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Rapid screening of antioxidant activity, fracture rate and scavenging of free radicals by hairy root of Periwinkle (*Catharanthus roseus* L. G. Don)

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ABSTRACT

Background & Aim: Oxidative stresses caused by free radicals are known to promote many diseases such as inflammation and cancer; therefore, any plant product that can inhibit free radical production can play an important role in preventing diseases. We aimed to compare the fracture rate and the scavenging activity of free radicals, and the antioxidant composition of *rol*B-transformed hairy root of *Catharanthus roseus* with those of the plant's natural root and leaf.

Experimental: Hairy root of the Madagascar periwinkle (*C. roseus*) were induced using *Agrobacterium rhizogenes* strain ATCC 15834 to transfer the *rol*B gene. PCR analysis was used to identify the gene's presence in the transformed hairy root. Folin-Ciocalteu reagent and HPLC were used to determine total phenolics and gallic acid. This study also entailed rapid screening of antioxidant by dot-blot DPPH staining on thin-layer chromatography (TLC), radicals fracture rate, free radical scavenging by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and nitric oxide-scavenging activities by Griess reagent.

Results: Our results showed that phenolic and gallic acid content in the ethanol extract of hairy root was significantly higher ($p \leq 0.05$) than that/ naturally found in the root and leaf of *C. roseus*. The hairy root extract had the highest dot-blot staining and fracture rate of radicals and the lowest IC_{50} for inhibition of $DPPH^{\bullet}$ and $^{\bullet}NO$. The transgenic hairy root of *C. roseus* showed high number of scavenging radicals, whereas the most active ethanol extract of hairy root registered by the highest level of gallic acid and total phenolic content.

Recommended applications/industries: The transformed hairy roots can be considered as a rich natural source of antioxidants.

1. Introduction

Catharanthus roseus L. G. Don (formerly *Vinca rosea* L., Apocynaceae) is commonly known as the Madagascar periwinkle. It is a perennial evergreen herb, 30-100 cm tall originally native to the island of Madagascar but now widely dispersed in the tropics (Lata, 2007). The significance of this plant is its ability

to synthesize a wide range of terpenoid indol alkaloids with medicinal values. These compounds have a broad application in the treatment of leukemia in children, and lymphocytic leukemia, Wilkins's tumor, neuroblastoma and reticulum cell sarcoma, Hodgkin's disease besides lympho sarcoma, choriocarcinoma (Aslam *et al*., 2010). Except alkaloids, other natural compounds in *C. roseus* have been less investigated. Oxidative stress occurs as the result of an imbalance between the internal production of free radicals and the antioxidant defense mechanisms of living organisms (Lobo *et al*., 2010). Active oxygen species at low or medium concentrations are integral to normal cellular responses and a healthy immune system. When the level of free radicals is increased and when both the enzymatic systems and low molecular antioxidants are not sufficient to protect the organism, free radicals formed in excess induce cellular damage, which contribute to the development of diseases well as the spoilage of food products (Shaw *et al*., 2005). Thus, antioxidants, as inhibitors of free radicals, boost the immune system and reduce the risk of cancer and other degenerative diseases. In recent years, the use of synthetic antioxidants and other chemical additives has been limited because of their as toxicity and possible role in carcinogenesis. Consequently, the discovery of natural alternatives, particularly plants, poses seminal area where much research is being conducted (Panicker *et al*., 2014; Esfa, 2012; Atta *et al*., 2007). The hairy root culture is as an important resource of valuable secondary metabolites owing to rapid growth, short doubling time, and the dependable synthesis of chemical compounds (Pistelli *et al*., 2010).

In the past two decades, considerable attention has been paid to the synthesis of these valuable hairy root compounds. The employment of cell suspension cultures from *C. roseus* has greatly expanded the production and enhanced the stability of secondary metabolites. In recent decades, this technique has extensively been used to produce more effective alkaloids and anti-cancer drugs from *C. rosus* (Goknay *et al*., 2009). Continuing to research the anti-oxidation potential of these hairy roots can lead to other, more efficacious, applications of this transgenic product. Among the natural products with antioxidant properties, phenolic compounds have been extensively studied. Because these compounds neutralize free radicals and inhibit lipid oxidation, they inhibit the formation of toxins such as malondialdehyde (MDA). Such phenolic compounds are particularly useful in the preservation of food products and in the pharmaceutical industry. Additionally, these phenolic compounds directly promote health and well-being through disease prevention (Stoilova *et al*., 2007). Multiple studies have shown that C. *roseus* has a high phenolic content (Pereira *et al*., 2010; Ferreres *et al*., 2008). A study conducted on aqueous extract of the plant root and leaf reported that the root of *C. roseus* has great antioxidant potential in scavenging free radicals (Pereira *et al*., 2010; Ferreres *et al*., 2008). To the best of our knowledge, no other report was found on the non-enzymatic antioxidant potential of plant hairy roots, and also their phenolic content. After considering the phenolic compounds found in hairy root, and the testing costs involved, gallic acid was the antioxidant compound chosen for quantitative analysis. Gallic acid is a type of phenolic acid with chemical formula $C_6H_2(OH)_3COOH$ and antioxidant, anti-inflammatory, antifungal and antitumor properties (Claudio *et al*., 2012). The present study assessed the antioxidant properties of transgenic hairy root of *C. roseus* by measuring the free radical scavenging activity and speed of radical destruction compared with the root and leaf.

2. Materials and Methods

2.1. Plant material

Using seeds from the roseus varieties of *C. roseus*, we cultivated the plants in pots inside a greenhouse under controlled conditions. A sample of the plant was identified by Dr Amin and a voucher specimen (6559-THE) was deposited in the herbarium, at the College of Pharmacy, Tehran University of Medicinal Science, Tehran, Iran.

2.2. Agrobacterium rhizogenes suspension preparation

A. rhizogenes strainATCC 15834 bacteria was cultivated in a new solid culture medium of LB (10 g L^{-1} bacto tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium

chloride plus agar 10 g L^{-1} in the acidity of 7) containing 50 mgL^{-1}, rifampicin and at temperature of 28ºC. A colony of grown bacteria was added to 5 mL liquid LB medium at 28ºC on 180 rpm shaker for 24 h in the dark. One milliliter of culture suspension was then added to 30 mL of the same medium for 24 h, and the concentration of bacteria was adjusted to 0.4 - 0.6 by spectrophotometer (UV/Vis T90 PG instrument) at 600 nm wavelength (Soleimani *et al*., 2012). The bacteria strain was provided from the National Research Center for Genetic Engineering and Biotechnology of Iran.

2.3. Induction and establishment of hairy root culture

The hairy roots were induced by injection of *A.rhizogenes* strain ATCC 15834 suspension culture to leaves. All explants were pre-cultured for 2 days on MS (Murashinge *et al*., 1962) basal medium. Insulin syringes were used for injection. In order to infect by injection, 5 µL of bacteria suspension were injected in to the dorsal vein of each leaf via insulin syringes angled at one or two positions. Samples were dried after 30 min with a sterile filter paper to remove surface pollution and transferred to petri dishes containing solid MS medium. Petri dishes were kept in darkness at 26ºC for 48 h, and the samples were transferred to the MS medium (no hormone, 3% sucrose, 500 mg L^{-1} cefotaxime and 0.8% agar). Roots appeared at the wound sites 10 - 15 days after infection, were allowed to grow about 2 cm, and were transferred to the MS medium supplemented with cefotaxime 500 mg L^{-1}. The first 45 lines obtained from *A. rhizogenes* infection were separated according to the growth rate. On the basis of macroscopic observations, one line from each typically morphology was selected. The hairy root showing the rapidest growth was chosen and cultured in 50 mL ½ MS liquid medium containing 3% sucrose and 500 mg L^{-1} cefotaxime on a 60 rpm shaker. To ensure a sufficient amount of hairy roots, a subculture was created once every 3 weeks.

2.4. PCR analysis

After the removal of bacteria, DNA was extracted via the CTAB procedure from hairy roots as well as from roots and leaves of *C. roseus* plant (Khan *et al*., 2007). PCRwas performed for 35 thermal cycles (denaturizing at 94 ºC for 1 min, primer annealing at 57ºC for 1 min, and primer extension at 72°C for 1 min) for *rol*B (forward primer 5´-ATGGATCCCAAATTGCTATTCCCCACGA-3' and reverse primer 5'-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3') specific primers (Rahnama *et al*., 2008). The amplified product was run on 1.2% (w/v) agarose gel at 100 V to separate the DNA fragments.

2.5. Extraction

The roots and leaves grown in a green house, the grown hairy roots transformed in the medium were adequately collected and dried in an oven at the temperature of 40°C for 72 h and then ground and powdered by a mill (IKA, Germany). Extraction was conducted through Sun et al. method (Sun *et al*., 2007) with a little modification. Briefly, the extract was taken from the powdered samples in each extraction with a 1:1 ratio by ethanol 80%, while being stirred at the temperature of 40°C for 2 h. After this period, the mixture was filtered and the residue was remixed with the same solvent mixture ratio and the operation was repeated for 2 h. The filtered solutions of both stages were mixed together. The extracts were condensed by the help of a rotary evaporator (Heidolph 4001, Germany) at the temperature of 40°C and were dried by a freeze dryer (Cherist 1-4 LD, UK). The extracts were kept at the temperature of -50°C until testing.

2.6. Antioxidant composition

2.6.1. Total phenolic content (TPC). Total phenolic compound content was determined by the Folin-Ciocalteau method (Singleton *et al*., 1999). In this way, 1 mL of ethanol was added to 10 mg of dried extract powder of each plant species and stirred well by vortex; then 200 µL of diluted folin reagent (1:10) was added to 40 µL of this extract and stirred well by vortex. Afterwards, 3120 µL of double-ionized water was added to it and then 600 µL of sodium carbonate 7.5% was added to it after 5 min. After 15 sec of being in vortex,

the samples were kept in a dark place at the temperature of 23°C for 90 min and the absorbance of samples was measured at the wavelength of 760 nm by the help of a spectrophotometer (UV/Vis T90 PG instrument Ltd). The standard diagram was drawn based on 50 – 1000 µg mL^{-1} tannic acid, and the amount of phenolic compounds in the plant was measured equivalent to the µg of tannic acid in 1 mg of dried extract (µg TAE mg^{-1} extract).

2.6.2. Gallic acid content (GAC). The chromatographic analysis was carried out on a Knauer HPLC system (Germany) equipped with a K-1001 pump and a UV-Vis detector (K-2600) at 271 nm, column: C-18 (4.6×250 mm, 5 µ particle size). An isocratic mobile phase of methanol: Ethyl acetate: water (25:50:70 v/v) with an elution volume of 0.7 mL min^{-1} was selected. Injection volume was 20 µL. The column temperature was maintained at 37°C. Area under each peak was calculated. To obtain the standard curves, five different concentrations (1 - 5 µg mL^{-1}) of gallic acid were injected, and the standard curves were plotted (Sawant *et al*., 2013).

2.7. Antioxidant activity

2.7.1. Rapid screening of free radical scavenging by dot-blot and DPPH staining. Screening of free radical scavenging of the ethanol extracts was eye-detected semi-quantitatively by a rapid DPPH staining TLC layer (silica gel 60 F254 on an aluminum backing; Merck). An aliquot (3µL) of each dilution of each sample with concentrations of 0.125 to 2 mg mL^{-1} was carefully placed on a TLC layer and allowed to dry for 3 min. The sheet bearing the dry spots was placed upside down for 10 sec in a 0.4 mM DPPH solution in ethanol. The excess solution was removed with tissue paper and the layer was dried with a hair-dryer (Chang *et al*., 2007).

2.7.2. Fracture rate of free radical activity. The fracture rate of $DPPH^{\bullet}$ activity of ethanol extracts was assessed using Brand-Williams et al. method (Brand-Williams *et al*., 1995). In this method, the concentrations of 6.25 to 200 µg ml^{-1} with the volume of 2 mL were prepared from each extract and poured into a test tube. 2 ml of ethanol $DPPH^{\bullet}$ with the concentration of 100 mM was added to each tube and stirred well in vortex for 15 sec; the absorption was read at the wavelength of 517 nm. The samples were kept in the dark place and the last absorption of each sample was measured after 30 min. The reaction rates were calculated using equation: Abs^{-3}-Abs_0^{-3} = -3kt. where k is the $DPPH^{\bullet}$ bleaching rate, while Abs_0 is the initial absorbance value, and Abs is the absorbance at increasing time. The antioxidant activity was expressed as Δ Abs/min/mg dry weight of extract.

2.7.3. Free radical scavenging activity (DPPH assay). The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity was assessed using Brand-Williams et al. method (Brand-Williams *et al*., 1995). The absorption was read at the wavelength of 517 nm after 60 min. Free radicals not treated with extracts served as controls. The following equation was utilized to calculate the percentage of free radical scavenging: Scavenging activity% = $(1 - A_s/A_0) \times 100$. Where A_s is the absorbance in the presence of extract or positive controls, while A_0 is the absorbance in the absence of extract and positive controls. IC_{50} was defined as the concentration of the sample, required for scavenging 50% of the DPPH free radicals (Molyneux *et al*., 2004).

2.7.4. Nitric oxide-scavenging activity. Investigation of nitric oxide radical inhibition was done by the help of Griess reagent (Govindarajan *et al*., 2003). In this experiment, the reaction mixture (3 ml) containing 2 ml sodium nitroprusside, (5 mM) and 0.5 ml phosphate buffer with 0.5 ml extract with concentrations of 25 to 200 mg ml^{-1} was incubated at room temperature for 150 min. Then 0.5 ml of reaction was mixed with 1 ml of sulfinic acid (0.33%) in 20% glacial acetic acid. After 5 min, 1 ml naphthyl ethylenediamine dihydrochloride 0.1% was added to and mixed with it, then placed at room temperature for 30 min. A pink color was formed in the solution. The absorption of this colored solution was measured at 540 nm against blank. The inhibition percentage was calculated according to the following formula. The IC_{50} concentration of sample is capable of inhibiting 50% of nitric oxide radical. Inhibition% = $[1-A_s/A_0] \times 100$. Where A_s is the absorbance in the presence of ethanol extract, while A_0 is the absorbance in the absence of ethanol extract.

2.8.Statistical analysis

The experimental results are expressed as mean with standard error. All measurements were taken in triplicate. The data were analyzed by an analysis of variance ($P \leq 0.05$) and the means were separated by

Duncan's multiple range tests. The IC_{50} values were calculated from a linear regression analysis.The statistical analysis was performed by SPSS

3. Results and discussion

3.1. Hairy roots induction& Molecular confirmation

Our morphological investigation revealed that *A. rhizogenes* strain ATCC 15834 was able to induce hairy roots from leaf explants of *C. roseus* during the 2 weeks. The hairy roots were long and very diffuse. In the control samples, no hairy roots were evident. PCR products amplified with *rol*B primers were detected in the hairy root and positive control (fragment with 500 bp). These results indicate that the *rol*B gene (500 bp), from the T-DNA of the *A. rhizogenes* Ri-plasmid, was integrated in to the genome of *C. roseus* hairy roots (Figure1). No bond was formed in the wells, which corresponded to our control samples (DNA extracted from natural root and leaf).

Various species of bacteria can transfer genes to higher plants (Broothaerts *et al*., 2005). In this experiment we used *A. rhizogenes* ATCC 1538. This bacterium harbors a Ri-plasmid involved in the root induction process; the root loci (*rol*) genes located in the TL-DNA region and the aux genes of the TR region (Baldi and Dixi, 2008). *A. rhizogenes* 15834 is the most common strain of bacteria used for hairy root induction (Setamam *et al*., 2014). Several other studies have reported on hairy roots induced by this strain (Gangopadhyah *et al*., 2010; Ahmadi Moghadam *et al*., 2014).

This study aimed to investigate and compare the antioxidant potential of transgenic hairy roots from the leaves of *C. roseus* infected with *A. rhizogenes* strain ATCC 15834with natural root and leaf of this plant. In our experiment, hairy roots appeared in the wound location 10 - 15 days after infection. In another study, exit points of hairy roots which induced by *A. rhizogenes* strain ATCC 15834 from the stem, hypocotyl and epicotyl, were reported after 10 days (Pratap *et al*., 2008). Transformation efficiency was 3% for whole leaves by the injection method. PCR analysis of the hairy roots verified transgenesis. That is, *rol*B gene (500 bp) from the T-DNA of the *A. rhizogenes* Ri-plasmid was integrated in to the genome of *C. roseous* leaves (Figure1). *rol*B is an important oncogene because when it is inactivated, transgenesis for root phenotype production will be disabled with the rest of the oncogene (Tzfira *et al*., 2006).

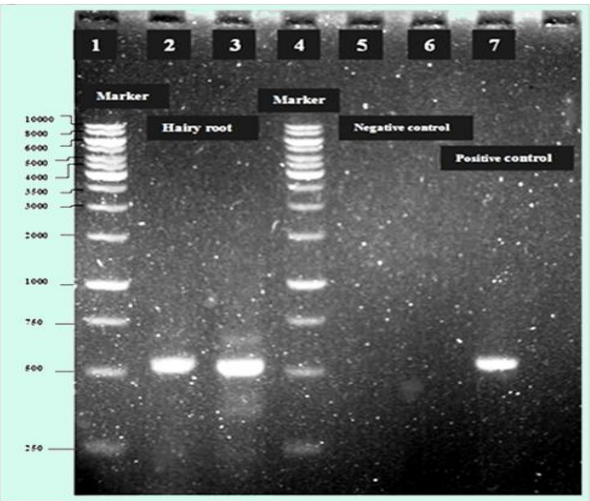

Fig 1. PCR analysis of hairy root culture of *C. roseu*s transformed *A.rhizogenes* ATCC 15834: lane 1 and 4, Marker (1kbp), lane 2 and 3, genomic DNA of hairy root culture showing amplified fragment of *rol*B (500bp), lane 5 and 6, genomic DNA from normal root and leaf culture separately, (negative control), lane 7- plasmid genome (positive control).

3.2. Antioxidant composition

The results showed the significant difference between the root,leaf, and hairy root for phenolic content. Phenolic content was higher in hairy roots than in root, and leaf, equal to µg of tannic acid per mg of dried weight of extract according to the standard curve of $y = 0.000492x + 0.04819$, $r^2 = 0.973$. Gallic acid content was higher in hairy root than in root, and leaf, equal to µg chatechin per mg of dried weight of extract according to the standard curve of $y = 34385.7x + 845900$, $r^2 = 0.992$ by HPLC method (Figure2).

3.3. Antioxidant activity

Figure 3 shows the stained silica layer revealing a purple background with bright yellow spots at the location where radical-scavenger capacity presented. The intensity of the bright yellow color depends upon the amount of radical scavenger present in the sample

(Huang *et al*., 2004). The ethanol extract of hairy root proved more active than root and leaf extracts.

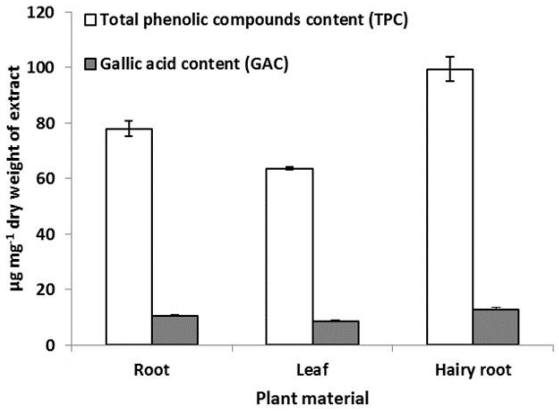

Fig 2. The phenolic and gallic acid content of ethanol extracts of hairy root, callus, root, and leaf of *C. roseus*. Data show means of three replicates with standard error.

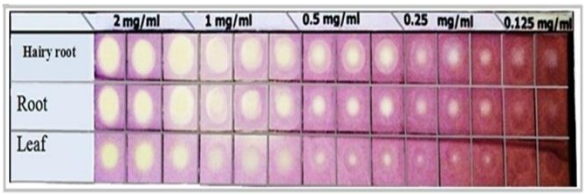

Fig 3. Dot blot assay by ethanol extracts of hairy root, root, and leaf of *C. roseus* on a silica sheet stained with a DPPH solution in ethanol, n=3.

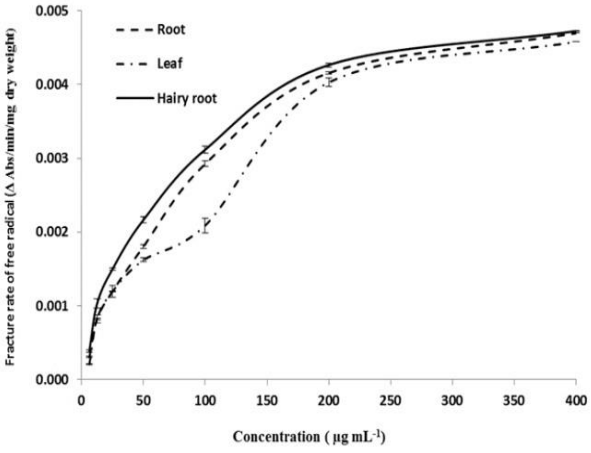

Fig 4. Fracture rate of free radicals (ΔAbs/min/mg dry extract) by ethanol extracts of hairy root, root, and leaf of *C. roseus*. Data show means of three replicates with standard error.

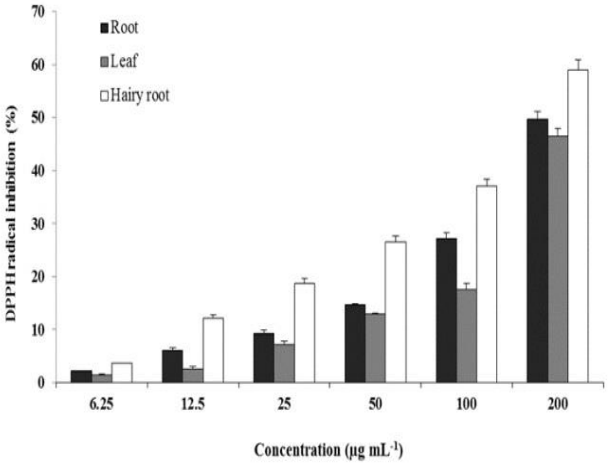

Fig 5. Scavenging of DPPH• activity by ethanol extracts of hairy root, callus, root, and leaf of *C. roseus*. Data show means of three replicates with standard error.

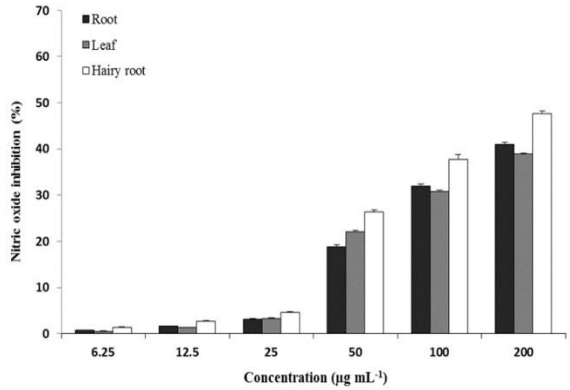

Fig 6. Nitric oxide radical scavenging activity by ethanol extracts of hairy root, root, and leaf of *C. roseus*. Data show means of three replicates with standard error.

Table 1. Pearson's coefficients of determining the relationship between phenolic and gallic acid content with antioxidant capacity of ethanolic extracts.

	phenolic	Gallic acid	DPPH	Nitric oxide
phenolic	1	0.956**	-0.921**	-0.959**
Gallic acid		1	-0.884**	-0.991**
DPPH			1	0.853**
Nitric oxide				1

** Significant at $p \leq 0.01$

Figure 4 shows the rate of destruction of free radicals significantly increased when adding a higher concentration of extracts. Free radicals were eliminated at a faster rate by ethanol extract of hairy root than extracts of *C. roseus* root and leaf.

As shown in Figure 5, the DPPH free radical trapping activity of extracts was significantly greater at higher concentrations. Hairy root extract showed the best performance in DPPH free radical trapping activity compared with *C. roseus* root, and leaf. At a concentration of 200 µg mL^{-1} of hairy root extract, it was able to harness more than 52% of the extant DPPH radicals, which was a notably high scavenging rate.

The hairy roots verified transgenesis, and enabled comparison of their antioxidant potential with the natural root and leaf. In our preliminary investigations of antioxidant potential, the investment spot technique was used semi-qualitatively based on our observation of free radical inhibition. The dot blot assay is another method to rapidly screen of antioxidant potential. In this process $DPPH^{\bullet}$ is a stabilized nitrogen center, where by dark purple color is created by dissolving the free radical in alcohol, which then turns to yellow during hydrogen or electron receiving. The compounds able to trigger this reaction can be considered antioxidants and radical scavengers (Dehpour *et al*., 2009).The DPPH radicals react with antioxidants present in hairy root, root and leaf extracts and become the stable molecule DPPHH, whose color changes from dark purple to bright yellow (Bendy *et al*., 2013). Ethanol extract from the hairy root showed greater potential than the other extracts for DPPH radical inhibition, as demonstrated by field color changes after staining. Hydrogen donation to free radical is one of the chemical mechanisms that empower antioxidants to inhibit of free radicals (Mohammadpour *et al*., 2012). In the present study the emergence of scavenging free radical through electron donation of antioxidant compounds was clearly exhibited. Other studies on different plants also show that field color changes were due to electron donation of antioxidant compounds (Bendy *et al*., 2013).The ethanol extracts of hairy root, root and leaf with the concentrations of 157.23 ± 5.09, 198.57 ± 0.94, and 223.86 ± 7.67 µg mL^{-1}, respectively, scavenged a half of DPPH radicals. Whereas no previous research has observed this phenomenon using plant hairy roots, according to the study on aqueous root and leaf extracts by pereira *et al*., (2010) and Ferreres *et al*., (2008), concentrations of 153 and 477 µg mL^{-1} were reported as for scavenging free radicals. This disparity may reflect differences in the contents of secondary metabolites under the influence of fluctuating environmental factors. At a concentration of 200 µg mL^{-1} of hairy root extract, it was able to harness more than 52% of the extant DPPH radicals, which was a notably high scavenging rate. In thermodynamic terms, assessing a reducing potential does not yield any information on reaction rate, but measuring the activity of a broken chain, can determine the destruction rate of radicals. The ethanol extract of hairy root was faster than other extracts in reducting and disabling DPPH free radicals .This finding may be due to more antioxidant compounds being present in the root hairs.

Figure6 shows that nitric oxide radical trapping increased significantly with higher concentrations of ethanol extracts. Hairy root extract showed the best performance in nitric oxide radical trapping activity compared with *C. roseus* root, and leaf. At a concentration of 200 µg mL^{-1} of hairy root extract, it was able to harness more than 47% of the extant nitric oxide radicals, which was a notably high scavenging rate.

In experiments examining the inhibition of nitric oxide radicals, sodium nitroprusside in an aqueous solution at physiological pH produces nitric oxide, which is measured by Griess reagent. Inhibitors of nitric oxide compete with oxygen to reduce production of nitric oxide; thus, any substance that decreases production of nitric oxide can be considered an antioxidant scavenging these radicals. From the stand point of observation, this event is accompanied by a

decrease in intensity of the azo dye (pink color), indicating reduction in absorption (Figure7). In addition to active oxygen, nitric oxide is also important in other pathological conditions such as inflammation and cancer (Lobo *et al*., 2010). Plant or plant products that can prevent formation of nitric oxide are pivotal in inhibition disease. Ethanol extracts of hairy root, root, and leaf with concentrations of 179.61±6.37, 218.63 ± 1.711, and 227.95 ± 4.44 µg mL^{-1} inhibited fully half of the nitric oxide radicals. Whereas no previous investigation observed these findings using plant hairy root, a study using aqueous root and leaf extract, at concentrations of 189 and 505 µg mL^{-1}, respectively, reported scavenging 25% of nitric oxide radicals (Pereira *et al*., 2010; Ferreres *et al*., 2008). The results showed that ethanol extracts, compared to the aqueous extract, proved significantly more potent in inhibiting nitric oxide radicals. This experiment effectively revealed the remarkable potential of roots in scavenging nitric oxide radicals. Figure6 shows that a hairy root extract concentration of 200 mg mL^{-1} was able to harness more than 47% of all nitric oxide radicals. Logic, therefore, would dictate that at higher concentrations, yet more of these radicals would be reduced. To evaluate the antioxidant compounds in root hairs, the presence of phenolic compounds was assayed. These compounds are well known for their profound antioxidant qualities (Pandy *et al*., 2009; Orcic *et al*., 2011). Studies on hairy root of other plants show that they are similarly rich in phenolic compounds (Parak *et al*., 2011). Phenolic content was measured using Folin reagent, which confirmed that hairy root phenolic content was higher than root and leaf because of their ability to synthesize a higher level of secondary metabolites (pirian *et al*., 2012).

Studies have, in fact shown that secondary metabolites are produced faster and more abundantly in hairy roots than in other normal parts of the plant. Karthikeyan et al, (Karthikeyan *et al*., 2010) reported that the total alkaloid content was 1% in a leaf, 2–3% in root, and up to 9% in hairy roots. Some studies show that alkaloid synthesis routes are closely allied with the synthesis of phenolic compounds (Verpoorte *et al*., 2002). Among these phenolic compounds, a higher level of gallic acid was found in hairy root versus root and leaf, which signals greater antioxidant properties. Table 1 shows how Pearson coefficients were applied in determining the relationship between phenolic and gallic acid content, measuring the antioxidant capacity of the extracts based on DPPH and nitric oxide inhibition.

4. Conclusion

The results of the present study revealed that the induced hairy roots of the leaves had more antioxidant and phenolic contents than the roots, and leaves of the plant. This can be ascribed to the enhanced synthesis of secondary metabolites such as phenolic compounds in hairy roots. The potent inhibition of free radical may also reflect the reducing power of these compounds. The potential inhibition of free radical by hairy root extract is of great importance. Therefore, from a pharmaceutical perspective, the extraction of their potent antioxidants can be very beneficial in inhibiting free radical, reducing the risk of degenerative diseases, and by virtue of their natural sources, replacing the current synthetic antioxidants.

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