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Agrobacterium-Mediated Genetic Transformation of *Withania coagulans* (Dunal) with *rol* A Genes and Its Antioxidant Potential

Samiya Rehman, Amjad Hussain,* Munzer Ullah, Enas Ali, Mariusz Mojzych, Syed Ali Raza Naqvi, Akbar Ali, Meher Ali, Ehab Gomaa, Sherif S. M. Ghoneim, Bushra Mirza, Ken Keefover Ring, Hidayat Hussain, Abdur Rauf, Najeeb Ur Rehman, and Faisal Attique

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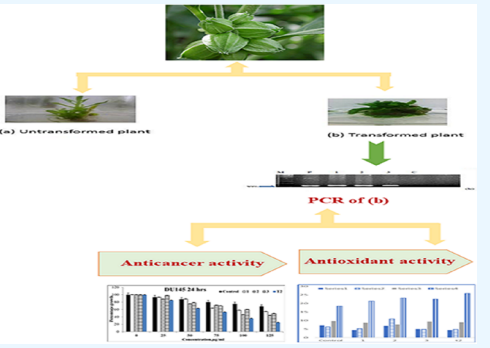
ABSTRACT: In ancient times, *Withania coagulans* Dunal was used as a therapeutic plant for the treatment of several diseases. This report aims to examine the effect of *Agrobacterium tumefactions*-mediated transformation of *W. coagulans* with the *rolA* gene to enhance secondary metabolite production, antioxidant activity, and anticancer activity of transformed tissues. Before transgenic plant production, the authors designed an efficient methodology for *in vitro* transformation. In this study, leaf explants were cultured on Murashage and Skoog (MS) media containing different amounts of naphthalene acetic acid (NAA) and benzyl adenine (BA). The best performance for inducing embryogenic callus was in MS medium containing 4 μM NAA and 6.0 μM BA, while the best results for shooting (100%) were obtained at 8 μM benzyl adenine. On the other hand, direct shooting was attained by subculturing leaves on MS medium supplemented with 8 μM benzyl adenine. Prolonged shoots showed excellent *in vitro* rooting results (80%) with 12 μM indole-3-butyric acid (IBA). The samples were precultivated for 3 days and were followed by 48 h infection with *A. tumefaciens* strain GV3101 having pCV002. Then, a vector expressed the *rol A* gene of strain *Agrobacterium rhizogenes*. Furthermore, three independent transgenic shoot lines and one callus line (T2) were produced and exhibited stable integration of transgene *rol A* genes, as revealed by PCR analysis. Transgenic strains showed a significant increase in antioxidant potential as compared to untransformed plants. Additionally, LC-MS analysis showed that the transformed strains have a higher withanolide content as compared to untransformed ones. Moreover, the reduced proliferation of prostate cancer cells was observed after treatment with extracts of transgenic plants. Furthermore, these transformed plants exhibited superior antioxidant capability and higher withanolide content than untransformed ones. In conclusion, the reported data can be used to select withanolide-rich germplasm from transformed cell cultures.



1. INTRODUCTION

Withania coagulans belongs to the *Solanaceae* family, a genus containing 23 species. Among these only two species, *W. coagulans* and *W. somnifera* are commercially and pharmaceutically substantial.[1] *W. coagulans* has commercial importance because the pulp and husk of its berries (fruits) have proficient milk coagulation characteristics and are also used in making vegetarian rennet for cheese.[2] *W. coagulans* holds a key position in the folk medicinal system from ancient times.[3] It is commonly used to treat tiredness, insomnia, consumption infirmity, and impotence,[4] fruit extracts of *W. coagulans* are known to possess significant cardioprotective and anti-inflammatory activities,[5] and its flowers are effective against diabetes.[6] *W. coagulansis is* frequently employed in conventional treatments for a broad range of diseases, such as malignancy.[7] For example, a leaf stalk extract has recently shown cytotoxic activity against breast tumor cell lines (Hela and MCF7 cell lines). Finally, a methanolic plant extract demonstrated anticancer efficacy against a renal epithelial cancer cell line MDA-MB-231[8] and aqueous extracts of *W. coagulans* exhibited antiapoptotic action against HeLa cell lines.[9]

The pharmacological activities of *W. coagulans* are mostly due to the class of naturally occurring steroidal lactones (C_{28}) called withanolides, in which C_{26} and C_{22} are oxidized to generate a six-membered lactone ring.[1] More than 40 withanolides have been isolated with coagulin L as the most abundant withanolide.[10] In addition, three anticancerous withanolides (withacoagulin G, H, and I) have also been

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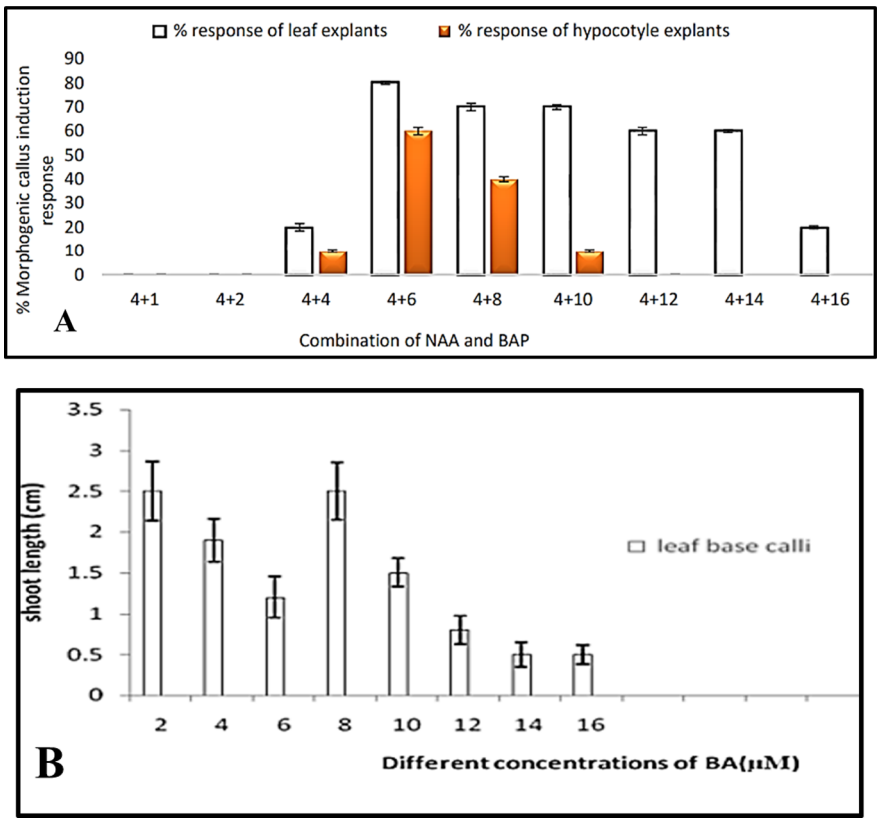

Figure 1. (A) Effect of different concentrations of NAA (μM) and BA on calli formation from leaf and hypocotyl explant. (B) Effect of different concentrations of BA (μM) on the shooting length of leaf-based calli.

reported.[11] Besides these three withanolides, several other withanolides (withaferien A, withanolide E) have been reported as anticancerous,[12] antiangiogenic,[13] anti-inflammatory[14] and cytotoxic properties.[15]

Plant-based antioxidant compounds eradicate reactive oxygen species from the human body, thereby preventing oxidative stress and as a result protecting the body from several diseases. These diseases that are produced as a result of antioxidant stress include cardiovascular diseases, cancer, and various autoimmune diseases.[16] Plant-based antioxidants help to maintain human health and preventing diseases. These plant-derived antioxidants include phenolics and flavonoids.

Currently, *Withania coagulans* are used extensively in medicine, providing advantages to human health and commercial use in the cheese-making industry. Naturally bioactive compounds (withanolides) are produced in limited quantity by the plant and are not cost-effective to produce commercially through chemical synthesis. Therapeutically important secondary metabolites, especially antioxidants like withanolides, need to be quantitatively increased in *W. coagulans* to enhance their medicinal and commercial importance. Thus, *in vitro* culture techniques offer a solution for the mass-propagation and large-scale production of its important secondary metabolites. *Agrobacterium*-mediated genetic transformation with *rol A* gene is an effective tool for the enhancement of secondary metabolites.[17] It has already been proven that *Rol A* genes increase the biosynthesis of secondary metabolites by activating the genes involved in the biosynthesis pathway of secondary metabolites.[18]

There is evidence of the transformation of *W. coagulans* by hairy roots, but there is no example of the transformation of *W. coagulans* with *Agrobacteriium tumefacians*. Other work has shown that increasing biomass was correlated with greater secondary metabolite production, such as withanolide D[19] and withaferin A[20] and withanolide, which were observed in has been reported in transformed root cultures of *W. somnifera*. Little is known about the mechanism how the *rol A* genes affect secondary metabolism but transformation with the *rol A* genes is useful to increase secondary metabolite synthesis in plants.

Populations of this medicinally and economically important plant are disappearing at alarming rates due to overcollecting, leading to this species being listed as endangered.[21] Micropropagation would be one way to help conserve this plant.[22] Additionally, the polygamodioecious nature of *W. coagulans* flowers reduces the chance of setting seed, increasing the chances of local species extinctions due to overexploitation.[23]

In the current study, the authors have determined the effect of transformation with the *rol A* gene on the antioxidant potential, withanolide content, and anticancer activity of *W. coagulans*. To date, only a few tissue culture studies on *in vitro* regeneration with less propagation response and transformation of *W. coagulans* have been reported, so the development of a more efficient micropropagation method coupled with transformation was needed. The present study has developed and optimized culture tissue and transformation methods for *W. coagulans* using genetically *W. coagulans* modified with the *rol A* gene. The withanolide profile in specified transformed lines of *W. coagulans* and control plants was compared to the anticancerous antioxidant activity of untransformed and transformed plants.

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2. RESULTS

2.1. Development of the Tissue Culture System for *Withania coagulans*. *2.1.1. Callus Induction.* Callus formation for hypocotyl and leaf explants began on different hormonal combinations in 12 to 15 days, and their callogenic response was recorded. Leaf explants had the highest embryogenic callus induction response (80%) at hormone concentrations of 4.0 μM NAA and 6.0 μM BA (Figure 1a, Table 1), whereas hypocotyl explants had the lowest (60%) at

Table 1. Callus Induction Response from Leaf Explants of *W. coagulans* Cultured on MS Medium Supplemented with NAA and BA

		Callus		
NAA:BA (μM)	CI (days)	CI (%)	mean length with SD	morphology
1:2	NA	no callus	(cm)	NA
1:4	NA	no callus	NA	NA
1:6	30	16	NA	yellow, fragile
1:8	NA	no callus	1 ± 0.1	NA
2:2	20	15	NA	yellowish green, fragile
2:4	20	40	1 ± 0.1	yellowish brown, fragile
2:6	12	30	2 ± 0.2	green, soft
2:8	30	15	1.5 ± 0.2	yellow, fragile
3:2	NA	no callus	1.5 ± 0.3	NA
3:4	NA	no callus	NA	NA
3:6	15	20	NA	yellowish green, soft
3:8	NA	no callus	2 ± 0.3	NA
4:1	NA	no callus	NA	NA
4:2	20	30	1 ± 0.4	yellowish green, soft
4:4	12	40	1 ± 0.4	dark green, soft
4:6	20	80	3.5 ± 0.5	dark green, hard, compact
4:8	20	64	5 ± 0.3	dark green, hard, compact
4:10	15	70	2.5 ± 0.3	dark green, hard, compact
4:12	15	62	2.5 ± 0.4	dark green, hard
4:14	15	60	2.5 ± 0.40.	dark green, hard

the same concentrations. At this concentration, the texture and shape of the calli were compact, green, and embryogenic. In addition, a combination of auxin and cytokinin in the MS medium strongly regulated the induction of morphogenic callus (Table 1).

2.1.2. Shoot Induction. Leaf-based green live calli were shifted to different shoot induction hormones in the MS media. Calli showed a response to selected PSI (shoot induction hormone) among different concentrations (shoot induction hormone). BA's highest multiple shoot induction activity was preferred by 8 μM benzyl aminopurine (BA) alone shown in (Figure 1).

The highest shooting response (100%) was recorded at 8 μM BA for leaf explant-based calli with a mean shoot length of 2.5 ± 0.11 cm and 30 shoots per callus (Figure 1B and 2C). Elongated shoots demonstrated *in vitro* rooting in half-intensity MS media supplemented with IBA after 3 weeks. The highest rooting efficiency (80%) and the best root number per shoot (12 roots/shoot) and root length (11.0 cm ±0.05) were achieved on 2.5 μM IBA supplemented half-intensity MS media. Figure 2C shows the shoot induction from leaf explants of the *W. coagulans* indirect trail via callogenesis.

2.1.3. Direct Shoot Formation. Explants were grown on MS media supplemented with varying doses of BA (2−16 μM) for direct shoot. The concentration of BA at 8 μM elicited the greatest reaction from the leaf explants during direct photosynthesis. Explants of the hypocotyl and leaves were used for direct shooting. In Figure 3, the 100% shoot response observed at 8.0 μM BA, with 2.5 ± 0.11 cm average shoot length and 30 shoots per leaf, comparable to the 38 shoots per explant observed in *W. somnifera* when treated with IAA (1.5 mg/L) and BAP (1.5 mg/L).[24]

Agrobacterium tumefaciens strain GV3101 with the pPCV002-A plasmid was effectively used to produce genetically modified *W. coagulans* expressing the *rol A* gene. Several infection and cocultivation factors in the transformation were found. These parameters included type of explant, bacterial infection period, acetosyringone concentration, and cocultivation duration for T-DNA transfer. Different infection periods of 10−30 min were used, and among them, an infection time of 10 min with leaf explants was the most efficient with a transformation efficiency of 44%. The most optimal concentration of 200 μM acetosyringone for 48 h gave maximum transformation efficiency of above 40%, while the cocultivation time of more than 48 h gave rise to bacterial contamination in further subculturing of transformed cultures.

2.2. Screening and Selection of Transformed Plants. Antibiotic application caused nontransformed buds to die, and only transgenic *in vitro* grown buds with the npt2 antibiotic resistance gene grew. A selection medium supplemented with 50 mg/L kanamycin was found best. The specific morphological changes associated with altered plants included curved stems, lower height, lack of true leaves, termination of apical meristem in swelling outgrowths, and elongated cotyledons. The transformed plants were intensely green in color as compared to control plants (Figure 4).

Integration of transgenes *rol A* of 304 bp and npt 11 of 781 bp was confirmed by PCR of extracted DNA from kanamycin-resistant transformed shoots (Figure 5).

The percentage of cocultivated explants that transformed based on the presence of *rol A* PCR-positive plants was 17% for leaves (Table 2). PCR yield and dilation of the 304 bp fragment of the *rol A* gene although no band was perceived in negative control plants.

2.3. Antioxidant Analysis of Transformed Plants. Simple bioassay procedures give crucial information about the bioactive components of the crude botanical extracts. In this study, methanol and chloroform extracts of four different lines of transgenic plants showed different antioxidant potentials. Levels of activities differed but all represented elevated potential (Figure 6). The transformed lines showed higher IC_{50} values, indicating less scavenging compared to the untransformed line. The abrupt behavior shows that the transformation might have intercepted the natural biological activity of the plant. Line 03 had the best free radical scavenging activity, showing the lowest IC_{50} value.

2.3.1. Free Radical Scavenging Activity. All samples showed free radical scavenging activity by converting the purple solution into a yellow one. The transformed lines

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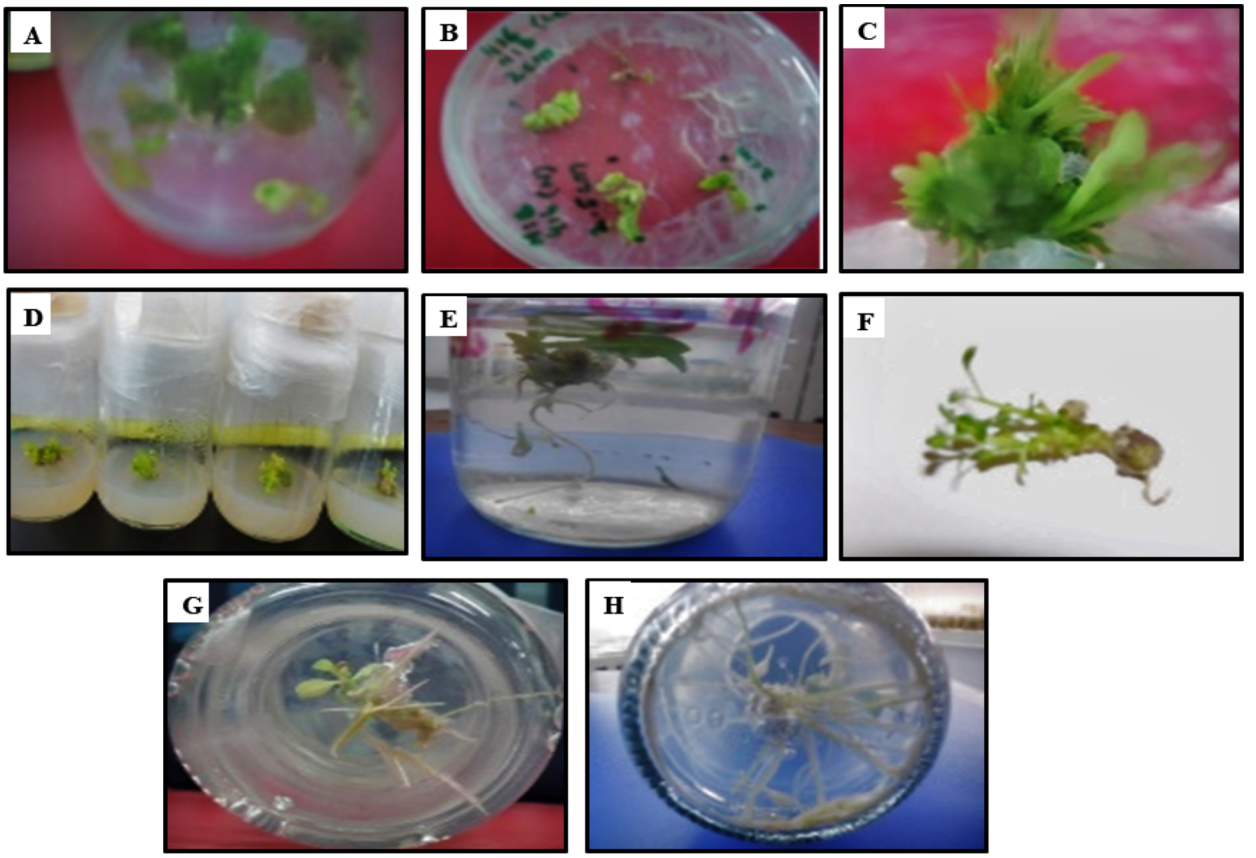

Figure 2. *W. coagulans* induces shoots from leaf explants through an indirect mechanism. (A) Callus induction, (B) embryogenic calli, (C) numerous shoot buds grow on the BA 8 (μM) callus medium. (D) Several shots on MS. (E, F) Rooting on 1/2 MS, IBA (2.5 μM). (G, H) 15 and 30 day older rooted shoot.

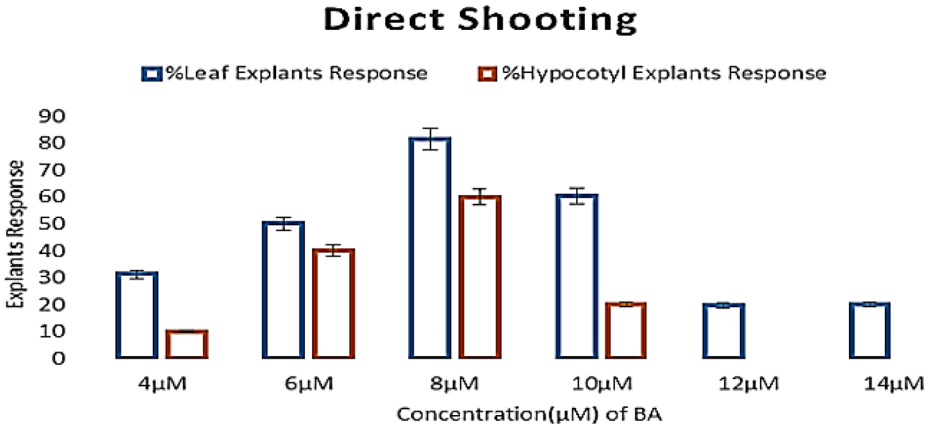

Figure 3. Direct shooting of *W. coagulans* with *Agrobacterium tumefaciens*-mediated transformation of the *rolA* gene.

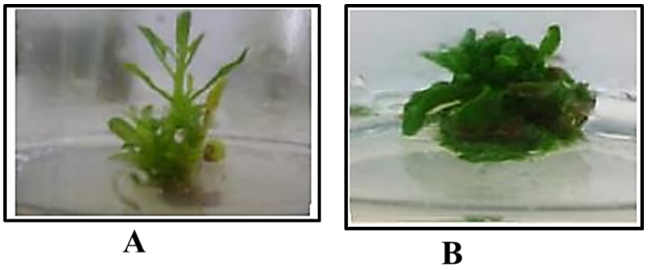

Figure 4. (A) Untransformed and (B) transformed *W. coagulans* plants

showed more IC_{50} values hence, less scavenging as compared to the untransformed line. The abrupt behavior shows that the transformation might have intercepted the natural biological activity of the plant. Line 03 also shows the best free radical scavenging activity by showing the lowest IC_{50} value. To conclude, our data suggest that *rol A* genes trigger the antioxidant potential of *W. coagulans*. Transformation of *W. coagulans* with the *rol A* gene gives a novel system for the enhanced synthesis of metabolites. Thus, the development of *rol A*-transformed *W. coagulans* offers unique opportunities for pharmacological preparations and also provides an alternative to field-cultivated plants. The study also invites tests of this

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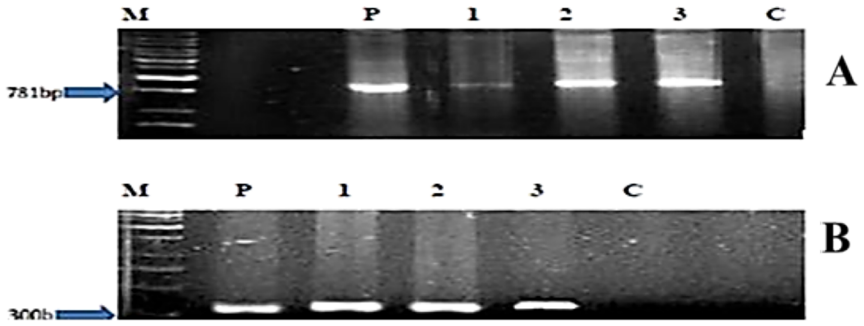

Figure 5. PCR analysis showing the amplified product of (A) npt11 and (B) *rol A* from transgenic *W. coagulans*. Lanes 1, 2, and 3 represent bands in the transgenic plants. Lane P represents the band obtained from plasmid DNA. Lane C represents the product from unsaturated plants. Lane M 1kb ladder from Sigma.

Table 2. Parameters Affecting Transformation of *Withania coagulans*

(A) Effect of type of explant on transformation efficiency			
type of explant	no. of explant used	no. of explants regenerated	transformation efficiency (%)
hypocotyl regions	150	55	36.6
leaf explants	200	64	32
calli	200	83	41.5
(B) Effect of acetosyringone concentration on transformation efficiency			
concentration of acetosyringone (μM)	no. of explants used	no. of explants regenerated	transformation efficiency (%)
100	150	42	28
200	150	67	44.66
300	150	37	24.6
(C) Effect of infection time on transformation efficiency			
infection time (min)	no of explants	no. of explants regenerated	transformation efficiency (%)
10	150	68	44
20	150	30	20
30	150	15	10

species with other *rol A* genes to check what changes in pharmacological activities they might bring.

2.4. LC-MS Analysis of Transformed Lines. *2.4.1. Identification of 12 withanolides.* To find 12 well-known and putative withanolides in *W. coagulans* extracts, LC-MS analysis was performed for selected transformed lines (Table 3). The extract named Control (untransformed) contained withanolides in lower amounts than samples 01, 02, 03, and T2. Total withanolide content was increased from μg per g of control extract to 86.94 μg per g of 03 transformed line extract, whereas it was increased up to 21393.93 μg per g in the case of t2, which is a callus developed from the 02 shoot line. Quantitatively individual withanolides are also enhanced in transformed lines, in contrast to control untransformed lines. Compared to the control extract, total withanolide content increased from more than 5-fold in the 03 transformed line extract and almost 1,260-fold in the T2 extract, which is a callus developed from the 02 shoot line. Individual withanolides were also enhanced in transformed lines in contrast to control untransformed lines. Withanolide G was not detected in the control but was found in small amounts in transformed line 01. Callus line T2 had the highest amount of individual and total withanolides. Coagulansin A, withacoagulin G, withacoagulin I, withacoagulin H, withacoagulin, withanolide E, withanolide D, and withanolide B were not found in the control but were present in T2 in high amounts (Table 3). Among individual withanolides, withanolide H was present in the highest quantity in the T2-transformed callus line.

2.5. Investigation of the *Withania coagulans* on the Prostate Cancer Cell Line in Its Transformed and Untransformed Stages. The MTT test was used for untransformed and transformed lines of *W. coagulans* extracts against androgen-independent (DU-145) and androgen-sensitive (C4−2) prostate cancerous cells. Transformed and untransformed *W. coagulans* extracts were used to treat prostate cancer cells (0−150 μg/mL for 24 and 48 h) and showed a time and dose-dependent gradual decrease in cell proliferation (Figure 7). Our findings revealed that C4−2 cells were marginally more susceptible to *W. coagulans* extracts than DU-145 cells. The data indicate that large amounts of withanolides in T2 relative to 01, 02, and 03 are responsible for its enhanced antiviability efficacy in comparison to specific prostate cancer cell lines (C4−2 and DU-145; Figure 3A−D). Transformed *W. coagulans* lines have sown higher antiprostate activity than untransformed *W. coagulans* (control). This result is also related to the withanolide contents of these analyzed plant lines. Higher withanolide content in the plant line gave rise to higher anticancer activity (Figure 7A−D).

3. DISCUSSION

The authors aimed to develop transformed *Withania coagulans* plants with *rol A* genes to improve their secondary metabolite content and biological activities for the benefit of mankind. Tissue culture is used to accomplish the replication of physiologically similar and healthy plant species that have poor natural propagation efficacy. In this study, the authors established a reliable method for the micropropagation of *W. coagulans*. The generation of morphogenic and embryogenic callus from leaf and hypocotyl explants enhanced the presence of auxin and cytokinin in MS media. The highest embryogenic callus-inducing response of leaf explants was found in NAA (4.0 μM) and BA (6.0 μM). This leads to the formation of compact, green, embryogenic calli, corresponding with the findings of Rani and Grover (1999)[25] and contradicting the finding of Kumar et al.[26] According to prior research that supports our results, *Toreniafournieri callus* induction required NAA (0.05 mg/L) with BA (3 mg/L) in many different combinations.[27] This study reveals that the identification of auxin to cytokinin results in the development of morphogenic calli in *W. coagulans*. The addition of BA at a concentration of 8 μM alone enhanced the induction of numerous shoots on

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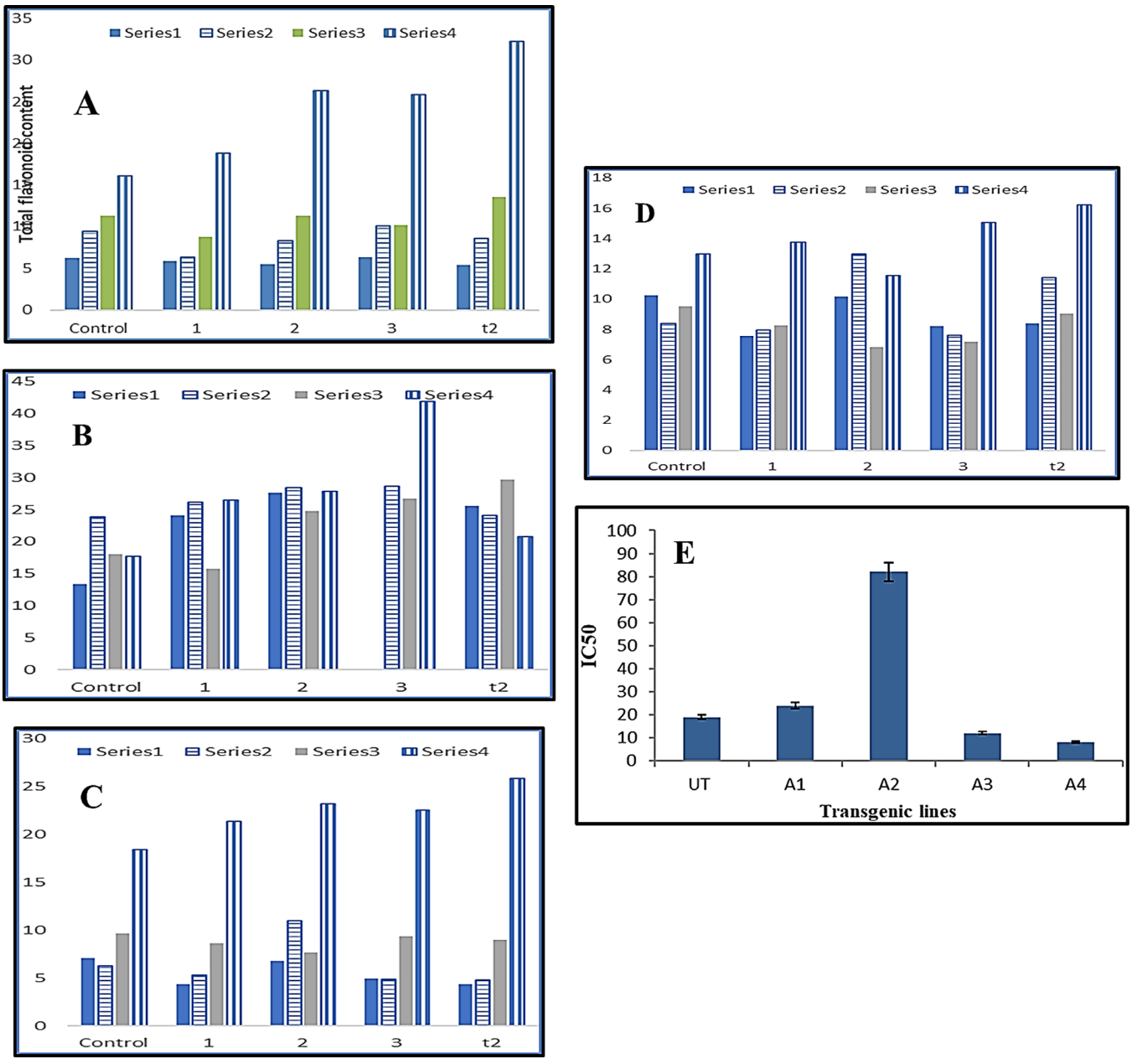

Figure 6. (A) Total flavonoid content (QE= quercetin equivalent). (B) Total phenolics content (GA= gallic acid equivalent). (C) Total antioxidant capacity. (D) Total reducing power. (E) Free radical scavenging activity. (UT: Untransformed lines 01, 02, 03; T2: transformed lines).

Table 3. Amounts of Withanolides (μgm per gm Extract) in Various *Withania coagulans* Extracts

sample/compound	RT (min)	Control	01	02	03	T2
coagulansin A	**9.70**	**0.00**	**0.00**	**0.00**	**0.00**	**244.98**
withanolide H	10.69	7.59	20.92	0.00	0.00	814.86
withacoagulin G	13.06	0.00	4.47	0.00	0.00	13.26
withacoagulin I	12.53	0.00	0.00	0.00	0.00	74.64
withacoagulin H	12.03	0.00	0.00	0.00	0.00	10.90
withanolide F	15.48	3.18	12.08	6.90	3.51	87.00
withacoagulin	16.73	0.00	21.11	30.88	23.52	144.47
unknown 1 (m/z 485) withanolide E	10.80	0.00	0.00	0.00	0.00	42.23
unknown 2 (m/z 469) withanolide A	11.76	6.29	27.50	30.04	29.92	642.85
unknown 3 (m/z 451) withanolide D	16.05	0.00	0.00	0.00	0.00	23.05
unknown 4 (m/z 469) withanolide B	17.34	0.00	10.21	19.12	7.46	41.69
total withanolide		;lds	96.29	86.94	64.41	2139.93

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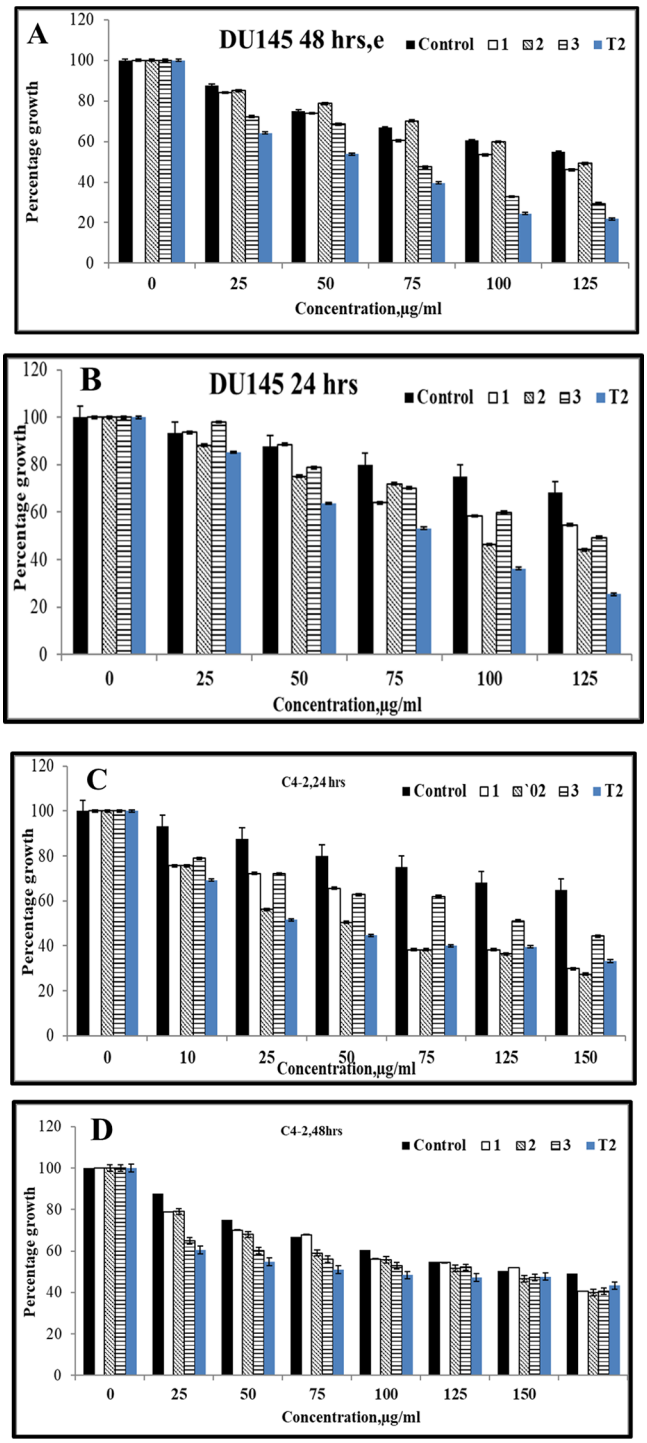

Figure 7. (A−D) Antiviability of transformed lines of *W. coagulans* against DU-145 and C4−2 for 24 and 48 h.

calli. According to 2009 research, BA significantly influenced *W. coagulans*' ability to produce numerous shoots, and it also significantly boosted the quantity and duration of newly differentiated shoots.[23] Several plant species need two mediums for micropropagation (shoot induction and shoot elongation), making micropropagation processes time-consuming and inefficient.[28] The average number of shoots with increasing cytokinin concentration decreased, but the length of shoots dropped.

The leaf explants had 100% shoot response with average shoot length and 30 shoots per leaf at 8.0 μM BA with 2.5 ± 0.11 cm (Figure 4), as compared to *W. somnifera*, showing 38 shoots per explant with IAA and BAP (1.5 mg/L) each.[25] Two measures (percent response and the number of shots) were low at low BA concentrations while raising the concentration enhanced the number of shots and percent response. The cloned stems and leaves are anchored on half MS- with IBA. The shoots became yellowish and perished when exposed to increased IBA (4 mg/L) concentration.

The transformation of a plant species is a crucial strategy for improving it since it enables the transmission and expression of new genes and aids in research into the activities of plant genes. The goal of this experiment was synthesizing an *Agrobacterium*-mediated effective transformation of *W. coagulans* by using *rolA* genes. For 5 to 10 min, bacteria were introduced to the explants used for transformation. Low transformation efficiency was caused by a shorter incubation period, while bacterial contamination of the explants was caused by a longer incubation period. Hence, the ideal infection window for effective transformation was identified.[2] The explants were placed for 7 min in the bacterial culture room before being transferred to the cocultivation medium. Acetosyringone and hormones are added to the cocultivation medium to increase bacterial pathogenicity.[29] The duration of cocultivation varied, but 48 h of cocultivation with bacteria produced the best results. The plants were transferred to the regeneration medium after cocultivation. The media for the screening of transformed cells contained kanamycin (50 mg/L).

It has been reported that the transformed plant has undergone significant morphological changes. These characteristics include reduced height, a bent stem, the lack of genuine leaves, termination of the apical meristem in swelling outgrowths, and enlarged cotyledons. Van Altvorst et al. (1992) reported that the *rol A* caused alterations, such as reduced height and enlarged vividly green leaves.[30] Arshad et al. (2014) observed comparable outcomes with *rol B*-transformed plants.[31] These morphological alterations may have been induced by hormone imbalance. Despite the development of various additional transformation techniques, *A. tumefaciens* remains a favored tool for the genetic modification of plant cells. As no other organism can cross-kingdom transmit genetic material.

Using the DPPH scavenging assay, we determined the scavenging activity of free radical *W. coagulans*. Free radical scavenging plays a vital role in chronic disorders like cancer and cardiovascular diseases. In our results, the scavenging effect depends on concentration and increases with increasing the concentration of plant extracts. Lower IC_{50} values show a better protective capability.

The antioxidant activity of plants is depicted in the best way by the flavonoid content of plants. The link between the antioxidant activity and flavonoid content is determined by a comparison of total flavonoids in transformed and untransformed plant lines 01, 02, 03, and T2. The untransformed line of *W. coagulans* has shown antioxidants of 40 ± 03QE/g. Line 04 (t2) showed the highest total flavonoid content 62 ± 3 QE/g, while the least activity was exhibited by 01. Line 04 was shown to be 1.62 times higher than the untransformed line. This greater antioxidant capability of the converted line t2 might be attributed to the existence of important quantities of phenols in 02. Most of the antioxidant activity of *ania* is

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because of high levels of phenols. Untransformed plants showed less activity compared to the transformed ones because transformation might have led to the enhanced production of phenols. The transformed *Rubia calli* showed increased antioxidant activity and suppressed reactive oxygen species. The study suggests that the antioxidant potential of all of the extracts may be the reduction of free radicals, chelation of metal ions, or the presence of phytoconstituents.

Reducing power of a plant is the plant's ability to donate electrons in a redox-type reaction so that free radicals are converted into a neutral or less reactive radical. The reducing power of these selected transformed lines 01, 02, and 03 and T2 are determined by their capacity to convert the Fe^{3+} ion to Fe^{2+}. The reducing power for the untransformed plant in this regard was noted to be less than that of the transformed but individual behavior of lines shows that the reducing power ability of the samples was somewhat equivalent. The reducing ability serves as an indicator of antioxidant ability.

All transformed lines had higher amounts of the 12 withanolides than the nontransformed line. Moreover, the total quantity of withanolide was greater in transformed lines than in untransformed lines (Table 3). Furthermore, several compounds (about 10 withanolides) were found missing in the control samples but present in substantial amounts in transformed lines. The quantitative and qualitative increases in the withanolide content were seen in the profiles of all transformed lines.

The amount of withanolide in the control was found to be 17.06 μg/g, whereas transformed lines showed significantly higher concentrations (96.29, 64.41, 86.94), and 2139.93 μg/g (01, 02, 03, and T2). The amount of bioactive asteroids produced by *Agrobacterium*-transformed Withania species has been shown to be much greater in previous research. Bandyopadhyay et al. (2006) found that *Agrobacterium rhizogens*-mediated transformation of *W. somnifera* resulted in greater levels of withanolides (withaferien A and withanolide D).[20] During the early stages of culture, with the highest concentrations of withaferin A being reached toward the conclusion of the culture period.[1] In our investigation, all transformed lines (01, 02, and T2) generated more bioactive withanolides than the control line (Table 3).

All genetically modified plant samples possess superior anticarcinogenic potential compared to untransformed lines. These findings were foreseen since transgenic plants possessed more withanolide than control. The highest levels of the specified 12 withanolides were found in the calli of transformed lines, in contrast to those of the untransformed line. Furthermore, if authors examine the overall quantity of withanolides and find that it is greater in all genetically transformed lines than in untransformed lines (Table 2). In our investigations, all the transformed lines (01, 02, and T2) exhibited a greater amount of bioactive withanolide than control lines, while among shoot lines 02 showed greater anticancer activity and t2 is a callus line showing the highest withanolide content and anticancerous activity as compared to all selected shoot lines (Table 2). It is observed that certain compounds (about 10 withanolides) are completely missing in the control lines but present a surplus in the transformed lines. The quantitative and qualitative withanolide content of each transformed line has increased. The total average withanolide amount in control was 17.06 μg/g but transformed lines contained 96.29, 64.41, 86.94, and 2139.93 μg/g, which is significantly greater than transformed lines (01, 02, 03, T2).

Hence, transformation with *rolA* genes served as a good tool for the induction and enhancement of antioxidant potential in medicinally important species.[32] Line 03 represented the best antioxidant potential, with the highest flavonoid content as well as free radical scavenging activity. Shoot line 03 (having the highest antioxidant potential and free radical scavenging activity) as compared to line 02 (having the highest anticancerous potential and withanolide content) has some phytochemicals such as flavonoids and phenolics in addition to withanolides causing higher antioxidant activities.

4. CONCLUSIONS

The transgenic plants had shown enhanced biological activities like anticancer effect, antioxidant activities, and secondary metabolites, which were withanolide in the recent case of genetic transformation of *W. coagulans*. The *rol A* gene was confirmed in transgenic plants; hence, our plant was successfully transformed with rol A genes and transformed lines were developed. Three transformed lines 01, 02, and T2 were developed, and their molecular-based PCR analysis has confirmed the insertion of the *rol A* gene. The increased quantities of secondary metabolites, notably withanolides, resulted in a considerable increase in biological activities, including improved antioxidant and anticancer properties. This increase in activities served as proof of the successful transfer of *rol A* gene in *W. coagulans*. The T2 is a callus form of the 02 transformed line that has produced the greatest amount of withanolides among all untransformed and transformed *W. coagulans* lines. T2 had the greatest effect in inhibiting the proliferation of prostate cancer strains and lines were more effective against the proliferation of C4−2 than the DU145 prostate cancer cell line. These findings indicated the potential for genetic change to improve the therapeutic value of *W. coagulans* and the involvement of withanolides in its increased anticarcinogenic capabilities.

5. EXPERIMENTAL SECTION

5.1. Tissue Culture Optimization of *W. coagulans*.

Authors followed two kinds of micro propagation approaches for *Withania coagulans* tissue culturing by following callus generation and tissue culture of *W. coagulans* by direct shoot regeneration.

5.1.1. Collection of Seed, Inoculation, and Sterilization. *Withania coagulans* seeds were assembled from the Musakhail District Mianwali, Pakistan. The seeds were disinfected by autoclaving for 20 s with 0.1% mercuric chloride under aseptic conditions before being rinsed thrice with autoclaved distilled water. After that, seeds were sterilized for 2 min by using 70% ethanol followed by culturing on a 3% agar plate. Then, Petri dishes were stored at 25 ± 2 °C in a dark environment for a couple of days after being transferred to the growth chamber. In a growth chamber, the cultures were kept at about 25 ± 2 °C under a 16 h photoperiod and uniform photosynthetic photon flux density (40 L mol m^{-2} s^{-1}). Finally, the seeds were cultivated in autoclaved soil in a greenhouse and the sterilization technique was identical to that used for sterilizing seeds previously.

5.1.2. Callus Induction. As an explant supply, 1 month-old seedlings were employed, and a section of 1.0 cm^2 leaf as well as 1.0 cm hypocotyl was produced. The leaf explants with borders were removed. Explants were grown on MS medium supplemented with sucrose (30 g L^{-1}) and various

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combinations of hormones (NAA, BA). Additionally, in the preautoclaving, the pH of the medium was adjusted to 5.8. The callogenic response of all of the explants was observed by monitoring explants for callus. As for the induction, the size of the callus was evaluated 4 weeks after culture (in triplicate) (Table 1).

5.1.3. Shoot Regeneration from Callus. To evaluate the response of shoot induction, soft, green, and embryogenic calli were transferred to MS media with varying doses of BA (2−16 μM). The medium for shoot regeneration was added with agar (0.8%) and sucrose (30 g L^{-1}). Twenty explants were examined in triplicate for each treatment. After 4 to 6 weeks, data was gathered on percent shoot induction (PSI), shoot lengths, and shoots per callus.

For PSI measurement, the following formula was determined

$$\mathrm{PSI} = \left[\sum \mathrm{RC}/n\right] \times 100$$

RC is the number of responding calli while n represents the total number of inoculation calli.

The shoots, after attaining heights of 2−3.5 cm approximately, were moved to half-strength MS media added with different concentrations of IBA. The generated roots at different concentrations were analyzed, and the concentration at which more roots were induced was regarded as best. The % response, number of roots per shoot, number of shoots per explant, and root length were all reported.

5.1.4. Direct Shoot Formation. The hypocotyl and leaf explants were cultivated under aseptic conditions on MS media supplemented with different doses of BA (benzyladenine) for a direct shot. For each concentration, the number of shoots per explant was recorded.

5.1.5. Root Regeneration and Acclimatization. When the shoots reached roughly 1−3.5 cm in height, they were cut from the base. Rooted plantlets were thoroughly cleaned with water to remove excess media before being transferred to plastic pots filled with autoclaved soil. The pots were acclimated for 1 week in a growth environment. After 1 week, the plants in polythene bags were transferred into a greenhouse kept at a temperature 25 ± 2 °C.

5.2. *Agrobacterium tumefaciens*-Mediated Genetic Transformation. *Agrobacterium tumefacians* strain GV3101, which has the pPCV002-A plasmid or vector encoding the *rol A* gene was used for transformation. The vector also included a selectable marker neomycin phosphotransferase II gene (NPTII) under the control of cauliflower mosaic virus (CaMV)35S promoter, conferring kanamycin resistance. In addition, *A. tumefaciens* was cultured overnight in 50 mL of LB broth after treatment with kanamycin (50 mg/L). The strains were grown in a liquid medium at 28 °C at 100 rpm in a dark environment.

5.3. Effect of bacterial culture density and varying infection time. The leaves, hypocotyl, and nodal explants are used for infection in transformation. These explants were cultured for 3 days prior to infection with suspension of Agrobacterium (OD600 = 0.4/0.5/0.6/0.7/0.8) for 10, 20, and 30 min. After bacterial infection, explants were dried and cocultured on MS media supplemented with acetosyringone (200 μM) for 12, 24, and 72 h in the dark. Explants were cleaned with cefotaxime solution, dried, and transferred to a mineral salt medium supplemented with appropriate hormones. For shoot induction and callus, a series of different antibiotics such as kanamycin (50 mg/L) and cefotaxime (250 mg/L) were used. In a growth chamber, these explants were kept at 25 °C for 16h with 60% relative humidity. After 2 weeks, explants were shifted to a fresh selection medium, and the amount of cefotaxime was decreased frequently after successive subcultures. The phenotypic characteristics of both transformed and nontransformed wild-type (WT) plants were investigated and recorded. These explants' transformation efficiency was evaluated.

5.4. Polymerase Chain Reaction of Transformed Plants. The incorporation of *NPTII* and *rol A* was confirmed using PCR analysis on transformed plants. Murray and Thompson's (1980) cTAB (cetyltrimethylammonium bromide) approach with minor changes was used to extract genomic DNA from transformed and untransformed plants for molecular diagnostics. *A. tumefaciens* DNA was also extracted by utilizing the alkaline lysis approach reported by Russell and Sambrook.[23] Employing the *NPTII-specific* primers, PCR methods were used to amplify the *NPTII* gene fragment (781bp). The forward primer has the sequence F5′-AAGATGGATTGCACGCAGGTTC-3′ while the reverse primer has the sequence R5′-GAAGAACTCGTCAAGAAGGCGA-3′, having the following sequences: forward primer F5′-AGAATGGAATTAGCCGGACTA-3′ and reverse primer R5′-GTATTAATCCCGTAGGTTTGTT-3′. The PCR reactions underwent a total of 35 cycles at 72 °C for the period of 10 min, 94 °C for about 35 s, 72 °C for approximately 45 s, and 53 °C (*rol A*), 54 °C(NPTII) for about 35 s. The amplified DNA samples were examined using 1% Aga rose gel electrophoresis (Syngene).

5.5. Analysis of the Antioxidant Potential for Transformed *W. coagulans* Plant Preparation of Extracts. Shoots and leaves of 4−5 months transformed plants (designated as 01, 02, 03, and t2) and untransformed plants (control plants) were harvested and air-dried for 2 weeks at room temperature. The test samples were then ground to a fine powder. The powdered samples were mixed with chloroform: methanol (1:1) thoroughly. The samples were subjected to repeated cycles of sonication and overtaxing followed by centrifugation at 14000 rpm for 5 min. Then, a rotary evaporator was used to concentrate the filtrate. The desiccated extract was solubilized in DMSO (100 mg/mL) and then sonicated for 5 min to remove lumps.[33]

5.5.1. Determination of Total Phenolic Content. The phenolic contents were calculated by the Folin−Ciocalteu method.[34] The extracts were prepared in 1 mg/mL concentration by mixing with 1.5 mL of 1:10 (v/v water) Folin−Ciocalteu reagent and incubated for 5 min. Then, 1.5 mL of Na_2CO_3 (75 g/L) was added and mixed well. For color development, the mixture was left for 90 min in a dark environment at room temperature. The absorbance was determined to be 765 nm. All the phenolic content was represented as % w/w of gallic acid equivalents.

5.5.2. Determination of Total Flavonoid Content. Total flavonoid contents of untransformed and transformed *W. coagulans* were calculated by aluminum chloride colorimetric process.[35] Each sample was individually mixed with 10% $AlCl_3$ (100 μL), 1 M KCH_3CO_2 (100 μL), CH_3OH (150 μL), and distilled H_2O (700 μL). After 30 min, the absorbance of the mixture was recorded at 415 nm by using a spectrophotometer. The standard curve for determining the total flavonoid content of *Withania coagulans* was calculated by utilizing quercetin solutions equivalent from 0.0 to 8.0 g/mL in methanol.

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5.5.3. Determination of Free Radical Scavenging Activity. The DPPH free radicals scavenging activity of the samples was evaluated by using the 96-well plates with minor modifications in the previously reported method.[11] DMSO and ascorbic acid were used as negative and positive controls, respectively. The DPPH assay was conducted by using 96-well plates, and the volume of the mixture was 200 μL. The volume of DPPH of 198 μL (3.2 mg/100 mL methanol) was used. The blends were mixed well and kept in the dark. After 1 h, the absorbance was calculated at 517 nm. The scavenging effect (%) of free radical by test samples was measured using the following formula and IC_{50} values were calculated using table curve software.

$$\%\text{age scavenging effect} = (1 - \text{As}/\text{Ac}) \times 100$$

where "As" and "Ac" are absorbance of the test sample and negative control, respectively.

5.5.4. Evaluation of Total Antioxidant Capacity. The total antioxidant capacity of *W. coagulans* was performed in a 96 well-plate by following the phosphomolybdenum colorimetric method.[36] The total antioxidant capacity reagent (198 μL) and the samples (2 μL) were added in various amounts; then, the mixture was at 90 °C for 90 min in a water bath. The mixture was cooled when it changed to dark blue. After that, the absorbance of the mixture was taken at 695 nm against blank. The total antioxidant capacity of the mixture was expressed as equivalent to that of ascorbic acid.

5.5.5. Determination of Reducing Power. The reducing power of the extracts of *W. coagulans* was determined by assessing the ability of the plant extract for the ferric Fe^{3+} ion to ferrous ion Fe^{2+}. A reducing power assay was performed by the method of the method of Sarikurkcu.[37] Initially, 1.25−10 mg/mL samples were added to the Eppendorf tubes along with 0.2 M phosphate (pH = 6). It was followed by the addition of potassium ferricyanide (500 μL) and then the mixture was incubated for 20 min at 40 °C. After that, trichloracetic acid (500 μL) was added and centrifuged for 10 min at 3000 rpm. Then, the supernatant was isolated (500 μL) and $FeCl_3$ (100 μL) was added; the color of $FeCl_3$ changed to blue on reduction. After that, the sample (200 μL) was poured onto a microtiter plate. The absorbances of the samples were recorded at 700 nm.

5.6. LC-MS Analysis of *W. coagulans* Plant Material. The greenhouse pots were used to collect transformed and untransformed *W. coagulans* plant lines. For both (transformed and untransformed plants), samples were dried to extract secondary metabolites from *W. coagulans* using chloroform−methanol.

5.6.1. Determination of Reducing Power. For this purpose, the quantification technique employed quercetin (supplied by Sigma-Aldrich) instead of withanolide standards, as shown in Table 1.

5.6.2. LC-MS Analysis. The final solution (2 μL) was injected by water ultrahigh pressure liquid chromatography (UHPLC) fitted with an electrospray ionization mass spectrometer (ESI-MS) and photodiode array detector. The *W. coagulans* leaf extracts were lyophilized until dry and then dissolved in methanol by vortex mixing. After that, the mixture was sonicated for 30 min at room temperature. The samples were centrifuged for 10 min at 14,000*g*, and the supernatant was injected into glass LC vials. To analyze the concentrations of the compounds in the final solutions, 2 μL of each sample was injected into Waters UHPLC coupled with PDA and ESI single quadrupole MS. A binary gradient 0.1% v/v aqueous solution of CH_2O_2 (mobile A) and CH_3CN with 0.1% v/v CH_2O_2 (mobile B) was used to separate the compounds on equity UPLC HSS C18 column (100 × 2.1 mm, 1.8 m particle size) at 40 °C with a constant flow rate of 0.5 mL min^{-1}. The gradient started at 10% B followed by an instantaneous ramp to 30% B for 6 min, then climbed to 35% for 10 min, 45% for 20 min, and then 90% B for 5 min, before reverting to baseline conditions after 30 min at 10% B. Throughout every run, the PDA was programmed to record ultraviolet (UV) data (210−450 nm). During MS operation, the source and desolvation temperatures were 150 and 250 °C, the nebulization gas flow was 500 L h^{-1}, and the capillary and cone voltages were 3 kV (negative ionization mode) and 30 V, respectively. In centroid mode, mass spectra (m/z = 200−1000) were collected. By adding up the integrated peak areas of the deprotonated (M−H) and formate adduct $(M-H+ HCOOH)^-$ forms of each chemical in the extracted ion chromatograms (EIC), authors were able to determine the quantities of the putative withanolides in milligrams of quercetin equivalents per gram of plant extract using a five-point standard curve of quercetin.

5.7. Analysis of *W. coagulans* on Prostate Cancer Cells. The MTT assay was used to test the anticancerous activity of *W. coagulans* plant extracts by using DU-145 and C4−2 prostate cancer cell lines. To examine the impact of *W. coagulans* extracts on cell development, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. In 96-well microtiter plates, cells (1×10^4 cells per well) were seeded in 1 mL of entire culture media supplemented with a concentration range of 10−250 g/mL *W. coagulans* extracts. After 24 h in a humidified 37 °C incubator, 200 μ L of MTT (5 mg/mL: 1 PBS) was applied. The DMSO (200 μ L) was added after 2 h centrifuged at 1800*g* for 5 min at 4 °C. The absorbance was measured at a wavelength of 540 nm range, and the growth inhibition impact of *W. coagulans* extracts was evaluated as a percentage of cell growth inhibition, with DMSO-treated cells serving as 100% control.

5.8. Statistical Analysis. MS Excel 10.0 software was used for graphing and statistical analysis. The cell growth % was calculated at a given concentration = mean (OD sample/mean OD control treated with DMSO) × 100. The GraphPad Prism (San Diego, CA) was used for all statistical analyses.

AUTHOR INFORMATION

Corresponding Author

Amjad Hussain − *Instituite of Chemistry, University of Okara, Okara, Punjab 56300, Pakistan;* orcid.org/0000-0003-1697-7580; Email: amjadhussain@uo.edu.pk

Authors

Samiya Rehman − *Department of Biochemistry, University of Okara, Okara, Punjab 56300, Pakistan*

Munzer Ullah − *Department of Biochemistry, University of Okara, Okara, Punjab 56300, Pakistan*

Enas Ali − *Faculty of Engineering and Technology, Future University in Egypt, New Cairo 11835, Egypt*

Mariusz Mojzych − *Department of Chemistry, Siedlce University of Natural Sciences and Humanities ul, Siedlce 08-110, Poland; Faculty of Health Sciences, Collegium Medicum, The Mazovian Academy in Plock, 09-402 Płock, Poland;* orcid.org/0000-0002-9884-6068

Syed Ali Raza Naqvi − *Department of Chemistry, Government College University, Faisalabad 38040, Pakistan*

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Akbar Ali − *Department of Chemistry, Government College University, Faisalabad 38040, Pakistan;* orcid.org/0000-0002-2914-0934
Meher Ali − *Department of Chemistry, Karakoram International University, Gilgit 15100, Pakistan*
Ehab Gomaa − *Department of Civil Engineering, College of Engineering, Taif University, Taif 21944, Saudi Arabia*
Sherif S. M. Ghoneim − *Department of Electrical Engineering, College of Engineering, Taif University, Taif 21944, Saudi Arabia*
Bushra Mirza − *Department of Biochemistry, Quaid-e-Azam University, Islamabad 44000, Pakistan*
Ken Keefover Ring − *Department of Botany and Geography, University of Wisconsin, Madison, Madison, Wisconsin 53706, United States*
Hidayat Hussain − *Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Halle (Saale) D-06120, Germany;* orcid.org/0000-0002-8654-8127
Abdur Rauf − *Department of Chemistry, University of Swabi, Swabi 94640, Pakistan*
Najeeb Ur Rehman − *Natural & Medical Sciences Research Center, University of Nizwa, Nizwa 616, Sultanate of Oman*
Faisal Attique − *Institiute of Chemistry, University of Okara, Okara, Punjab 56300, Pakistan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c07069

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