW; Fresh weight

Effect of different elicitors on antioxidant activity of chickpea sprouts

DPPH scavenging activity in the chickpea sprouts is shown in table 2. The highest DPPH radical scavenging activity was observed at MeSA (1%) + P (IC_{50} value 23.98 mg/g FW) treated sprouts followed by the MeSA (0.5%) + P (IC_{50} value 29.81 mg/g FW) treatment whereas sprouts treated with NE1 (IC_{50} value 150.00 mg/g FW) showed least DPPH radical scavenging activity as compared to control (IC_{50} value 160.89 mg/g FW) (Table 2). The similar results were obtained in the studies of Swieca and Dziki (2015) [33] where the quenching and reducing ability of elicited wheat flour sprouts increased on elicitation. The increase in antioxidant activity is usually positively correlated with phenolics content. From our study it was also observed that the MeSA (1%) + P treatment showed highest reducing power (IC_{50} value 92.46 mg/g FW) followed by MeSA (1%) (IC_{50} value 112.49 mg/g FW) whereas least reducing power was observed in sprouts treated with NE1 (IC_{50} value 162.05 mg/g FW). An increase in the content of phenolic acids such as hydroxycinnamic acids may be responsible for the enhanced reducing potential of seedlings since phenolic acids have the ability to reduce iron ions. Similar findings were also observed by Swieca (2016) [34] in a study on buckwheat sprouts where elicitation improved the antiradical and lipid protecting ability by increasing the phenolic content. A significant increase in the chelating activity of sprouts was observed after treatment with H_2O_2 for 24 h. Chelation activity of elicited chick pea was in the range of 20.23 to 81.46%. Highest metal ion chelation activity (81.46%) was observed in MeSA (1%) +P treated chickpea sprouts followed by MeSA (0.5%) + P (62.61%) whereas NE1 treated sprouts showed the least chelation activity (25.10%) and the chelating activity in treatments NE1 and P (0.1mM) was found to be at par with the control (Table 2). The antioxidant potential was also checked by FRAP activity. It was in range of 2.17 to 4.20 µM Fe^{2+} /g FW. Treatment MeSA (1%) + P showed the highest FRAP value (4.20 µM Fe^{2+} /g FW) followed by MeSA (0.5%) + P (3.72 µM Fe^{2+} /g FW). Least FRAP activity was observed in the treatment NE1 (2.52 µM Fe^{2+} /g FW) (Table 2). An increase in FRAP values after sucrose and mannitol treatments is also observed in other studies (Guo *et al.,* 2011) [35]. Thus elicitation is a simple and cost-effective method for the enrichment of bioactive components of sprouts and enhancing their antioxidant activity.

Table 2: Antioxidant activity of chickpea sprouts

Treatments	DPPH IC_{50} (mg/g FW)	Reducing Power IC_{50} (mg/g FW)	FRAP (µM Fe^{2+}/g FW)	Metal ion chelation (%)
Control	160.89 ±12.79	192.94 ±5.03	2.17±0.22^a	20.23±0.71^a
NE1	150.00 ±15.34	162.05 ±3.02	2.52±0.09^a	25.10±3.00^a
NE2	112.09 ±11.23	147.71 ±4.69	3.05±0.25^a	29.14±1.03^b
SA (10 mM)	127.72 ±14.76	168.42 ±7.67	2.37±0.06^a	34.47±3.08^b
SA (20 mM)	123.31±6.41	138.26 ±6.41	2.69±0.10^a	37.71±3.14^b
MeSA (0.5%)	122.43 ±3.50	117.50 ±2.25	2.66±0.06^a	34.64±2.89^b
MeSA (1%)	102.25 ±14.47	112.49 ±3.72	2.97±0.22^a	50.45±7.22^b
P (0.1mM)	66.42± 14.24	186.90 ±4.26	2.41±0.19^a	23.77±4.7^a
NE1+P	36.67 ±0.97	159.42 ±2.42	2.61±0.34^a	31.67±1.02^b
NE2+P	32.75 ±1.32	141.44 ±4.66	3.06±0.27^b	39.77±4.91^b
SA (10 mM)+P	39.61 ±1.17	164.28 ±9.58	2.69±0.10^a	44.47±3.08^b
SA (20 mM+P)	35.74 ±3.62	131.59 ±0.14	2.79±0.16^a	61.77±2.11^b
MeSA (0.5%)+P	29.81 ±1.05	120.83 ±6.77	3.72±0.45^b	62.61±3.49^b
MeSA (1.0%)+P	23.98 ±1.77	92.46 ±5.44	4.20±.07^b	81.46±1.85^b

*The superscript alphabet b shows the values significantly different from control (($p \leq 0.05$).
NE1; Neem extract (1:1 dilution of NE2), NE2; Neem extract (100 g fresh leaves of Neem /250 mL Distilled water), SA; Salicylic acid, MeSA; Methyl salicylate, P; Precursor (L-phenylalanine), FW; Fresh weight

Effect of different elicitor on different enzymatic activity of chickpea sprouts

In this study, the results revealed increased activities of PAL and TAL (the key enzymes of secondary metabolism). After elicitation, the PAL activity in the chickpea sprouts was varied from 7.28 to 20.89 µg *t*-cinnamic acid/hr/mg protein (Table 3). Highest PAL activity was observed in MeSA (1%) + P treatment (20.89 µg *t*-cinnamic acid/hr/mg protein) followed by MeSA (1%) (17.63 µg *t*-cinnamic acid/hr/mg protein) whereas the NE1 treated sprouts showed least PAL activity (8.61 µg *t*-cinnamic acid/hr/mg protein). Similarly the TAL activity was also elevated in the elicited chickpea sprouts. TAL activity was in range of 6.79 to 33.34 µg *p*-coumaric acid/hr/mg protein as shown in table 3. Highest TAL activity was shown by chickpea sprouts elicited with MeSA (1%) + P treatment (33.34 µg *p*-coumaric acid/hr/mg protein) followed by MeSA (0.5%) + P (26.35 µg *p*-coumaric acid/hr/mg protein). The key role of these enzymes in phenolic synthesis is well-documented (Randhir *et al*., 2004) [36]. This increase in enzyme activity may be the biochemical mechanism which is responsible for increased concentrations of phytochemicals and antioxidant activity. Swieca, (2015) [31] found the similar results in the elicited lentil sprouts and reported a direct relationship of enhanced phenolic content and antioxidant activity with the increase in the activity of PAL and TAL produced by phenylpropanoid pathway. In another study by Swieca *et al.* (2014) [29], an improved phenolic content and antioxidant activity of lentil sprouts was observed on elicitation with UV-B treatment and feeding with the precusrors of phenylpropanoid pathway.

The present results also show a significant increase in peroxidase (POD) activity on elicitation in all the studied sprouts. In elicited chickpea sprouts POD activity was varied from 40.91 to 100.47 Units/min/mg protein. Chickpea sprouts showed highest POD activity in MeSA (1%) + P treatment (100.47 Units/min/mg protein) followed by SA (20 mM) + P (90.21 Units/min/mg protein) shown in table 3. Similar results have also been found by Randhir and Shetty, (2007) [37] in which the GPX activity for ascorbic acid treated dark germinated fava bean sprouts was increased as compared to control. Elicitation induces production of ROS which act as signaling compounds for the secondary metabolic pathways

leading to enhanced phenolic content and antioxidant activity. The increase in POD activity in response to elicitation may be due to the role of peroxidases in combating biotic and abiotic stresses due to their role in defence processes including cell wall modifications and metabolism of auxin and ethylene (Gaspar *et al*., 1991) [38].

Table 3: Phenylalanine ammonia-lyase, tyrosine ammonia-lyase and peroxidase activities chickpea sprouts

Treatments	PAL activity (µg t-cinnamic acid/hr/mg protein)	TAL activity (µg *p*-coumaric acid/hr/mg protein)	Peroxidase activity (Units/min/mg protein)
Control	7.28±1.82^{a}	6.79±3.58^{a}	40.91±3.15^{a}
NE1	8.61±0.12^{a}	7.83±2.05^{a}	62.23±4.4^{b}
NE2	9.75±1.04^{a}	13.75±7.7^{a}	75.07±7.64^{b}
SA (10 mM)	9.39±3.56^{a}	12.72±10.38^{a}	62.70±6.16^{b}
SA (20 mM)	10.45±1.21^{a}	15.87±3.35^{a}	75.60±10.89^{b}
MeSA (0.5%)	13.39±1.17^{b}	17.27±.37^{a}	77.53±5.66^{b}
MeSA (1%)	17.63±2.23^{b}	20.95±0.67^{b}	83.73±13.7^{b}
P (0.1mM)	7.60±3.36^{a}	14.71±2.69^{a}	53.40±7.91^{a}
NE1+P	8.69±3.00^{a}	17.80±10.63^{a}	79.00±9.54^{b}
NE2+P	8.82±3.73^{a}	18.63±8.32^{a}	84.37±3.1^{b}
SA (10 mM)+P	10.27±1.83^{a}	21.71±9.14^{b}	74.97±3.11^{b}
SA (20 mM)+P	10.59±0.85^{a}	24.06±1.05^{b}	90.21±7.36^{b}
MeSA (0.5%)+P	15.18±1.81^{b}	26.35±10.68^{b}	85.60±7.11^{b}
MeSA (1.0%)+P	20.89±1.14^{b}	33.34±5.52^{b}	100.47±7.06^{b}

*The superscript alphabet b shows the values significantly different from control (($p \leq 0.05$).
NE1; Neem extract (1:1 dilution of NE2), NE2; Neem extract (100 g fresh leaves of Neem /250 mL Distilled water), SA; Salicylic acid, MeSA; Methyl salicylate, P; Precursor (L-phenylalanine)

Conclusion

Our study reveal that elicitation is a feasible approach to improve the phytochemical composition and bioactive properties of sprouted chickpea, 1% methyl salicylate (MeSA) plus precursor (0.1 mM L-phenylalanine) was found to be the most effective treatment for improving the bioactive properties in chickpea. To improve the health promoting phytochemical levels and bioactivity of low-processed food (sprouts) elicitation is the relatively easy and cheap approach in the edible sprout productions industry.

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