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Transformation of Lettuce with *rol ABC* Genes: Extracts Show Enhanced Antioxidant, Analgesic, Anti-Inflammatory, Antidepressant, and Anticoagulant Activities in Rats

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Abstract Lettuce is an edible crop that is well known for dietary and antioxidant benefits. The present study was conducted to investigate the effects of *rol ABC* genes on antioxidant and medicinal potential of lettuce by *Agrobacterium*-mediated transformation. Transgene integration and expression was confirmed through PCR and real-time RT-PCR, respectively. The transformed plants showed 91–102 % increase in total phenolic contents and 53–65 % increase in total flavonoid contents compared to untransformed plants. Total antioxidant capacity and total reducing power increased up to 112 and 133 % in transformed plants, respectively. Results of DPPH assay showed maximum 51 % increase, and lipid peroxidation assay exhibited 20 % increase in antioxidant activity of transformed plants compared to controls. Different in vivo assays were carried out in rats. The transgenic plants showed up to 80 % inhibition in both hot plate analgesic assay and carrageenan-induced hind paw edema test,

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while untransformed plants showed only 45 % inhibition. Antidepressant and anticoagulant potential of transformed plants was also significantly enhanced compared to untransformed plants. Taken together, the present work highlights the use of *rol* genes to enhance the secondary metabolite production in lettuce and improve its analgesic, anti-inflammatory, antidepressant, and anticoagulatory properties.

Introduction

Lactuca sativa (lettuce) is one of the top five routinely consumed edible crops in the USA [1]. It has a good taste, contains few calories, is nutritious, and is a good source of vitamins K, A, and C and potassium. Its leaves and stem contain many active constituents such as lactucerin (which plays a part on analgesia, hypnosis, and treatment of neurasthenia) and mannitol (which affects blood circulation and also acts as diuretic) [2]. The seed oil of lettuce has been reported for sedative, hypnotic, analgesic, and anticonvulsant properties [3]. Phenolics and flavonoids, which are commonly distributed in plants, have been stated to exert free radical scavenging abilities as well as anticarcinogenic, anti-inflammatory, and anxiolytic properties [4–6]. Significant levels of phenolic compounds and their antioxidant activities have been reported in lettuce [7, 8]. Although phytochemical levels are comparatively less in lettuce, its high antioxidant potential reveals the likelihood of increasing the health benefits after enhancing the quantity of its phenolic constituents [7]. Phenolic compounds of lettuce differ among different cultivars like oak leaf, loose leaf, butter head, romaine, and iceberg [7, 9]. However, the quantity of its phenolic acids and flavonoids seems to be sensitive to environmental factors. Furthermore, Ferreres et al. [10] and DuPont et al. [9] stated that storage and post-harvest processing result in substantial losses in phenolic acid and flavonoids in numerous cultivars of lettuce, which raises the need for either enhancing the metabolite concentration in natural plants or seeking the alternative sources of these antioxidants.

Lettuce has been a plant of interest for the genetic transformation due to its rapid growth and ease of transformation. Although *L. sativa* is rarely a natural host of *Agrobacterium* species, it is susceptible to artificial inoculation by *A. tumefaciens.* Previously, lettuce has been successfully transformed to express vaccines for foot and mouth disease [11], vesicular stomatitis virus infection [12], and cholera toxin [13] as well as for the expression of human lactoferrin, thymosin [14], nattokinase [15], lysine [16], *Escherichia coli* heat-labile enterotoxin subunit B [17], vitamin C [18], β-carotene, and folic acid [19, 20]. Sobolev et al. [21] transformed *L. sativa* L. with asparagine synthetase A gene from *E. coli*, and they found a significant accumulation of inulin in transgenic plants. The CHI gene from Chinese cabbage was expressed by Han et al. [22] in lettuce plants of the cultivar Chungchima which showed up to 4-fold increase of flavonoids whereas total phenol contents were increased up to 3.5-fold. Recently, the role of endogenous ascorbic acid as a browning-preventing agent has been determined by transformation of L-galactono-1,4-lactone dehydrogenase gene in lettuce [23].

It has been reviewed [24] that transformation with *rol* genes enhances the amount of secondary metabolites in hairy roots. Various studies have proved *rol* genes as powerful inducers of secondary metabolites [25–27]. It has been shown that the positive health effects of dietary vegetables are mainly because of their secondary metabolites including flavonoids

[28]. Flavonoids have been shown to minimize the risk of numerous diseases like cancer, age-related diseases, atherosclerosis, and anti-inflammatory and cardiovascular diseases [29–31]. Although various studies have been conducted to introduce foreign genes into the genome of the lettuce, little or no attention has been given to enhance its intrinsic secondary metabolites for improved medicinal properties.

The aim of the present study was to enhance the secondary metabolites of *L. sativa* L. (cv. Grand Rapids), particularly antioxidants such as phenolics and flavonoids by the expression of *rol ABC* genes. *Agrobacterium*-mediated transformation was confirmed by polymerase chain reaction (PCR). Transformation resulted in the enhancement of secondary metabolites of lettuce. In vitro and in vivo analyses of lettuce extracts showed a significant increase in antioxidant, analgesic, and anti-inflammatory activities as compared to untransformed wild-type (WT) lettuce.

Methods

Bacterial Strain and Plasmid

Agrobacterium tumefaciens strain GV3101 containing plasmid pPCV002-ABC kindly provided by Dr. A. Spena (Max-Planck-Institute fur Zuchtungsforschung, 5000 Koin 30, FRG) [32] was used for transformation. The T-DNA region of the plasmid pPCV002-ABC contained the coding sequence of *rol ABC* gene under the control of native promoter-terminator (Supplementary Fig. 1). This strain was grown overnight in Luria broth medium. After inoculation, bacterial cultures were maintained at 28 °C and 120 rpm in a shaking incubator. Bacterial cells were harvested by centrifugation at 3500 rpm for 10 min, and pellets were resuspended in LB medium to a density of about 5×10^{10} CFU/ml.

Transformation and Regeneration

The seeds of *L. sativa* L. (cv. Grand Rapids) were purchased from a seed store in Rawalpindi, Pakistan. The surface sterilization of seeds was performed with 70 % ethanol for 1 min and 10 % sodium hypochlorite for 45 s. Sterilized seeds were germinated on ½ MS medium [33]. For transformation, 15-day-old grown plants were used. Cotyledonary nodes and internode explants were prepared and pre-cultured for 2 days on shooting media [34]. Afterwards, explants were infected with bacterial strain containing the desired construct for 5 min, dried on an autoclaved filter paper for 2–3 min, and then placed on MS shooting media supplemented with 200 μM acetosyringone. After 2 days of incubation in the dark at 28 °C, explants were washed with antibiotics and placed on selection medium (containing 0.1 mg/L NAA, 0.5 mg/L BAP, 50 mg/L kanamycin, and 300 mg/L cefotaxime). Explants were subcultured to fresh selection media after every 14 days. Regeneration occurred within 1 month, and after 3 to 4 cycles of selection, completely transformed plants were regenerated on selection medium. After transformation, regenerated plantlets were used directly for analysis.

Molecular Analysis by PCR

DNA of transformed and untransformed WT plants was extracted by CTAB method [35]. Plasmid DNA from GV3101, to be used as positive control, was isolated by alkaline lysis method. PCR analysis was carried out using a programmed DNA thermal cycler (Biometra, USA). PCR

amplification conditions were followed as reported previously [36]. The details of primer sequences and conditions of PCR amplification of desired genes are presented in Supplementary Table 1.

Expression Analysis of *rol* Genes

Semi-quantitative reverse transcriptase PCR was performed to analyze expression of *rol A*, *rol B*, and *rol C* genes by RevertAid M-MuLV (Moloney murine leukemia virus) reverse transcriptase kit (Thermo Scientific no. K1622), according to the manufacturer's instructions. Variation found in the *rol* gene transcripts was further confirmed by real-time qPCR which also confirmed the transgene copy number. RT-qPCR was performed according to previously reported method [37]. The amplification reaction was performed by gene-specific primers of *rol A*, *rol B*, and *rol C* genes. The β-actin gene was used as internal control [38]. The RT-qPCR reactions were run in triplicate with mean values and standard deviation calculated for all cycle thresholds (Ct). For each gene, the relative expression levels were normalized with respect to the housekeeping gene β-actin.

Preparation of the Extract

Biomass was extracted from 8-week-old regenerated untransformed (WT) and transformed aerial parts of *L. sativa* to perform antioxidant assays. Both types of plants were regenerated from internodes and were of the same age to make the comparison. One gram of dried material of each type was extracted with 20 ml methanol at room temperature in a sonication bath (Kerry Ultrasonic, UK) for 1 h separately. The extracts were filtered using Whatman no. 1 filter paper and concentrated in vacuo to generate the crude extracts. For experiments, extracts were prepared as 50 mg/ml in distilled water.

Measurement of Total Phenolic Contents

Total phenolic contents (TPC) were measured according to the reported method [8] with some modifications, by using Folin-Ciocalteu reagent. In the experiment, 4 µl of each extract (50 mg/ml in distilled water) was transferred into each well of 96-well plates and 98 µl of Folin-Ciocalteu reagent diluted with distilled water (10-fold) was added. The resulting solution was mixed well and kept at 25 °C for 5 min, and then 98 µl of 6 % Na_2CO_3 was added. The resulting reaction mixture was kept at 25 °C for 90 min, and the absorbance was measured at 725 nm using a micro-plate reader. The TPC were expressed as gallic acid equivalents. Distilled water was used as negative control.

Measurement of Total Flavonoid Contents

For total flavonoid content estimation, aluminum chloride colorimetric method was followed [39] with some modifications. Four microliters of samples (50 mg/ml in distilled water) was independently mixed with 10 µl of 10 % $AlCl_3$, 10 µl of 1 M potassium acetate, and 176 µl of distilled water in each well of 96-well plates. This reaction mixture was kept at 25 °C for 30 min. Absorbance of the reaction mixture was measured at 405 nm. The total flavonoid contents (TFC) were expressed as quercetin equivalents, and distilled water was used as negative control.

Measurement of Total Antioxidant Capacity

Total antioxidant capacity (TAC) was measured by phosphomolybdenum method described by Phatak and Hendre [40]. In the reaction mixture, 4 µl (50 mg/ml in distilled water) of extract was added with 196 µl of reagent solutions (28 mM sodium phosphate, 4 mM ammonium molybdate, and 0.6 M sulfuric acid). Then, the mixture was incubated for 90 min at 95 °C in a water bath. Absorbance was taken at 630 nm after cooling the reaction mixture at room temperature. In blank reaction, 4 µl of distilled water was used instead of extract. TAC of each extract was expressed as ascorbic acid equivalent.

Measurement of Total Reducing Power

Total reducing power (TRP) was measured by the method described by Moein et al. [39]. The TRP of the extracts was determined by using 20 µl (50 mg/ml in distilled water) of extract mixed with 490 µl of 0.2 M of phosphate buffer and 490 µl of 1 % potassium ferricyanide. The reaction was incubated for 20 min at 50 °C. Then, 500 µl of 10 % trichloroacetic acid was added to the reaction mixture; the mixture was then centrifuged for 10 min at 3000 rpm. After taking 500 µl of the upper layer of the mixture in new Eppendorf tube, 100 µl of 0.1 % ferric cyanide was added. Absorbance was measured at 630 nm. One hundred microliters of distilled water was used to make the instrument blank. TRP of each sample was expressed as ascorbic acid equivalent.

2,2-Diphenyl-1-Picryl-Hydrazyl Free Radical Scavenging Assay

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging capacity of the extracts was measured by using the method reported earlier [8]. DPPH solution (316 µM) was prepared in methanol. Two microliters of the test sample and 98 µl of DPPH solution were added in each well of 96-well plates. It was then incubated for 1 h at 37 °C. Using a micro-plate reader, absorbance was measured at 515 nm. Each extract was tested at the concentrations of 1000, 500, 250, and 125 µg/ml. Ascorbic acid and distilled water were used as positive and negative controls, respectively. Percentage scavenging was calculated by Eq. (1), and IC_{50} values were determined by TableCurve software.

$$\text{Percentage scavenging} = [1\text{-absorbance of extract/absorbance of control}] \times 100 \quad (1)$$

Lipid Peroxidation Assay

The lipid peroxidation activity was evaluated by using previously reported method [41]. Lipid peroxidation was induced in liposomes prepared from egg lecithin through ultrasonic irradiation by adding 490 µl of 400 mM $FeCl_3$ and 490 µl of 200 mM ascorbic acid. In this mixture, 20 µl of the each sample was added with the final concentrations of 1000, 500, and 250 µg/ml. Distilled water was used as negative control. The samples were incubated for 60 min at 37 °C. Then, 1 ml of stopping solution comprising 0.375 % (w/v) thiobarbituric acid, 1.5 % (v/v) trichloroacetic acid, and 0.25 M HCl was added in the mixture to inhibit the reaction. These mixtures were placed in a boiling water bath for 15 min, cooled, and centrifuged at 4000 rpm. Then, 200 µl of the resulting solution was picked and added in each well of 96-well plates. Absorbance was measured at 532 nm. The percentage inhibition was calculated by Eq. (2), and IC_{50} value was determined with TableCurve software.

$$\text{Percentage inhibition} = [\text{Ac-As/Ac}] \times 100 \qquad (2)$$

where Ac means absorbance of the control and As means absorbance of the test sample.

DNA Damage Assay

DNA protection activity of the extracts was measured in vitro by using the method as previously reported [42, 43]. The reaction mixture was prepared in a PCR tube with total volume of 15 μl, having 3 μl pBR322 plasmid DNA (0.5 μg), 4 μl of 30 % H_2O_2, 3 μl of 2 mM $FeSO_4$, and 5 μl of plant extracts at final concentrations of 10, 100, and 1000 μg/ml. A positive control was used which contained pBR322 DNA treated with 2 mM $FeSO_4$ + 30 % H_2O_2, and untreated pBR322 DNA was used as negative control. Extract and pBR322 were also used as a control to check the natural damaging or protective effect on DNA. Then, the mixture was incubated for 1 h at 37 °C. All reaction mixtures were subjected to 1 % agarose gel electrophoresis in 1× TBE buffer with a 1-kb ladder. In each well, 10 μl of reaction mixture was loaded. Gels were analyzed by scanning with Gel Doc (Bio-Rad) computer program. Thickness and intensity of bands were visually compared with positive and negative controls for evaluation of DNA protection.

Animals and Treatment Groups

Albino rats of either sex between 150 and 200 g of weight were used in the experiments. Rats were kept in standard aluminum cages and bred with water ad libitum and standard diet in the primate facility of the Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. The study design was approved by the Institutional Animal Ethics Committee, and all provisions were carried to minimize animal sufferings. The experiments were performed at the same time each day, and rats were fasted overnight prior to testing to avoid the variability. Five rats were used per treatment. Extracts and standard drugs were administered orally with the concentrations of 500 and 10 mg/kg of the rat body weight, respectively, to each group.

Hot Plate Analgesic Assay

Analgesic activity was determined by hot plate method which is based on stimulation of pain by heat and was first reported by Eddy and Leimbach [44]. Prior to oral gavage, rats were placed on a hot plate at 55 ± 2 °C to determine the jumping and paw licking response which was noted down as initial reaction time (It). After 30 min of dosage, each rat was placed on the hot plate (55 ± 2 °C) and the basal response time was recorded by observing paw licking and jumping reaction (whichever seemed first) which was taken as final point (Ft). The reaction time in seconds was noted down at the intervals of 0.5, 1, and 2 h after dosage with a cutoff period of 30 s. Percentage analgesic activity was calculated by Eq. (3).

$$\%\text{activity} = \left[\left(\frac{\text{Ft−It}}{\text{It}} \right) \times 100 \right] \qquad (3)$$

Carrageenan-Induced Hind Paw Edema Test

Anti-inflammatory effect was evaluated by using carrageenan-induced hind paw edema test [45]. After 1 h of oral dosage, edema was induced by injecting 100 μl of carrageenan

prepared in 1 % in saline into the subplanter region of the left hind paw. The paw volume was measured quickly before and after the carrageenan injection by using a plethysmometer (UGO Basile 7140), which served as the control readings of paw. Regular interval readings (1 h each) were taken by measuring the paw volume up to 4 h. The percentage edema inhibition was determined by Eq. (4).

$$\%\text{inhibition} = \left[\left(\frac{C-S}{C} \right) \times 100 \right] \tag{4}$$

where C is edema of control rats and S is edema of treatment rats.

Forced Swimming Test

The forced swim test was used to determine the antidepressant activity of *L. sativa* as described previously [46]. One day before the experiment, rats were engaged in water containing a vertical cylinder (18 cm diameter, 40 cm height, and 15 cm water; retained at 25 °C) separately and were forced to swim. In 5–6 min, the animal became steady and stayed motionless for almost 80 % of the time. After 15 min, rats were evacuated, dried, and placed back to their cages. This whole procedure is called pre-swimming. On the experiment day, after 30 min of dosage, rats were again placed in the water-filled vertical cylinder and the camera was positioned to the side of the cylinder. Video recording was started, and the rats were placed in the cylinder. After 6 min, recording was stopped; the rats were removed from the cylinder, dried, and placed back to their cages. When the experiment was completed, all videos were observed carefully (last 4 min of a total recorded video) to estimate the total immobility time.

Capillary Tube Anticoagulant Test

Capillary tube method [2] was used to determine the anticoagulant activity of the lettuce plant extracts and exudates. In this method, tail of the rat was swiped with spirit and then was pierced by the lancet. The tail was squeezed to get more blood to be filled into a capillary tube. The time of appearance of the drop of the blood on the cut tail was noted. The capillary tubes were sealed and immersed in water bath at 37 °C. After 1 min, the tube was taken out and small pieces of the capillary tube were broken at every 10 s until a fibrin thread is seen between the two broken ends. The time interval between the appearance of the blood drop and the thread formation was the clotting time.

Statistical Analysis

The data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. Results are represented as mean ± S.D., and $p < 0.05$ was considered as significant. Percentage change of transformed lines (TransL) in comparison with untransformed plants (WT) was calculated by Eq. (5).

$$\text{Percentage change} = \left(\frac{\text{TransL}-\text{WT}}{\text{WT}} \right) \times 100 \tag{5}$$

Results

Transformation and Regeneration of Lettuce Plants

Lactuca sativa (cv. Grand Rapids) was transformed with *A. tumefaciens* GV3101 harboring the *rol ABC* genes. Transformation experiments were carried out with 400 explants. Transformation efficiency was calculated as 50 %; however, only three transformed lines survived on selection medium till maturity. These lines were originated from cotyledonary nodal parts. This higher regeneration potential of cotyledonary nodal parts might be due to the presence of intercalary meristems distributed in leaves [47]. However, phenotypic alterations were observed in all transgenic plants. All transformed plants indicated a reduction in inflorescence and stem heights, internodal lengths, and leaf area as compared to untransformed plants (Fig. 1).

Confirmation of Transgene Integration and Expression

PCR was performed to confirm the integration of *rol* genes in the genome of *L. sativa* (cv. Grand Rapids) which showed the amplified products of 308 bp for *rol A*, 779 bp for *rol B*, 540 bp of *rol C*, and 780 bp of *nptII* gene as shown in Fig. 2a–h. Respective amplified products were also obtained from plasmid DNA of GV3101-*ABC*. No amplification was obtained with untransformed WT plant samples. It is evident from Fig. 2 that amplified products were of exact sizes and no non-specific bands were obtained in PCR. RT-qPCR confirmed the presence of *rol* gene transcripts in all transgenic plants (Fig. 2). For each

Fig. 1 Transformed plants of *Lactuca sativa* L. **a** Transformants of *rol ABC* genes, **b** callus of *rol ABC*-transformed explants, **c** untransformed plant, and **d** transformed plant
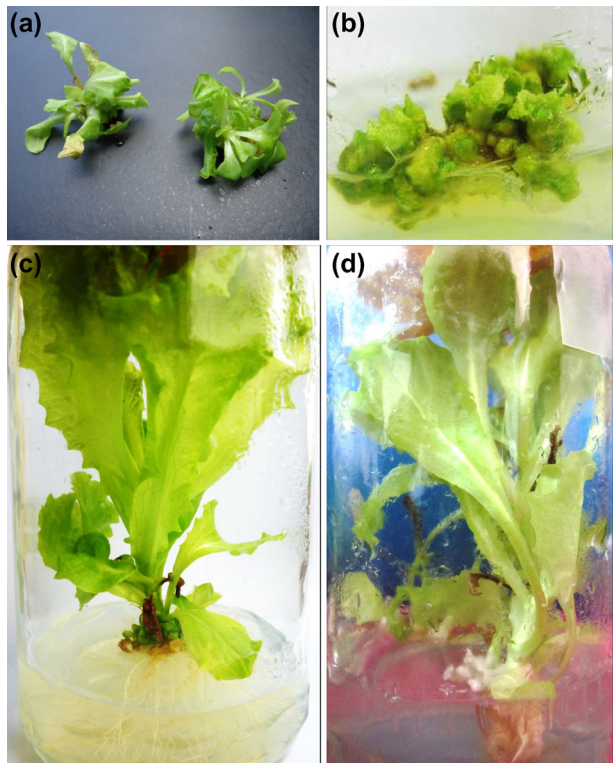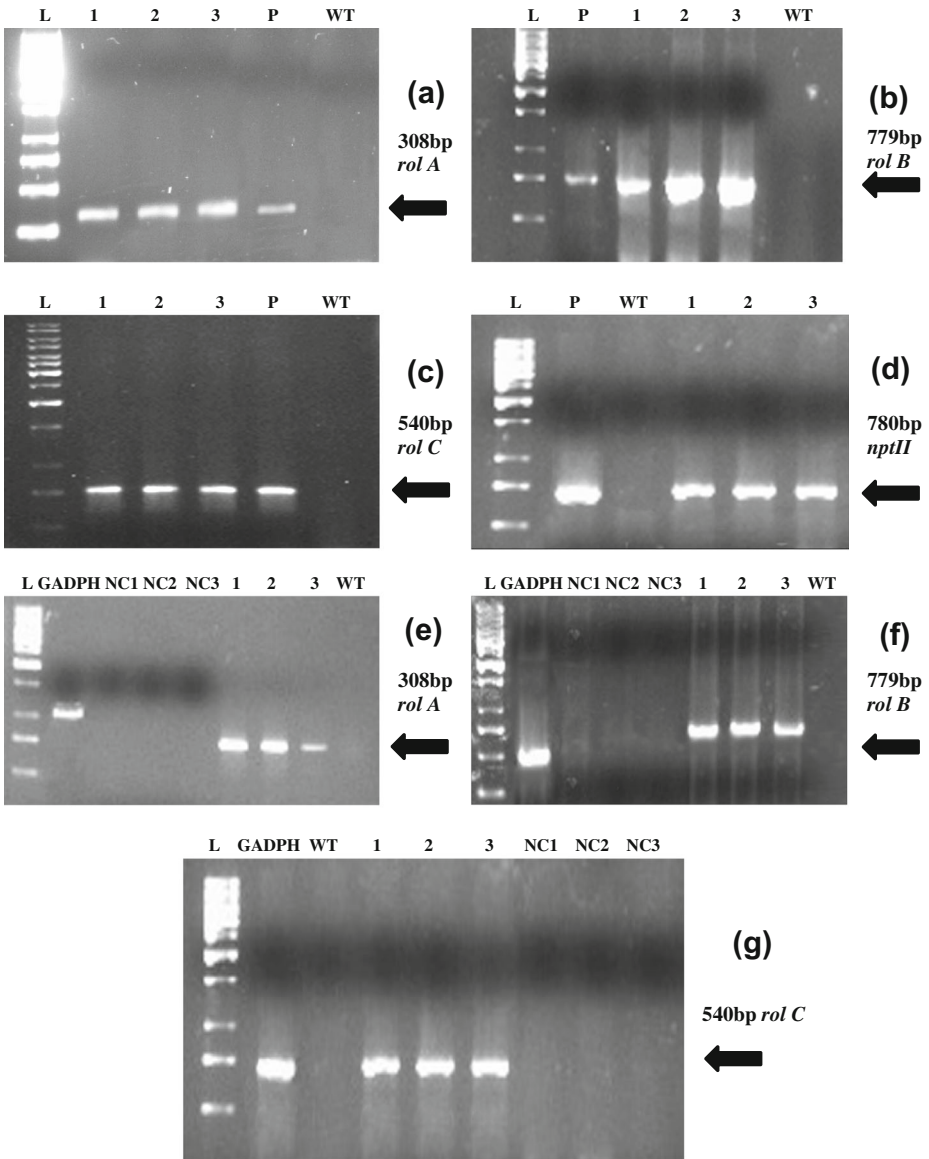
Fig. 2 PCR-amplified products. **a** *rol A* gene, **b** *rol B* gene, **c** *rol C* gene, and **d** *nptII* gene. Semi-quantitative RT-PCR showing the relative expression of **e** *rol A* gene (transgenic lines *1–3*), **f** *rol B* gene (transgenic lines *1–3*), and **g** *rol C* gene (transgenic lines *1–3*). *WT* stands for wild-type untransformed plant of *Lactuca sativa* L. *Lane P* refers to the plasmid DNA, and *lane L* corresponds to the 1-kb ladder DNA (Fermentas). *NC* is reaction for each of the RNA samples without reverse transcription of the respective gene, and GADPH (496 bp) was used as positive control for the semi-quantitative RT-PCR

reaction, RNA sample without reverse transcription was used as negative control (NC) and β-actin was used as the housekeeping gene. The results exhibited that the transcripts of *rol A* gene in each line were not uniform; the transgenic lines *rol ABC1* and *rol ABC2* both showed higher levels of transcripts of *rol A* gene as compared to *rol ABC3* line. The *rol B* gene also

showed the same pattern. In comparison, in three transgenic lines of *rol ABC*, similar levels of transcripts of *rol C* gene were obtained (Figs. 2 and 3).

Total Phenolic Contents and Total Flavonoid Contents

A comparison of TPC and TFC in untransformed and transformed plants of *L. sativa* (cv. Grand Rapids) is shown in Fig 4a. TPC were expressed as milligrams of gallic acid equivalent per gram of dry weight. The results showed that there was a 102.5, 100.4, and 90.9 % increase in TPC in transgenic lines of *rol ABC1*, *rol ABC2*, and *rol ABC3*, respectively, in comparison to untransformed extracts. On the other hand, TFC were expressed as milligrams of quercetin equivalent per gram of dry weight. All three transgenic lines showed almost the same level of enhancement with a maximum increase of 65.4 % for *rol ABC1* followed by 64.7 % for *rol ABC2* and 53.1 % for *rol ABC3* as compared to untransformed plants. Results showed a good correlation between TPC, TFC, and the antioxidant capacity of the respective transformed plants.

Total Antioxidant Capacity and Total Reducing Power

TAC of *L. sativa* (cv. Grand Rapids) extracts was determined spectrophotometrically through phosphomolybdenum method. The extracts of *rol ABC1* transformants showed 112.6 % increase, *rol ABC2* showed 100.1 % increase, and *rol ABC3* exhibited 73.9 % increase in TPC as compared to untransformed plants. On the other hand, TRP was determined by potassium ferricyanide method. All extracts showed good TRP activity which is represented as the number of equivalents per gram of dry weight of ascorbic acid (Fig 4b). When TRP was compared, 133.2, 98.0, and 61.5 % increase in reducing power were measured for the transformed plants of *rol ABC1*, *rol ABC2*, and *rol ABC3*, respectively.
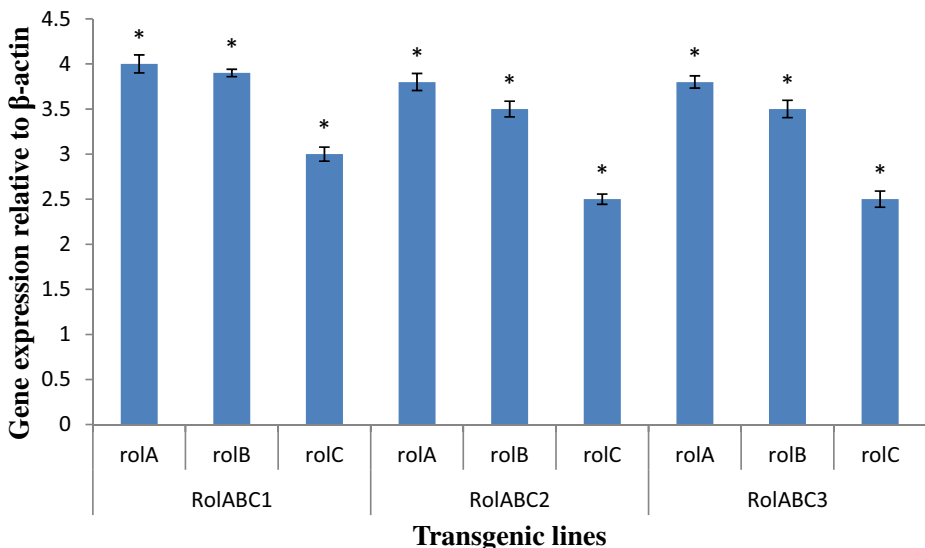

Fig. 3 Expression level of *rol* genes through real-time qPCR with respect to constitutively expressed β-actin. Values are expressed in mean ± S.D. *$p < 0.05$ statistically significant

DPPH Free Radical Scavenging Assay

The screening of the lettuce extracts using the DPPH free radical method proved to be effective for the determination of comparative enhancement of antioxidant potential in the transformed lines of *rol ABC* genes. Ascorbic acid served as positive control (IC_{50} 0.01 mg/ml). Significant DPPH radical scavenger activity was evident for all transformed extracts than for untransformed plants (Fig 5a). The extracts of *rol ABC1*-transformed plants showed the highest DPPH radical scavenging capacity with an IC_{50} value of 0.21 mg/ml which is 50.8 % more compared to untransformed plants with an IC_{50} of
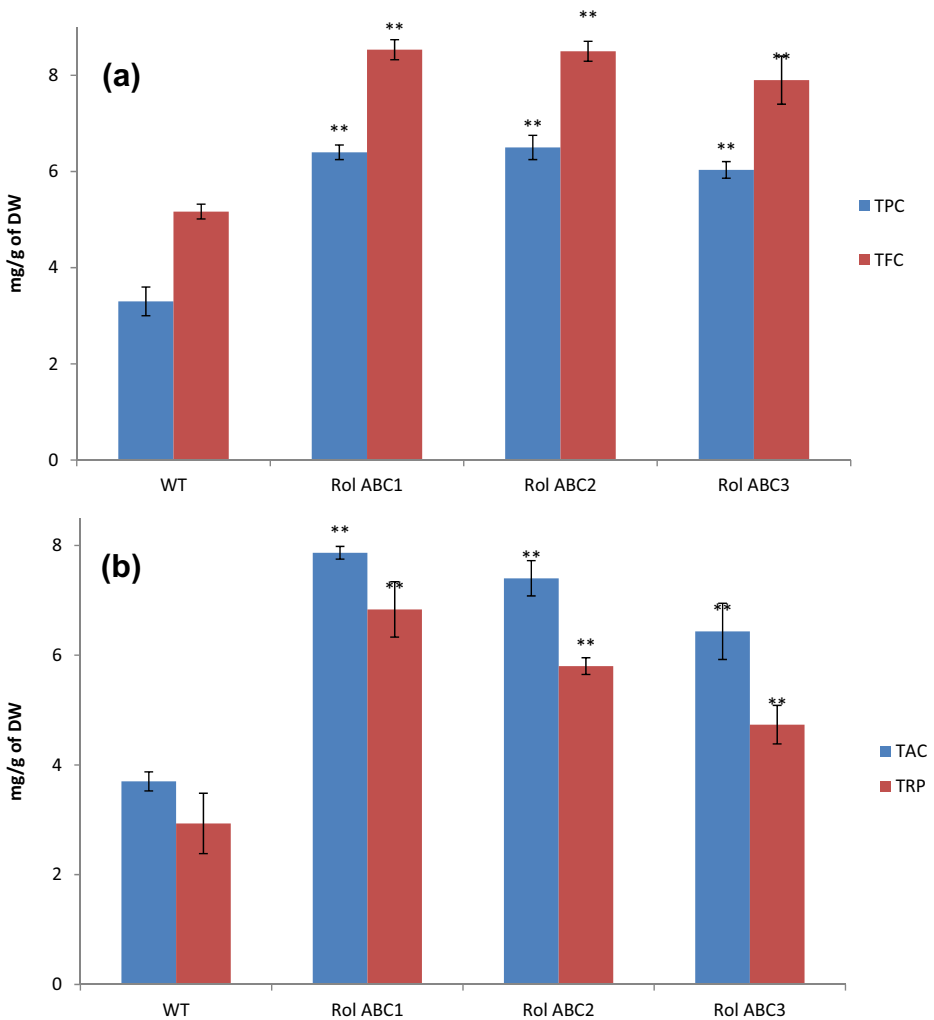
Fig. 4 Results of phytochemical analysis and antioxidant determination of *Lactuca sativa* L. **a** Represents total phenolic contents (TPC) and total flavonoid contents (TFC) while **b** represents total antioxidant capacity (TAC) and total reducing power (TRP), where *Rol ABC* means transgenic lines (*1–3*) of *rol ABC* and *WT* means wild-type (untransformed) plant. Values are expressed in mean ± S.D., in milligrams per gram of dry weight (DW). **$p < 0.01$ statistically significant

0.43 mg/ml. The extracts of *rol ABC2* and *rol ABC3* transformants showed 98.0 and 61.5 % increase as compared to extracts of untransformed plants (Fig 5a).

Lipid Peroxidation Assay

Lettuce extracts were tested for the inhibition of lipid peroxidation by the thiobarbituric acid assay. Vitamin E was used as a positive control (IC_{50} 0.003 mg/ml). The extracts of *rol ABC1*, *rol ABC2*, *rol ABC3*, and untransformed plants showed antioxidant activity with IC_{50} 0.52, 0.52, 0.64, and 0.65 mg/ml, respectively (Fig 5b). Only 20 % increase in activity was observed in transformed plants of *rol ABC1* and *rol ABC2*, while in the case of *rol ABC3*, the increase was only 5 % as compared with untransformed plants.
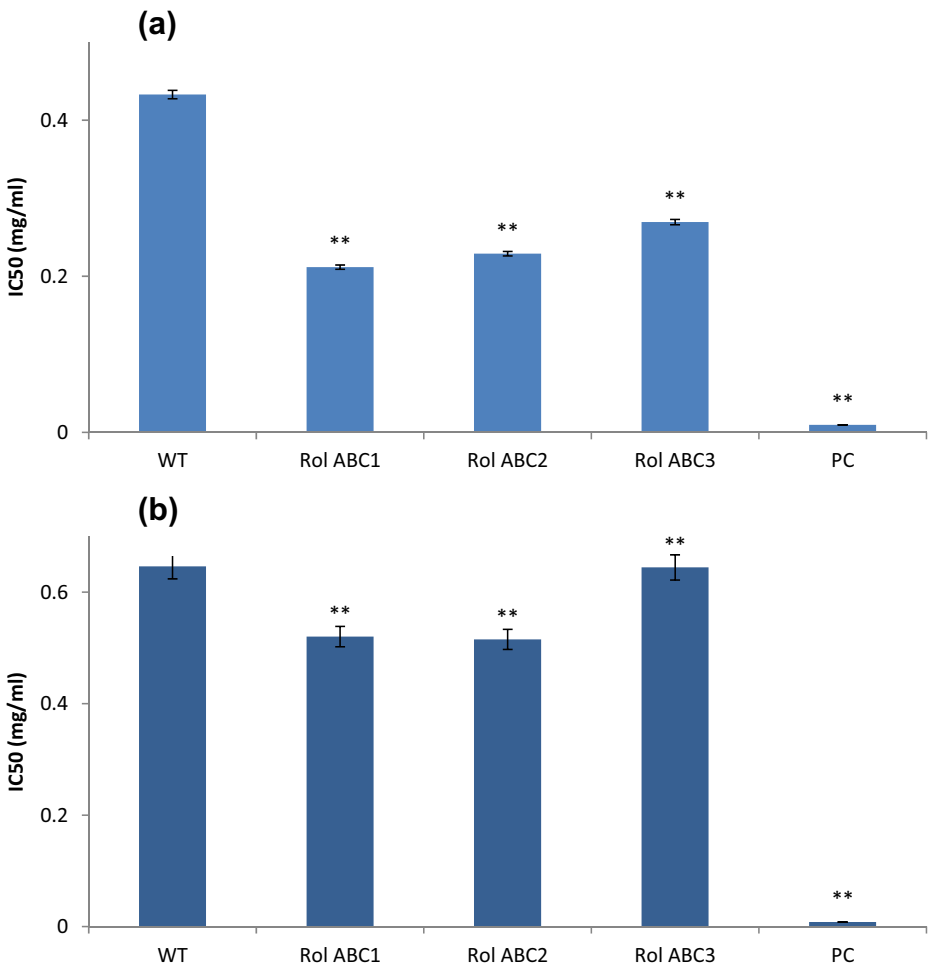

Fig. 5 Results of antioxidant activities of *Lactuca sativa* L. The figure represents the results of **a** DPPH and **b** antilipid peroxidation assay (LPA), where *Rol ABC* means transgenic lines (*1–3*) of *rol ABC*, *WT* means wild-type (untransformed) plant, and *PC* means positive control which is ascorbic acid in the case of DPPH and vitamin E in the case of LPA, respectively. Values are expressed in mean ± S.D. **p < 0.01 statistically significant

DNA Damage Assay

The scavenging effect of untransformed and transformed extracts of lettuce plants was further evaluated by DNA damage assay by examining the intensity of bands formed on 1 % agarose gel. Results are shown in Fig 6a, b. All the transformed extracts exhibited significantly high protection at 1000 and 100 μg/ml concentrations, while good protection was prominent at concentration of 10 μg/ml. The retention of the relaxed form of DNA with more intense bands in transgenic lines compared to controls indicated that there was a significant increase in DNA protection activity.

Analgesic Assay

In hot plate assay on the extracts of *L. sativa* (CV. Grand Rapid)-transformed *rol ABC* (transgenic lines 1–3), genes showed significant enhancement in analgesic activity. Percentage inhibition (Fig 7a) was calculated which showed a gradient increase in activity up to 1 h of treatment followed by a decrease in activity at 2 h. Saline and aspirin were used as negative and positive controls which showed 21.2 and 95.1 % activity, respectively. The untransformed extract (WT) showed 47 % analgesic activity. The highest activity was displayed by *rol ABC1* with 85.8 % reduction in analgesia which is about 82.5 % higher than that of untransformed plants. In the case of *rol ABC2* and *rol ABC3*, extracts showed 54.5 and 47.1 % increase in analgesic activity, respectively, in comparison to untransformed extracts.

Anti-Inflammatory Study

In addition to analgesic activity of extracts, the anti-inflammatory effect of transformed plants was also evaluated. The change in percentage inhibition (Fig 7b) of edema was
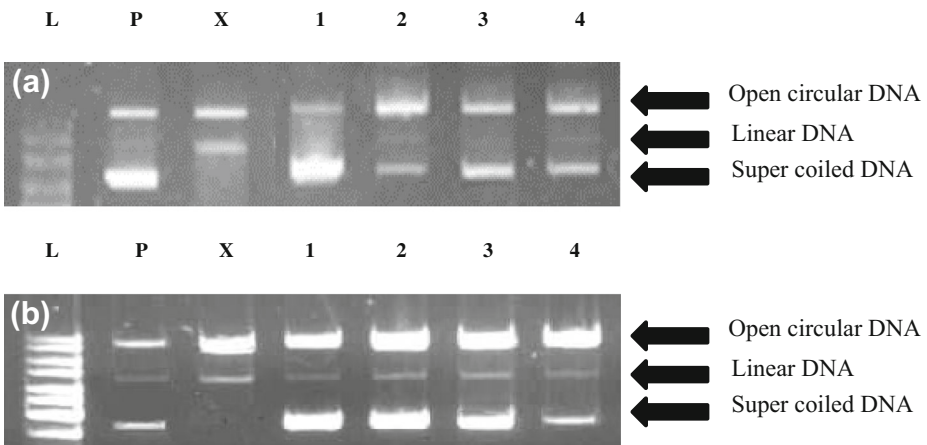

Fig. 6 Results of DNA damage assay. **a** Effect of the extracts of untransformed plants on pBR322 plasmid DNA. *L* DNA ladder (1 kb), *P* pBR322 plasmid, *X* pBR322 plasmid treated with $FeSO_4$ and H_2O_2 (positive control), *lane 1* pBR322 plasmid + 1000 μg/ml of extract (control for the pro-oxidant effect of the extract on DNA), *lane 2* plasmid + 1000 μg/ml of extract + $FeSO_4$ + H_2O_2, *lane 3* plasmid + 100 μg/ml of the extract + $FeSO_4$ + H_2O_2, *lane 4* plasmid + 10 μg/ml of the extract + $FeSO_4$ + H_2O_2. **b** Effect of plant extract of *rol ABC1* on pBR322 plasmid DNA. *L* DNA ladder (1 kb), *P* pBR322 plasmid, *X* pBR322 plasmid treated with $FeSO_4$ and H_2O_2 (positive control), *lane 1* pBR322 plasmid + 1000 μg/ml of *rol ABC1* (control for the pro-oxidant effect of the plant extract on DNA), *lane 2* plasmid + 1000 μg/ml of *rol ABC1* + $FeSO_4$ + H_2O_2, *lane 3* plasmid + 100 μg/ml of *rol ABC1* + $FeSO_4$ + H_2O_2, *lane 4* plasmid + 10 μg/ml of *rol ABC1* + $FeSO_4$ + H_2O_2
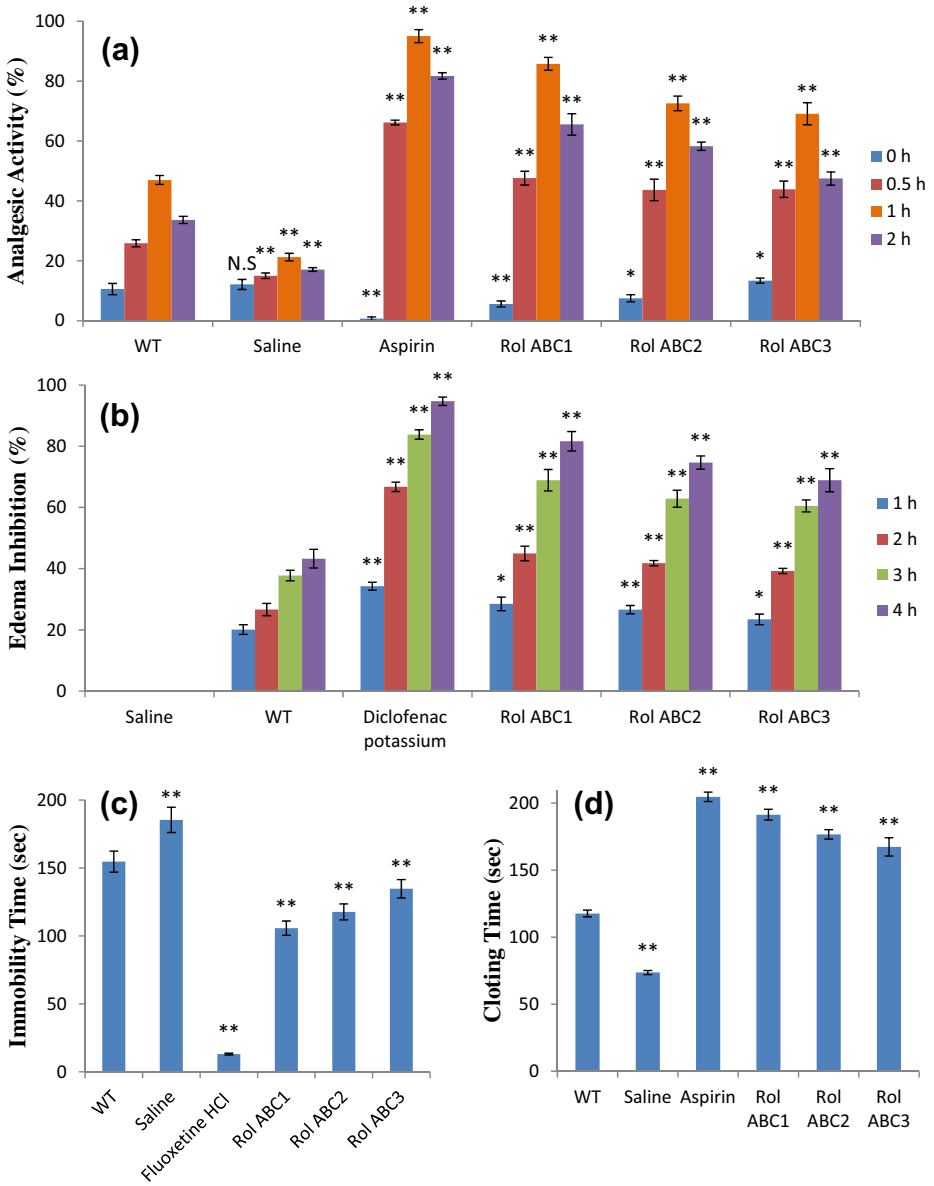
Fig. 7 Results of in vivo assay of *Lactuca sativa* extracts. The figure represents the results of **a** analgesic, **b** anti-inflammatory, **c** antidepressant, and **d** anticoagulant activity, where *Rol ABC* means transgenic lines (*1–3*) of *rol ABC* and *WT* means wild-type (untransformed) plant. Every group ($n = 5$) is expressed in mean ± S.D. *$p < 0.05$, **$p < 0.01$ statistically significant

determined after the injection of carrageenan at 1, 2, 3, and 4 h. A gradient increase in activity was monitored along with time maximum at 4 h. Diclofenac potassium and saline served as positive and negative controls with percentage inhibition of 94.7 and 0 %, respectively. The maximum activity (81.6 %) was exhibited by extracts of *rol ABC1*

as compared with untransformed extracts (43.3 %). The extracts of *rol ABC2* and *rol ABC3* showed 66.3 and 60.1 % reduction in edema, respectively.

Antidepressant Study

The potential antidepressant effect of *L. sativa* transformed with *rol ABC* genes after oral administration was studied by the forced swimming test, and results are presented in Fig. 7c. In this experiment, the duration of immobility time in comparison with saline (negative control; 183 ± 4.5 s) and fluoxetine HCl (positive control; 14 ± 0.5 s) were evaluated. Results showed that a slight increase in activity was exhibited by different transgenic lines with a percentage increase of 31.4, 23.6, and 12.5 % for *rol ABC1*, *rol ABC2*, and *rol ABC3* lines, respectively, when compared with untransformed plants (Fig. 7c).

Anticoagulant Study

Anticoagulant properties of extracts of transformed lettuce plants were also investigated. The transgenic line *rol ABC1* exhibited the highest anticoagulant potential with the clotting time of 191 ± 3.4 s as compared with untransformed extracts (117 ± 2.8 s; Fig. 7d). Aspirin and saline were used as positive and negative controls, with the clotting time of 205 ± 4.4 and 73 ± 3.1 s, respectively. Overall, the *rol ABC1*, *rol ABC2*, and *rol ABC3* transgenic lines displayed anticoagulant activities with 62.7, 50.2, and 42.3 % increase, respectively, as compared to untransformed lettuce.

Discussion

Previously, a number of studies have been done on the transformation of lettuce using *Agrobacterium rhizogenes*. Here, we have developed an efficient protocol for the *A. tumefaciens*-mediated transformation of lettuce cv. Grand Rapids. The transformation resulted in a significant increase of antioxidants such as phenolics and flavonoids. Different in vitro and in vivo assays showed that transformed lettuce extracts had more antioxidant, analgesic, anti-inflammatory, antidepressant, and anticoagulant properties as compared to untransformed lettuce. To the best of our knowledge, this is the first report of lettuce transformation with *rol* ABC genes and for enhancement of antioxidants.

In the present study, *A. tumefaciens* harboring *rol ABC* genes was used for the transformation of lettuce, which was confirmed by PCR. The expression of *rol* genes was studied by semi-quantitative reverse transcriptase and real-time PCR [48]. The amplification of transcripts with desired fragment sizes was observed in all samples of transgenic lines. After transformation, some morphological differences were observed in transgenic plants in comparison to untransformed plants. These changes were identical in all regenerated transformed lines, which shows that the changes induced were due to transformation and not because of somaclonal variation. Previously, a similar effect has been observed by Curtis et al., who showed that the transformation of lettuce with *rol AB* resulted in adverse phenotypic effects such as shorter stems and internodal parts, reduction in inflorescence and leaf area, leaf wrinkling, and lower pollen viability [49]. The effect was also shown to appear in subsequent generation. In general, *rol* genes have been shown to affect the morphology of different plants as reviewed by Casanova et al. [50].

Transgenic lines of *rol ABC* were investigated for their TPC, TFC, TRP, TAC, and antioxidant activity by using DPPH, lipid peroxidation, and DNA damage assay. Our results show that transformation with *rol* genes had an enhancing effect on phytochemicals such as antioxidant as compared to untransformed plants. The results of TPC, TFC, TAC, and TRP in these transformed plants presented a statistical significance between higher antioxidant activities and enhanced secondary metabolites including phenolics and flavonoids. Moreover, investigation of the relationship among different metabolites showed a strong positive correlation with TPC and TFC [51]. The phytochemical studies on this plant have already reported that it contains a number of flavonoids [7, 52]. Flavonoids are known to have free radical inhibitor effect and scavengers of lipid peroxidation [53]. The phenolic compounds in general and the flavonoid compounds in particular are considered to be the vital antioxidants which show scavenging activity on the free radicals [28, 54]. The overall results showed that transformed plants were rich in radical scavengers such as flavonoids and phenolic acid derivatives as compared to untransformed plants. It can be suggested that the DPPH scavenging potential of the plant is related to the presence of phenolic compounds [55, 56]. On the other hand, lipid peroxidation is triggered by the production of free radicals from numerous sources containing organic hydroperoxides and iron-containing and redox-cycling compounds. The transformed extracts showed significant enhancement in activity as compared to untransformed plants. Previous studies have shown that extracts of lettuce revealed prominent oxygen radical scavenging capacity [7] and inhibition of lipid oxidation in lower-density lipoproteins [57]. DNA damaging activity is based on the ability of extracts to protect the attack of •OH produced from the Fenton reaction which breaks supercoiled plasmid DNA into open circular or linear forms [58]. In our results, transformed plants showed up to 50 % increase in the protection of DNA than the untransformed plants. The protective effect of extract on DNA may be due to the presence of phenolics and flavonoids which can prevent the production of reactive oxygen species (ROS) by complexing cations that participate in hydroxyl radical formation [59].

The hot-plate tests are useful in elucidating centrally mediated antinociceptive responses, which focus mainly on changes above the spinal cord level [60], possibly acting on a descending inhibitory pain pathway [61]. Additionally, it had several advantages, particularly the sensitivity to strong analgesics and limited tissue damage. It is interesting to note that the results of analgesic and anti-inflammatory activity highly correlate with each other which suggest their strong positive correlation between these two properties. *Lactuca sativa* has been reported to contain triterpenoids which are well known for their anti-inflammatory properties [62]. It is possible that transformation had some effects on the production of triterpenoids which may have in turn enhanced analgesic and anti-inflammatory potential. However, exact mechanisms need to be further investigated. Additionally, antidepressant and anticoagulant activities were also evaluated for the all the transgenic lines. The results showed a slight increase in antidepressant activity while the enhancement in anticoagulant activity was quite good with 42–62 % increase in different transgenic lines. The results showed delay in formation of hemostatic plug, which suggests that the plant has an effect on coagulation system [63]. The *rol ABC1* line revealed a significant reduction in the clotting time which reflects that there was an enhancement in one or more of the clotting factors involved in the intrinsic pathway which eventually leads to supplementary clot formation [64].

Conclusion

Transformation of *L. sativa* L. (cv. Grand Rapids) with *rol ABC* genes has shown that the antioxidant, analgesic, and anti-inflammatory activities of transgenic lines were significantly enhanced with the increased levels of total phenolics and flavonoids. The transgenic lines which produced more phenolics and flavonoids also possessed higher activities, while these activities remained lower for the lines which showed comparatively less increase in their total phenolic and flavonoid contents. Overall, it can be concluded that transformation with *rol* genes can be used as tool to enhance the level of useful secondary metabolites such as antioxidants in medicinally important plant species.

Authors' Contributions All authors contributed to the work. BM conceived the study design and supervised the study. MTW contributed in the study design and transformation. HI and ED conducted transformation and assays. All authors drafted the manuscript and approved the final version.

Compliance with Ethical Standards The study design was approved by the Institutional Animal Ethics Committee, and all provisions were carried to minimize animal sufferings.

Competing Interests The scholarship was provided to HI by the Higher Education Commission (HEC), Pakistan, to conduct the study. The funding body has no role in study design, collection, analysis, interpretation of data, and writing of the manuscript and in the decision to submit the manuscript for publication.

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