

Ajuga Bracteosa Transgenic Regenerants Display Better Pharmacological Potential

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Abstract

Ajuga bracteosa Wall. ex Benth is an endangered medicinal herb used against different ailments in folklore medicines. Here, we aimed to create a new insight to the fundamental mechanisms of genetic transformation in the ethnomedicinal usage of this plant. We transformed the plant with *rol* genes of *Agrobacterium rhizogenes* and raised the regenerants from the hairy roots. The transgenic regenerants were screened for *in vitro* antioxidant activities, a range of *in vivo* assays, and linked the activities with elemental analysis, polyphenol content and different phytochemicals found through HPLC. Among 18 polyphenolic standards, kaempferol was found most abundant in all transgenic lines (up to 101.26 ± 6 $\mu\text{g}/\text{mg}$). Furthermore, among all tested plant extracts, transgenic line 3 (ABRL3) showed maximum phenolics (13.39 ± 2 μg GAE/mg) and flavonoids content (4.75 ± 0.16 μg QE/mg). ABRL3 also demonstrated potent total antioxidant capacity (8.16 ± 1 μg AAE/mg), total reducing power, (6.60 ± 1.17 μg AAE/mg), DPPH activity ($\text{IC}_{50} = 59.5 \pm 0.8$ $\mu\text{g}/\text{mL}$), hydroxyl ion scavenging ($\text{IC}_{50} = 122.5 \pm 0.90$ $\mu\text{g}/\text{mL}$), and iron chelating power ($\text{IC}_{50} = 154.8 \pm 2$ $\mu\text{g}/\text{mL}$) among all plants. Transformed plant extracts also produced significant analgesic, anti-inflammatory, anticoagulant, and antidepressant properties in *in vivo* mice model as compared to control untransformed plant material. Additionally, no abnormal behavior or lethality was observed in any animal tested. In conclusion, transgenic regenerants of *A. bracteosa* pose better pharmacological properties under the effect of *rol* genes as compared to wild type plants.

Introduction

Medicinal plants have been used in traditional medicines for thousands of years and they contain a wide variety of biologically active plant products called secondary metabolites. In the last decade, the potential toxicity of synthetic drugs led to a resurgence of the use of these metabolites as a facile and economical alternative approach [1]. According to the World Health Organization (WHO), approximately, 80% population of developing countries depends on plant products to alleviate and treat serious ailments [2].

Free radicals, such as reactive oxygen and nitrogen species, produced in the human body can be a root cause of aging, rheumatism, malignancies, diabetes, cardiovascular, and neurodegenerative disorders [3]. Higher rate of incidence of these diseases triggered the research of natural antioxidants and various studies have suggested that dietary polyphenols are the most abundant antioxidants in nature [4]. These polyphenols can directly scavenge free radicals, chelate metal ions, and inhibit their pro-oxidant activities, thus reducing the risk of chronic metabolic diseases. Moreover, polyphenols also possess anti-inflammatory, anti-thrombotic, and analgesic activities [5]. Likewise, different trace elements not only improve plant immunity but also provide scaffolding for antioxidant enzymes as cofactors in humans. Hence, it is dire need to thoroughly investigate different herbal extracts for their antioxidant properties to develop new drugs [6].

Ajuga bracteosa is considered elixir to a variety of ailments and it is found in hilly areas of Pakistan, Nepal, Kashmir, India, and the Himalayan region. Extensive literature survey reveals that different extracts of *A. bracteosa* have a variety of pharmacological activities. It is used to cure skin infections, respiratory

issues, digestive problems, malaria, protozoal diseases, diabetes mellitus, hepatitis, arthritis, epilepsy, inflammation, neurological disorders, and cancer [7,8,9,10]. This medicinal importance is due to a repertoire of metabolites such as essential oils, ecdysteroids, terpenoids, phenolics, flavonoids, and withanolides characterized in this plant [11].

Root oncogenic loci (rol) genes are well known for the upregulation of secondary metabolism [12]. Different *rol* genes have different induction capacity of secondary metabolites [13] and a lot of the pharmacological activities are linked to the amount of the secondary metabolites produced in the medicinal plants [14]. We hypothesize that the amount of these secondary metabolites biosynthesized *de novo* in *A. bracteosa* could be enhanced and hence the better pharmacological effects could be attained. Considering that, we transformed *A. bracteosa* with *rolABC* genes of *Agrobacterium rhizogenes* and hairy roots were produced. To resolve the issue of organ specificity of certain metabolites, intact plants (regenerants) were regenerated from these transgenic hairy root lines and their extracts were screened for antioxidant activities. Multiple *in vivo* activities including analgesic, anti-inflammatory, antidepressant, and anticoagulant activity were evaluated using BALB/c mice. In the continuation of our previous study [15], estimation of essential elements of transformed regenerated plants of *A. bracteosa* by atomic absorption spectrophotometry and HPLC fingerprinting of polyphenol content was performed to support our results.

Materials And Methods

Source of plant

The plant material was collected from the premises of Quaid-i-Azam University, Islamabad, Pakistan. These plants were identified by Prof. Dr. Rizwana Aleem Qureshi (Taxonomist), Department of Plant sciences, Quaid-i-Azam University, Islamabad. A voucher specimen number (HPM-460) was deposited in the herbarium of Quaid-i-Azam University. Fresh green plants were surface sterilized and tissue cultured on Murashige and Skoog (MS) medium. Transgenic hairy roots were generated through *rolABC* containing *A. rhizogenes* mediated transformation. Intact plants were regenerated from hairy roots by following the previously optimized method in our lab [16]. Polymerase chain reaction (PCR) was performed for the confirmation of transformation and stable integration of *rol* genes in transformed roots and regenerants [15,16]. Untransformed tissue cultured plants and transgenic regenerants of *A. bracteosa* plants were used in this study.

Elemental Analysis

For the digestion of leaf samples, an earlier reported method [17] was employed with slight modifications. Powdered leaf samples were weighed (100 mg each) and heated in an oven at 110 °C in a china dish for the removal of moisture. The dried samples were heated at 550 °C for 4 h and then cooled at room temperature. Then Nitric acid (6 M; 10 mL) was added for acid digestion. After filtration, the solution was diluted up to a 25 mL mark with deionized water. Reference standards of 12 micro and macro elements (Sigma-Aldrich, USA), were used to quantify the essential elements and to find the possible accumulation

of hazardous heavy metals in *A. bracteosa*. Estimation of all elements was carried out on Fast Sequential Atomic Absorption Spectrometer (Varian 240AA FS-Australia). The operating parameters for working elements were optimized according to the manufacturer's recommendations.

Crude extracts preparation for biological activities

Aerial parts of three independent transgenic lines (ABRL1, 2, and 3) and *in vitro* grown untransformed *A. bracteosa* plants (WT) were shade dried after rinsing with water. Leaves were ground and 1 g powdered material was allowed to set in a mixture of methanol: chloroform (1:1; 5 mL). After 1 h the mixture was sonicated for 10 min followed by 20 min of shaking and the process was repeated three times. Finally, the plant extracts were filtered and pooled filtrates were dried, weighed, and stored at room temperature for further analysis.

Phytochemical profiling

Qualitative assays

Crude extracts of *A. bracteosa* were evaluated for the presence of major families of secondary metabolites such as alkaloids, glycosides, flavonoids, phenols, tannins, saponins, terpenoids, coumarins, betacyanin (β -cyanin), anthocyanin, and sterols using standard qualitative procedures based on coloring reaction and/or precipitation [18].

Quantitative assays

Estimation of total phenolics and flavonoids content

The total phenolic content (TPC) was calculated by Folin-Ciocalteu (FC) assay using gallic acid as standard [19]. Briefly, 20 mg of each dried extract was dissolved in 1 mL of DMSO. Gallic acid (1 mg, Merk) was dissolved in 1 mL of DMSO and further diluted. In this experiment 5 μ L of plant extract was mixed with 98 μ L of 10 times diluted FC reagent in 96 well plate (Thermo Scientific). After 5 min, 98 μ L of 6% sodium carbonate was added and incubated at 25 °C for 90 min. Finally, the absorbance was measured at 630 nm with a microtiter plate reader (Elx 800). TPC was expressed as gallic acid equivalents (μ g GAE/mg dry weight of plant extract). Total flavonoid content (TFC) was determined by already optimized colorimetric method [20] with slight modifications. 5 μ L of each extract (20 mg/mL) was mixed with equal volumes of potassium acetate (1 M) and 10% aluminium chloride (20 μ L each). Then 155 μ L of distilled water subsequently added and mixture was left at 37 °C for half an hour. After incubation, absorbance was determined at 405 nm and TFC was expressed as quercetin equivalents (μ g QE/ mg extract).

Quantification of polyphenols by RP-HPLC

For RP-HPLC analysis, polyphenols were extracted from aerial parts of wild plant and transgenic lines according to previously reported procedure [21]. HPLC-DAD system (Agilent technology; Germany) was

attached to an analytical column (Sorbex RXC-8) with the dimensions, 5 µm, 4.6× 250 mm, for separation of polyphenols. This separation was achieved using two mobile phases; water: methanol: acetonitrile: acetic acid (85: 10: 5: 1) were present in mobile phase A while mobile phase B included methanol: acetonitrile: acetic acid (60: 40: 1). The process was operated by regulating the gradient program as follows; (t (min), % B) (0, 50) (20, 50) (25, 100) (40, 100) with 1ml/min flow rate at 350 bars. 20 µL of each crude extract and standard was injected. Identification and quantification of polyphenols were carried out by comparison of the peaks of each metabolite with particular retention time in the standards.

***In vitro* antioxidant assays**

Potent antioxidant activities from crude extracts was detected by various antioxidant assays [22]. For Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, ascorbic acid (Merk, Germany) was used as positive control. Phospho molybdate method was used for determination of total antioxidant activity. This TAC was reported as ascorbic acid equivalent (µg AAE/mg DW). Reducing power activity was recorded as ascorbic acid equivalent. The OH scavenging potential of extracts was determined by classical thiobarbituric acid (TBA) and deoxyribose based method. In this study, Gallic acid was used as positive control. The Ferrous ion (Fe⁺⁺) chelating ability of plant extracts was also investigated. DMSO was used as negative control in all antioxidant assays.

Experiment design for *in vivo* assays

For the experiment, healthy male BALB/c albino mice (4-6 weeks; 25-30 g) were procured from the NIH, Islamabad, Pakistan. Test mice were maintained under controlled environmental conditions (23.0 ± 2.0 °C temperature; 60 - 70% of relative humidity; 12 h light/dark cycle) in the Primate facility Quaid-i-Azam University (QAU), Islamabad, Pakistan. All the mice were fed with pellet diet and had free access to water *ad libitum*. All the experiments were approved by the Animal Ethical Committee, QAU (BEC-FBS-QAU2019-157).

Plant extracts and standard compounds were prepared in normal saline (0.9% NaCl) and administered orally according to mice body weight. In these experiments, 30 male mice were randomly divided into 6 groups as following:

Normal control group: 200 mg/kg normal saline.

Positive control group: 10 mg/kg standard drugs.

Test group-1: 200 mg/kg crude extract of wild type plant (WT).

Test group-2: 200 mg/kg crude extract of ABRL1.

Test group-3: 200 mg/kg crude extract of ABRL2.

Test group-4: 200 mg/kg crude extract of ABRL3.

Acute toxicity test

Before performing *in vivo* experiments, animals were tested for toxic effects of *A. bracteosa* plant extracts. The oral administration of plant crude extracts (up to 1g/kg) proved to be non-toxic and did not produce mortality for the next 24 h. Guidelines 425 of the Organization for Economic Corporation and Development (OECD) were strictly followed during the study.

In vivo biological activities

For all *in vivo* assays, previously reported methods [8] were employed and normal saline was used as negative control. For analgesic activity, the standard drug, aspirin was used as a positive control. The anti-inflammatory activity of *A. bracteosa* crude extracts were assessed through carrageenan-induced hind paw edema model. Here, Diclofenac potassium was used as positive control. Tail suspension test, a simple and inexpensive method, was used to assess anti-depressant activity. Data collected in TST were expressed as arithmetic means of immobility time for each experimental group. Whereas, capillary tube was used to analyze the anticoagulation activity of the crude extracts of *A. bracteosa* in mice.

Statistical analysis

All the experiments were performed in triplicates. One way analysis of variance (ANOVA) was used to find variability between data. IC₅₀ values were calculated through GraphPad Prism Software Version 7.0 (GraphPad Prism® Software, Inc. San Diego, CA, USA) by plotting transformed inhibitors vs log values of inhibition. PAST 4.03 was used for principal component analysis (PCA). $p > 0.05$ was considered significant and data was represented as mean of 3 values \pm SD (Standard Deviation).

Results

Elemental analysis

The elemental screening of transformed and untransformed *A. bracteosa* digested extracts revealed the presence of 10 elements, commonly found in plants, as listed in Table 1. Data shows that transgenic line 3 (ABRL3) is enriched with 3 macro-elements: sodium $3.94 \pm 2 \mu\text{g}/\text{mg}$ dry weight (DW), potassium $13.09 \pm 2 \mu\text{g}/\text{mg}$ DW, and calcium $1.95 \pm 0.5 \mu\text{g}/\text{mg}$ DW. While magnesium is more abundant in transgenic line 1 ($2.15 \pm 0.3 \mu\text{g}/\text{mg}$ DW). Likewise, the highest amounts of all micro-elements are found in ABRL3 except chromium which is more prevalent in ABRL1. On the other hand, untransformed wild type (WT) plant extract has the least concentrations of all elements ranging from $0.004 \pm 0.01 \mu\text{g}/\text{mg}$ to $8.06 \pm 0.3 \mu\text{g}/\text{mg}$ of dry sample. We also observed that cadmium and lead are below the detection limit in all samples.

Qualitative screening

Qualitative analyses showed the presence of medicinally important phytoconstituents in the methanol: chloroform extracts of *A. bracteosa* summarized in Table 2. These findings suggest that alkaloids,

phenolics, flavonoids, and glycosides are present in all extracts in a larger amount. Whereas tannins and saponins are found in moderate amounts. Anthocyanin, β -cyanins, coumarins, and sterols were absent in all extracts while, terpenoids were identified only in transgenic lines.

Quantitative analyses

Determination of TPC and TFC

TPC and TFC were calculated as μg of gallic acid and quercetin equivalents/mg dry extract by using calibration curves of gallic acid ($y = 0.0163x - 0.0130$, $R^2 = 0.9983$) and quercetin ($y = 0.0517x + 0.0172$, $R^2 = 0.9991$) respectively (Figure 1). In this study, ABRL3 has maximum quantity of phenolics ($13.39 \pm 2\mu\text{g GAE/mg DW}$) followed by ABRL1 ($11.28 \pm 1\mu\text{g GAE/mg DW}$), and ABRL2 ($8.31 \pm 1.5\mu\text{g GAE/mg DW}$). Whereas, WT contains minimum amount of phenolics ($4.803 \pm 0.04 \mu\text{g GAE/mg DW}$). Similarly, TFC ranges from the highest value in ABRL3 ($4.75 \pm 0.16 \mu\text{g QE/mg DW}$) to the lowest value in WT ($1.55 \pm 0.08 \mu\text{g QE/mg DW}$).

RP-HPLC

Among the eighteen polyphenolic standards tested through RP-HPLC, twelve were detected in all extracts of *A. bracteosa*. Kaempferol was most abundant in all transgenic samples ranging from $78.6 \pm 5 \mu\text{g/mg}$ in ABRL2 to $101.26 \pm 6 \mu\text{g/mg}$ in ABRL3. WT contained the highest amount of ferulic acid ($75.55 \pm 3 \mu\text{g/mg}$) and the lowest amount of rutin ($0.63 \pm 0.5 \mu\text{g/mg}$). Cinnamic acid was least abundant in all transformed lines with $4.36 \pm 0.5 \mu\text{g/mg}$ in ABRL1, $5.47 \pm 0.2 \mu\text{g/mg}$ ABRL2, and $6.09 \pm 0.3 \mu\text{g/mg}$ in ABRL3 (Table 3). Overall, ABRL3 has a predominantly higher content of all polyphenols screened in this study as represented by chromatogram in Supplementary Figure 1.

In vitro antioxidant assays

TAC and TRP

Total antioxidant capacity (TAC) and total reducing power (TRP) of samples against ascorbic acid equivalent are given in Figure 2. Results showed that ABRL3 exhibited the maximum total antioxidant capacity ($8.16 \pm 1 \mu\text{g AAE/mg DW}$) while minimum antioxidant capacity was shown by WT ($4.18 \pm 0.16 \mu\text{g AAE/mg DW}$). TAC was found to decrease in the order ABRL3 > ABRL1 > ABRL2 > WT. All the transgenic lines showed significantly increased antioxidant activity ($p < 0.01$) compared to wild plants. Data also shows that ABRL3 exhibited the highest reduction power of $6.60 \pm 1.17 \mu\text{g AAE/mg DW}$ followed by $5.8 \pm 1 \mu\text{g AAE/mg DW}$ in ABRL1. TRP followed the as that of TAC in all plant extracts. However, TRP was significantly higher ($p < 0.01$) in transgenic regenerants than wild control plants.

DPPH Radical scavenging assay

The percent scavenging activity of the extracts were evaluated using DPPH free radical scavenging assay and IC_{50} values were calculated (Figure 3a). IC_{50} values varied in a concentration-dependent manner however, all extracts showed higher IC_{50} values than ascorbic acid (IC_{50} $39.3 \pm 1 \mu\text{g/mL}$), used as positive control. The minimum IC_{50} values were exhibited by ABRL3 ($59.5 \pm 0.8 \mu\text{g/mL}$) followed by ABRL1 ($97.64 \pm 0.5 \mu\text{g/mL}$). Overall, order of IC_{50} values was $ABRL3 < ABRL1 < ABRL2 < WT$. The DPPH radical scavenging activity of different extracts presented good correlation with TPC ($R^2 = 0.9627^{***}$, $p < 0.001$) and moderate correlation with TFC ($R^2 = 0.7627^{**}$, $p < 0.01$) as shown in Figure 3d.

Hydroxyl ion scavenging assay

All extracts of *A. bracteosa* scavenged $\cdot\text{OH}$ radicals while lowest IC_{50} values were recorded for ABRL3 ($122.5 \pm 0.90 \mu\text{g/mL}$) and ABRL2 ($129.7 \pm 2 \mu\text{g/mL}$) followed by ABRL1 ($138.4 \pm 1 \mu\text{g/mL}$), whereas, the highest IC_{50} was observed for WT ($1056.9 \pm 4 \mu\text{g/ml}$). IC_{50} of all extract samples were significantly different from the standard gallic acid ($81.1 \pm 4 \mu\text{g/mL}$) as given in Figure 3b. A highly significant correlation was observed with TPC ($R^2 = 0.9312^{***}$, $p < 0.001$) and moderate correlation with TFC ($R^2 = 0.8158^{**}$, $p < 0.01$) (Figure 3d).

Ferrous ion chelating activity

In this study, the finest values for IC_{50} were exhibited by ABRL3 ($154.8 \pm 2 \mu\text{g/ml}$) followed by ABRL2 ($179.2 \pm 1 \mu\text{g/mL}$). Overall, order of IC_{50} of $ABRL3 < ABRL2 < ABRL1 < WT$ was observed (Figure 3c). The iron chelating activity of various extracts showed good correlation with TPC ($R^2 = 0.9159^{***}$, $p < 0.001$) and moderate correlation with TFC ($R^2 = 0.8243^{**}$, $p < 0.01$) as given in Figure 3d.

***In vivo* assays on BALB/c mice**

Analgesic activity

The crude extracts of transgenic *A. bracteosa* plants showed a delayed latency period and increased analgesic activity in BALB/c mice. Aspirin (positive control) and crude extracts displayed a time-dependent activity on a hot plate by suppressing nociceptor activity in mice (Figure 4a). Maximum activity was observed after 1 h of oral dose in ABRL3 ($87.3 \pm 3\%$) followed by aspirin ($85.66 \pm 4\%$). Whereas, normal saline-treated mice (negative control) produced the least significant analgesic effect ($13 \pm 4\%$). ABRL1 and ABRL2 also demonstrated increased activity ($76 \pm 3\%$ and $74 \pm 2\%$) compared to the wild type control group ($41 \pm 3\%$).

Anti-inflammatory activity

Pain and inflammation are often linked to each other. Therefore, crude extracts were also tested for anti-inflammatory activity against carrageenan-induced hind paw edema in BALB/c mice. Observations were made after 1h, 2h, and 3h of oral dose for edema treatment. Diclofenac potassium was used as a positive drug and showed $77.6 \pm 3\%$ activity after 3h post dosage. ABRL3 revealed the highest anti-

inflammatory activity ($82.3 \pm 2 \%$) while WT manifested the lowest activity ($46.6 \pm 2 \%$) among all tested samples (Figure 4b).

Antidepressant activity

The potential antidepressant activity of *A. bracteosa* crude extracts was evaluated in mice by tail suspension test and results are displayed in Figure 4c. All transgenic lines exhibited enhanced antidepressant activity compared to wild type and positive control (Fluoxetine-HCl). ABRL3 demonstrated lowest immobility time of 38.3 ± 2 sec followed by 45.7 ± 3 sec in ABRL1 and 63.3 ± 4 sec in ABRL2.

Anticoagulant activity

Anticoagulant activity of wild type and transgenic lines of *A. bracteosa* is shown in Figure 4d. Transgenic lines delayed blood clotting from 2.46 min in negative control to 4.51min, 5.40 min, and 5.41 in ABRL1, ABRL2, and ABRL3 treated mice respectively. WT crude extracts were least effective among all plant extracts with a clotting time of 3.3 min.

Discussion

The plant kingdom consist of countless species with number of uses in medicines. They contain valuable secondary metabolites demonstrating anti-inflammatory, anticancer, antioxidant antidiabetic and/or antimicrobial properties [23]. The growing demand for these plant secondary metabolites forces the use of new green biotechnology tools to create new, more productive in vitro transgenic plant cultures [24]. This study reports the examination of the potential role of *ro/ABC* genes in increasing the production of secondary metabolites and compares the pharmaceutical efficacy of transgenic regenerants and *in vitro* grown untransformed *A. bracteosa* plants.

Minerals are requisite for disease resistance and normal physiology of the human body; however, high concentrations could be a health risk. Major elements including sodium, potassium, calcium, and magnesium are involved in protein synthesis, nerve transmission, bone development, muscle contraction, and enzyme activation [25]. Here we found that all studied elements were below the maximum permissible limit as recommended by WHO and European pharmacopeia [26]. Among 10 detected elements, potassium was most abundant in all samples. However, the Na/K ratio was below 1 which is required to maintain normal blood pressure [27]. Only minute amounts of 6 trace elements were detected. These trace elements have well reported anti-inflammatory, antianemia, antidiabetic, and anticancer properties [28].

Next, phytochemical analysis was carried out to investigate the effects of transformation on secondary metabolite production. Qualitative assays screened several medicinally active molecules in *A. bracteosa* based on their polarities. Considerable amounts of alkaloids, phenolics, flavonoids, and glycosides were present in all extracts. These phytoconstituents possess antimicrobial, antioxidant, anti-inflammatory, and anticancer properties [29]. Saponins and tannins used as astringents, hepatoprotective,

cardioprotective, and anticancer agents [30] were present in moderate quantities. The presence of terpenoids in transgenic plants can impart antioxidant, antibacterial, antiviral, chemoprotective, and neuroprotective characters [31,32] to these transgenics.

The antioxidant activity of phenolics and flavonoids primarily depends on the presence of hydroxyl groups [33]. Current study demonstrated significantly higher values of TPC and TFC in transformed regenerants as compared to untransformed plants. Our observations are in accordance with the studies reporting enhanced TPC and TFC of *Artemisia dubia*, *A. annua*, and *Lactuca sativa* plants transformed with *rolABC* genes [20,34,35].

RP-HPLC delineated a marked surge in polyphenols of transformed plants than their untransformed counterparts. These differences support our previous findings indicating that *rolABC* genes enhanced the expression of biosynthetic pathway genes in transformed regenerants of *A. bracteosa* [15]. We found that ABRL3 presented highest expression of β -hydroxy β -methylglutaryl-CoA reductase (HMGR), farnesyl diphosphate synthase (FDS), 4-hydroxy-3-methyl-but-2-enyl pyrophosphate synthase (HDS), and phenylalanine ammonia lyase (PAL) genes owing to significant phenolic content and antioxidant activity of this line. Higher expression of the metabolic pathway genes can easily be correlated to the higher production of phytoecdysteroids and hence the polyphenols contents. These polyphenols are reported to prevent aging and age related disorders such as cancer, cardiac malfunctions, diabetes, neurodegenerative problems, and inflammatory disorders [3]. Their protective mechanisms involve suppression of oxidative stress, maintenance of Nuclear Factor Kappa B (NF- κ B) and interleukins (IL-1 β), activation of gluconeogenesis, inhibition of angiogenesis, and cyclooxygenases (COX-1 and COX-2) [36]. Our results show that six polyphenol markers, namely, plumbagin, thymoquinone, catechin, emodin, gentisic acid, and luteolin were below the detection limit in all crude extracts. However, some earlier studies [10] reported catechin in ethyl acetate and aqueous fractions of *A. bracteosa*. These findings are likely to be related to diverse nature of metabolites and polarity in different solvent systems [37].

All extracts were evaluated for TAC, TRP, and antioxidant activity through DPPH, hydroxyl radical scavenging and iron chelating power assays. Transgenic lines fared better for reducing power and antioxidant activity than untransformed plants. Similar pattern of results was found in *Artemisia annua* [38] and lettuce [39] plants transformed with different *rol* genes. We observed a significantly positive correlation between antioxidant activities and plant phenolics. Whereas moderate correlation with TFC suggests that antioxidant properties are attributed to higher phenolics than flavonoids content [10].

Hot plate assay is simple and sensitive method to assess anti-nociceptive drugs to relieve pain [22]. The results of analgesic activity showed that transgenic plant extracts protected mice from visceral pain and produced comparable effects to standard drug aspirin. Nonsteroidal anti-inflammatory drugs (NSAIDs) can be used to treat inflammation associated with pain. However, their overuse can lead to serious side effects. Therefore, herbal products are used as an alternative to antagonize inflammatory agents [22]. In

current study *A. bracteosa* extracts reduced the carrageenan induced edema in mice following the same mechanism as NSAIDs. Different studies suggest that withanolides and phenolics present in *A. bracteosa* inhibit activity of prostaglandins and COX [40,41]. Terpenoids, phytoecdysteroids, and phenolics attribute to anti-depressant and anticoagulant properties of transformed *A. bracteosa* plant extracts. Thus, this plant can be a potent agent to treat neurodegenerative and thrombolytic disorders [8,20]. Our findings are in agreement with former results [39] verifying that *rol* genes increased pharmaceutical value of methanolic extracts of transformed lettuce in rats.

Conclusions

In conclusion, present findings suggest that transgenic *A. bracteosa* plants are a rich source of essential elements and polyphenols. Overall, all transgenic lines showed significant antioxidant, analgesic, anti-inflammatory, antidepressant, and anticoagulant activities as compared to the untransformed wild type plant extracts. We could see that *rol* genes influenced positively the production of active secondary metabolites and this could be linked to the enhanced activities exhibited by the transgenic plant extracts. This study provides more rationalized scientific reasons for the folklore use of this plant. However, further investigations are needed to understand the underlying protective mechanisms.

Declarations

Ethics approval and consent to participate

Animal study was approved by the Animal Ethical Committee, Quaid i Azam university, Islamabad, Pakistan (BEC-FBS-QAU2019-157).

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

SR and BM designed the project. SR carried out the experimental work. NA and RM analyzed the data. FM and AK helped in HPLC analysis of transgenics. SR wrote the manuscript while WKK and BM proofread and critically evaluated it. BM supervised the entire study. All authors have read and approved the final manuscript.

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Tables

Table 1 Quantity of different elements in aerial parts of *Ajuga bracteosa*

Elements ($\mu\text{g}/\text{mg}$)	λ_{max} (nm)	Slit width (nm)	Samples				WT= <i>in vitro</i> grown
			WT	ABRL1	ABRL2	ABRL3	
Sodium	589.0	0.5	2.86 \pm 0.2	3.54 \pm 0.8	3.07 \pm 1	3.94 \pm 2	
Potassium	766.5	1.0	8.06 \pm 0.3	12.00 \pm 1	12.62 \pm 3	13.09 \pm 2	
Calcium	422.7	0.5	0.50 \pm 0.06	1.69 \pm 0.5	1.32 \pm 0.4	1.95 \pm 0.5	
Magnesium	285.2	0.5	0.91 \pm 0.1	2.15 \pm 0.3	1.12 \pm 0.2	2.12 \pm 0.7	
Zinc	213.9	1.0	0.25 \pm 0.05	0.40 \pm 0.1	0.24 \pm 0.04	0.47 \pm 0.03	
Iron	248.3	0.2	0.25 \pm 0.1	0.33 \pm 0.2	0.46 \pm 0.1	0.44 \pm 0.2	
Manganese	279.5	0.2	0.004 \pm 0.01	0.02 \pm 0.01	0.01 \pm 0.03	0.02 \pm 0.01	
Nickel	232.0	0.2	0.09 \pm 0.02	0.18 \pm 0.1	0.19 \pm 0.1	0.23 \pm 0.2	
Copper	324.8	0.5	0.005 \pm 0.01	0.01 \pm 0.02	0.01 \pm 0.03	0.02 \pm 0.01	
Chromium	357.9	0.2	0.16 \pm 0.03	0.45 \pm 0.1	0.43 \pm 0.2	0.36 \pm 0.1	

untransformed *Ajuga bracteosa* plant extract, ABRL1-3=crude extracts of transgenic line 1, 2 and 3 of *A. bracteosa*. Data is represented as mean \pm SD (n=3)

Table 2 Phytochemical constituents of *Ajuga bracteosa*

Phytochemicals	Samples			
	WT	ABRL1	ABRL2	ABRL3
Alkaloids	++	++	+++	+++
Glycosides	+	+++	++	+++
Flavonoids	+	++	++	+++
Phenols	++	++	++	++
Tannins	+	+	+	+
Saponins	+	+	++	++
Terpenoids	-	+	+	+
Coumarins	-	-	-	-
β -cyanins	-	-	-	-
Anthocyanin	-	-	-	-
Sterols	-	-	-	-

(+) present (++) moderate concentration (+++) high concentration (-) absent. WT= *in vitro* grown untransformed *Ajuga bracteosa* plant extract, ABRL1-3=crude extracts of transgenic line 1, 2 and 3 of *A. bracteosa*

Table 3 Polyphenolic composition of crude extracts of *Ajuga bracteosa*

Serial no.	Compound name	λ_{\max} (nm)	Extracts ($\mu\text{g}/\text{mg}$ dry extract)			
			WT	ABRL1	ABRL2	ABRL3
1	Vanillic acid	257	8.98 \pm 1	15.87 \pm 3	15.49 \pm 2	16.33 \pm 1
2	Rutin	257	0.63 \pm 0.5	4.49 \pm 1	9.24 \pm 2	14.86 \pm 2
3	Plumbagin	257	Nd	Nd	Nd	Nd
4	Thymoquinone	257	Nd	Nd	Nd	Nd
5	Gallic acid	279	4.59 \pm 0.3	14.99 \pm 2	15.01 \pm 3	16.67 \pm 1
6	Catechin	279	Nd	Nd	Nd	Nd
7	Syringic acid	279	10.79 \pm 0.8	13.93 \pm 2	12.41 \pm 1	17.78 \pm 3
8	Coumaric acid	279	1.92 \pm 0.7	15.39 \pm 3	14.02 \pm 1	23.45 \pm 2
9	Emodin	279	Nd	Nd	Nd	Nd
10	Gentisic acid	325	Nd	Nd	Nd	Nd
11	Caffeic acid	325	13.39 \pm 2	25.51 \pm 3	22.01 \pm 2	30.18 \pm 4
12	Ferulic acid	325	75.55 \pm 3	77.17 \pm 4	76.86 \pm 4	78.05 \pm 3
13	Cinnamic acid	325	3.19 \pm 0.7	4.36 \pm 0.5	5.47 \pm 0.2	6.09 \pm 0.3
14	Luteolin	325	Nd	Nd	Nd	Nd
15	Apigenin	325	8.20 \pm 2	20.84 \pm 5	23.12 \pm 3	32.29 \pm 4
16	Myricetin	368	4.14 \pm 0.7	13.66 \pm 3	11.6 \pm 2	13.37 \pm 4
17	Quercetin	368	4.68 \pm 0.3	6.44 \pm 0.8	7.52 \pm 1	9.19 \pm 0.5
18	Kaempferol	368	17.6 \pm 2	83.9 \pm 4	78.6 \pm 5	101.26 \pm 6

WT= *in vitro* grown untransformed *Ajuga bracteosa* plant extract, ABRL1-3=crude extracts of transgenic line 1, 2 and 3 of *A. bracteosa*. Data is represented as mean \pm SD (n=3)

Figures

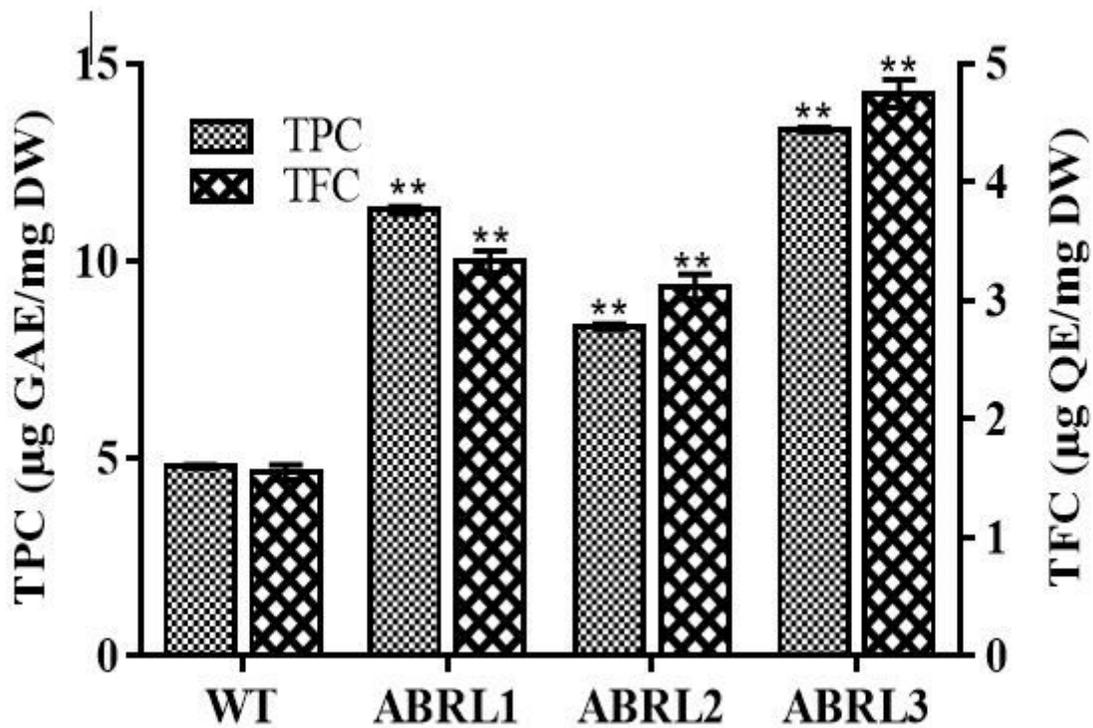


Figure 1

Phytochemical analysis and antioxidant potential of crude extracts of *Ajuga bracteosa*. (A) Total phenolic and total flavonoids content. WT= wild type untransformed *A. bracteosa* plants, ABRL1-3= Transgenic lines 1-3 of *A. bracteosa*. Each value represents mean \pm SD (n=3). **p < 0.01 statistical significance

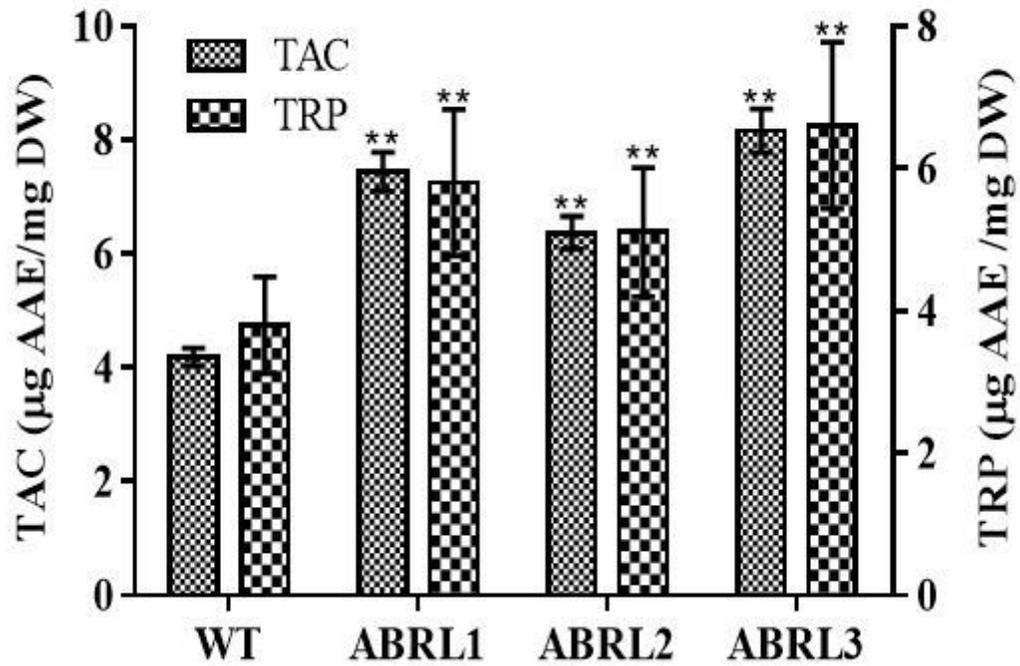


Figure 2

Total antioxidant capacity and total reducing power. WT= wild type untransformed *A. bracteosa* plants, ABRL1-3= Transgenic lines 1-3 of *A. bracteosa*. Each value represents mean \pm SD (n=3). **p < 0.01 statistical significance

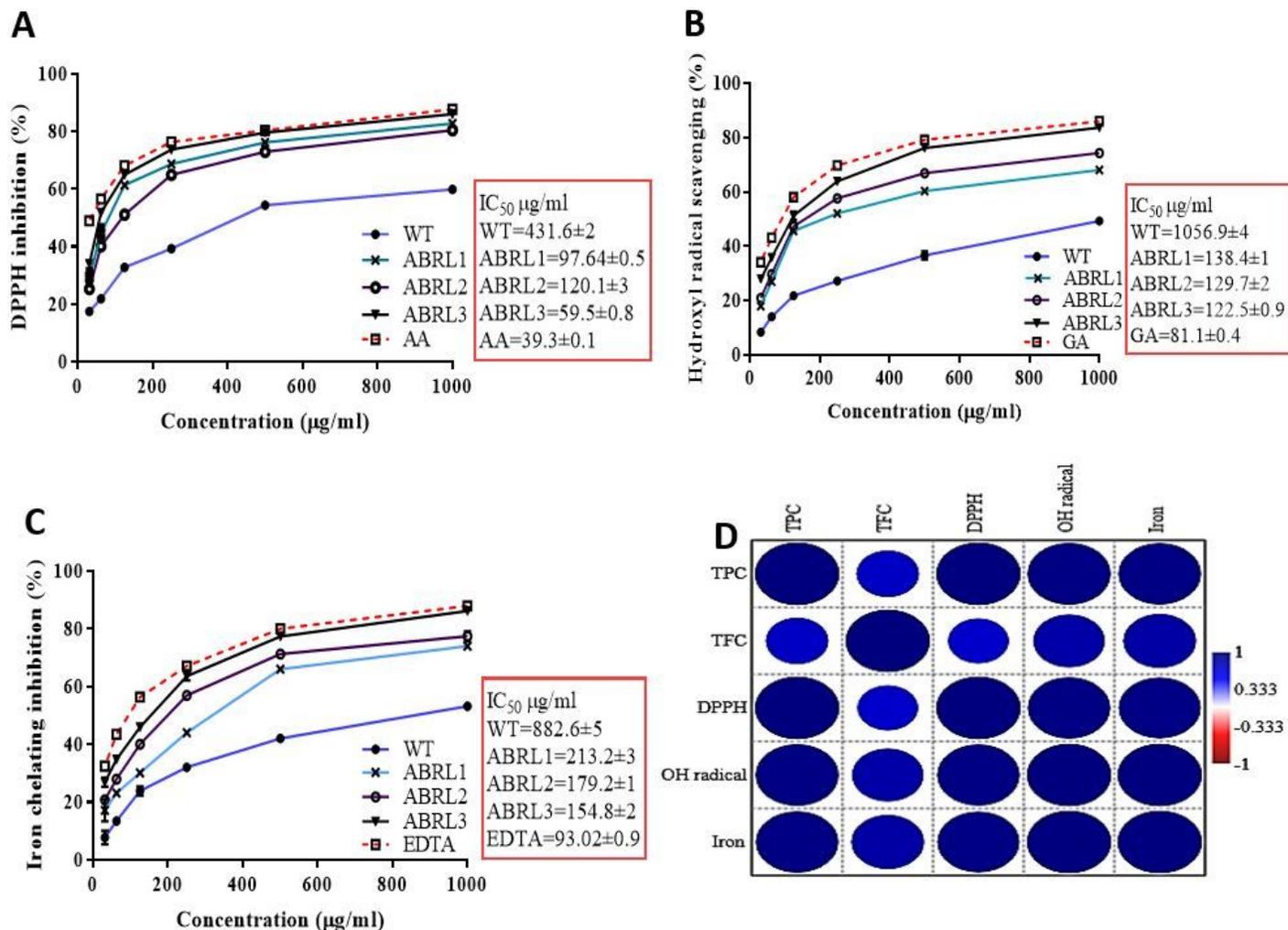


Figure 3

In vitro Antioxidant activities of crude extracts of *Ajuga bracteosa* at different concentration. (A) DPPH scavenging activity. (B) Hydroxyl ion scavenging activity. (C) Iron chelating power along with IC₅₀ values. (D) Correlation between TPC, TFC and IC₅₀ values of antioxidant assays. WT= wild type untransformed *A. bracteosa* plants, ABRL1-3= Transgenic lines 1-3 of *A. bracteosa*. Each value represents mean \pm SD (n=3). *p < 0.05, **p < 0.01 statistically significant

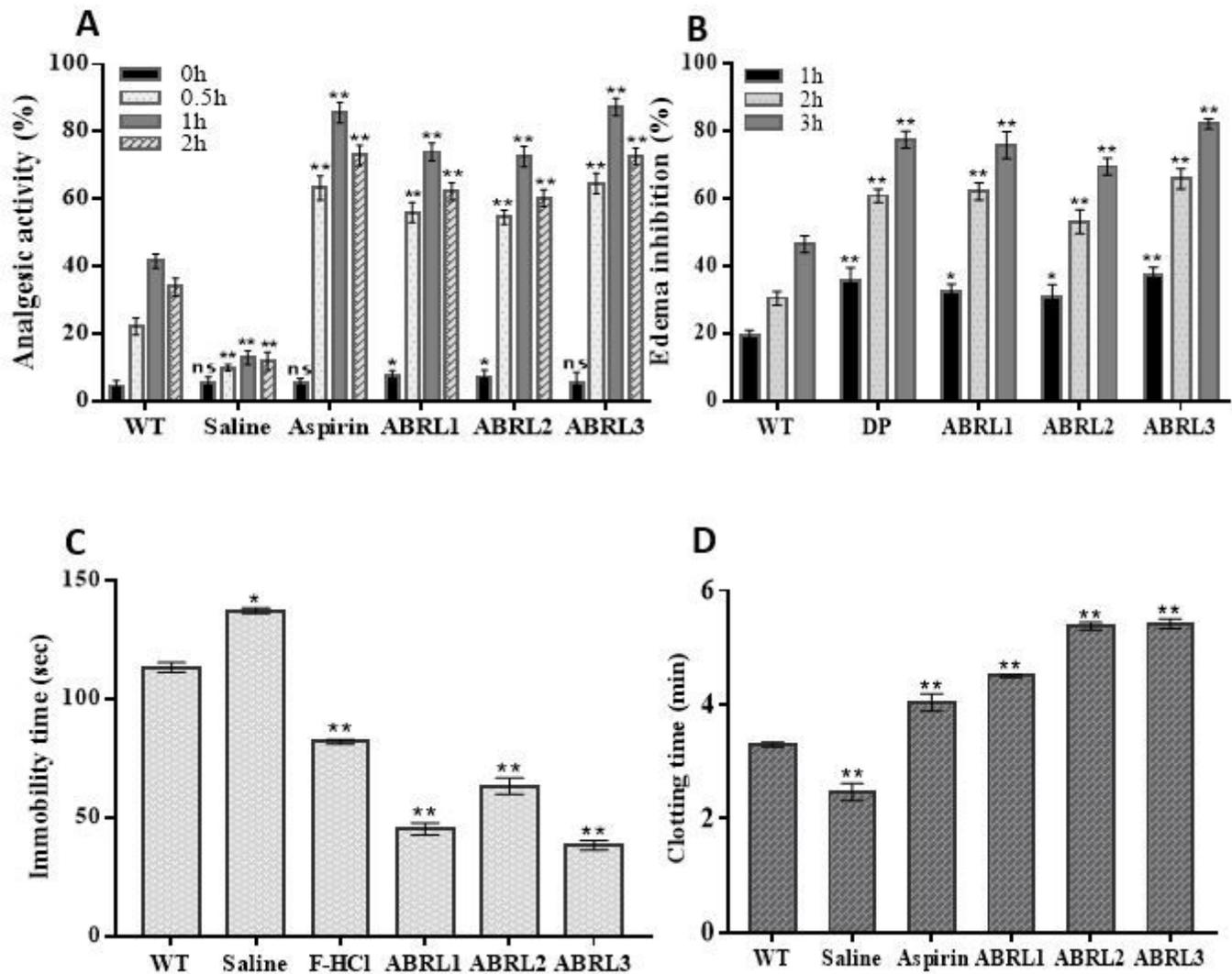


Figure 4

In vivo activities *Ajuga bracteosa* crude extracts. (A) Analgesic activity. (B) Anti-inflammatory activity. (C) Anti-depressant activity. (D) Anti-coagulant activity. F-HCl= Fluoxetine HCl , DP= Diclofenac Potassium WT= wild type untransformed *A. bracteosa* plants, ABRL1-3= Transgenic lines 1-3 of *A. bracteosa*. Each value represents mean \pm SD (n=5). ns shows non-significant, *p < 0.05, **p < 0.01 statistically significant data

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