Korean J. Medicinal Crop Sci. 12(5) : 406 - 414 (2004)

Isolation and Biological Activity of Resveratrol-3-O-β-D-Glucoside in Transgenic *Rehmannia glutinosa* L. Transformed by Peanut Resveratrol Synthase Gene (RS3)

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ABSTRACT : Resveratrol, which is both a phytoalexin with antifungal activity and a phytochemical associated with reduced cancer risk and reduced cardiovascular disease, is synthesized in a limited number of plant species including peanut. Resveratrol synthesis is catalyzed by the enzyme stilbene synthase including resveratrol synthase (RS). Resveratrol synthase gene (RS3) obtained from peanut, *Arachis hypogaea*, Fabaceae has been transferred into chinese foxglove, *Rehmannia glutinosa* by using *Agrobacterium* mediated transformation. RS t-DNA introduced to chinese foxglove (*R. glutinosa* L) by transformation and its reaction product, resveratrol-3-O-β-D-glucoside was isolated and characterized using HPLC. Also its biological effects was tested in inhibition of the lipid peroxidation of mouse LDL by glycosylated stilbenes derivatives obtained from transgenic plants. Resveratrol-3-O-β-D-glucoside isolated from transgenic *R. glutinosa* L. showed antimicrobial activity of the growth inhibition zone against *Escherichia coli* and *Salmonella typhimurium*. Therefore, this compound can be contributed to be useful as a phytoalexin for plant health as well as a phytochemical for human health.

Key words : *Rehmannia glutinosa* L., resveratrol synthase, resveratrol-3-O-β-D-glucoside

INTRODUCTION

In plants, stilbenes and their derivatives are regarded as phytoalexins that contribute to the defense against fungal infection (Hain *et al.*, 1993). Stilbenes have significant roles in the resistance of wood against microbial degradation. Phytoalexins are benzo-γ-pyronderivatives such as stilbene which are ubiquitous in photosynthesizing cells and they have been used for centuries in folk medicine to treat human diseases such as inflammation, allergy, headache, parodentosis, virus and fungal infection, stomach or duodenal ulcers, and even cancer (Adrian *et al.*, 2000; Palomino *et al.*, 2000). Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is found in plants, such as mulberries, grapes, and peanuts (Sobolev & Cole, 1999; Zhu *et al.*, 2000). Resveratrol and hydroxylated stilbenes are accumulated in plants in response to attacks by pathogens such as *Plasmospora viticola* or *Botrytis cinerea* (fungal infections) the causal agents for downy mildew and grey mould, respectively

Much attention has been focused on resveratrol and RS (resveratrol synthase) genes because of their implication as an important part of the plant defense

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Received October 4, 2004 / Accepted October 13, 2004

system. RS genes were transferred into the plants in which RS is absent in order to provide a defense system against fungal infections. Production of resveratrol has been associated with an increased resistance to various fungal pathogens in transgenic tobacco (Hain *et al.*, 1990; 1993), tomato (Thomzik *et al.*, 1997), rice (Stark-Lorenzen *et al.*, 1997), and wheat (Fettig & Hess, 1999). This finding has directed a lot of attention toward the RS gene expression in other medicinal plant tissues.

Increasing interest in monitoring the presence of resveratrol in plants was caused by so called the "French paradox". It has been found that in some parts of France, the death rate caused by coronary artery diseases was lower in spite of high consumption of fats. The consumption of wine was one of the dietary factors that may partially explain the low mortality caused by atherosclerosis (Renauld & De Lorgeril, 1992). The wide range of biological effects exhibited by resveratrol are thought to be due to its powerful antioxidant properties (Belguendouz *et al.*, 1997) and its ability to interact with redox sensitive cell signalling pathways (Bertelli, 1998). The ability of resveratrol to scavenge reactive oxygen species (ROS) *in vitro* has been well documented. For example, resveratrol was observed to prevent oxidative DNA damage induced in the kidney by $KBrO_3$ (Cadenas & Barja, 1999) and acts as a potent scavenger of peroxyl radicals in an isolated rat heart ischaemia reperfusion model (Ray *et al.*, 1999). Recently, the cancer chemopreventive activity (Palomino *et al.*, 2000) of resveratrol has been related to its ability to trigger apoptosis (Carbo *et al.*, 1999; Huang *et al.*, 1999), a mechanism of programmed cell death which has been linked to ROS and lipid peroxides (Hale *et al.*, 1996). In contrast, resveratrol has been shown to prevent apoptosis in K562 cells via the direct inhibition of arachidonate-metabolising enzymes such as lipoxygenase and cyclooxygenase (Maccarrone *et al.*, 1999). Cis- and trans- resveratrol and their corresponding resveratrol glucoside present in plant have been linked to this effect by virtue of their *in vitro* inhibitory activities on the oxidation of human low-density lipoproteins (Frankel *et al.*, 1995), antiplatelet properties (Bertelli *et al.*, 1995) and eicosanoid synthesis (Pace-Asciak *et al.*, 1995). On the other hand, as a medicinal natural product, resveratrol is known to be a cancer chemopreventive agent (Jang *et al.*, 1997) as well as an agonist for the estrogen receptor (Gehm *et al.*, 1997) and as a phytochemical of human health benefits are revealed as its association with reduced cardiovascular disease has been demonstrated (Fremont, 2000).

Resveratrol is known to occur in wine in aglycon and glycoside form, which is called as resveratrol-3-O-β-D-glucoside. Resveratrol-3-O-β-D-glucoside can be found in grape products in the concentration usually significantly higher than the aglycon. The relative distribution between the glycosilated and aglycon forms in wines is dependent on a number of factors, especially on viticultural and enological techniques used (Gu *et al.*, 2000). Also resveratrol level depends on a number of factor such as variety, geographical location, climate condition, fungal infection, ultraviolet light exposure, and enological techniques in grape (Siemann & Creasy, 1992; Jeandet *et al.*, 1995; Romero-Prez *et al.*, 1996). Also, it has been demonstrated that the addition of β-glucosidase (Jeandet *et al.*, 1994) and transgenic yeast expressing a gene encoding a glycosyl-hydrolase during vinification increased resveratrol contents (Gonzlez-Candelas *et al.*, 2000).

HPLC techniques have become the dominant and more frequently used method of resveratrol determination. LC methods with gradient elution and UV or fluorimetric detection have been reported for the determination of resveratrol and resveratrol-3-O-β-D-glucoside in biological samples, especially in grape berries and wines. Recently, resveratrol has been assayed by the direct injection without purification and pre-concentration steps. LC with electrochemical detection has been shown to be selective and sensitive enough for the determination of resveratrol and other phenolic compounds in natural sources (Zhu *et al.*, 2000; Melzoch *et al.*, 2000) or various medicinal plants (Lim *et al.*, 2004a)

Recently, we described that cDNA fragment RS3 (AF227963, *Arachis hypogaea*) transformed in medicinal plant *Rehmannia glutinosa* L using *Agrobacterium* (Lim *et al.*, 2004b). By succession, the gene expression

of resveratrol synthase identify and characterize its reaction product after transformation using HPLC. Also its biological effects were tested, and concluded that could be useful as a phytoalexin for plant health, as well as a phytochemical for human health.

MATERIALS AND METHODS

Extraction and isolation of compound predominantly in the EtOAc fraction of transgenic *Rehmannia glutinosa* L.

Leaves of control (non-transgenic) and transgenic *Rehmannia glutinosa* L. grown in a green house were harvested, dried (1 kg) and homogenized with 70% Acetone (500 mℓ×2). The extract was concentrated at 40℃ in vacuo and resulting aqueous extract (150 mℓ) was extracted with n-haxane (150 mℓ×2), methylene chrolide (150 mℓ×2) and EtOAc (150 mℓ×2), successively. The EtOAc fraction (1 g) was dissolved in MeOH-H_2O (1:1) (1 mℓ) and chromatographed on a Sephadex LH-20 column (1.5×60 cm) and eluted using a stepwise of MeOH in H_2O at a flow rate of 1 mℓ · min^{-1}. The eluted extracts with MeOH-H_2O (7:13) were contained a major compound. Recrystallization was peformed about matrix of crystalline and deposit which was happened during column chromatography process to get pure crystal formed compounds. The effluent that impurities have mixed much to separate pure crystal compound, small amount of mixing solvent (acetone and H_2O) added and dissolved perfectly. The distilled water added in this mixture and do leaving alone in cold temperature (4℃), so that deposit may be formed. Same manipulation was repeated three and four times. A major compound which on TLC (25DC-Plastic-folien Cellulose F, Merck) developed in BuOH-HOAc-H_2O (3:1:1, v/v, solvent A) and 6% HOAc (solvent B), then developed compound was observed under the UV lamp (254, 365 nm), and gave a violet colouration after spraying with vanillin-HCl-ethanol (4.8 g:12 mℓ:480 mℓ) reagent. Also, two-dimensional TLC was performed using solvent A and B for purity of isolated compound confirmed. Resveratrol-forming stilbene was quantified through HPLC on a model LC-10A liquid chromatography (Shimadzu Co., Kyoto, Japan) by method described as above literature (Lim *et al.*, 2004a)

Antioxidant effect of resveratrol-forming stilbene by measuring inhibition of lipid peroxidation and DPPH free radical scavenging assay

Antioxidant effect of these compounds (resveratrol, resveratrol-3-O-β-D-glucoside) isolated from transgenic *R. glutinosa* L. and standard compound (trans-resveratrol, astringin, Sigma) by measuring the inhibition of lipid peroxidation induced by Cu^{2+} in fresh mouse LDL (low density lipoproteins) and DPPH free radical scavenging assay. The standard compounds were purchased from Sigma (USA) and Polyphenol laboratories (USA). Inhibition of lipid peroxidation was determined by measuring the production of thiobarbituric acid-reactive substance, called TBARS method (Buege & Aust, 1978). LDL samples were solublised with 0.15 N NaOH. LDL oxidation was determined using TBARS and conjugated diene levels directly. Thiobarbituric acid reactive substances (TBARS) measurements were made by incubating the LDL with TBARS solution (0.12 M TBA in 15% TCA and 1% HCl) for 30 min at 95℃. Conjugated diene levels were monitored at 234 nm using an UV spectrophotometer (HITACHI Instrument, Inc). *In vitro* oxidation of LDL was induced by 5 M CuSO4 and conjugated diene formation was analysed by following the absorbance at 234 nm at 5 minute intervals for 3 hours. The peak of diene conjugation was assessed 110 minutes after the start of *in vitro* oxidation of LDL with $CuSO_4$. Appropriate standard curves were prepared and monitored oxidation of LDL after added each stilbene compounds in various concentrations. Radical scavenging activities of the isolated compounds from transgenic plants were measured using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH). Radical scavenging activity of stilbene compounds was measured according to Yoshida *et al.*, (1989). Stilbene compounds at four different concentrations in MeOH (4 mℓ) were added to a solution of 0.15 mM DPPH (1 mℓ) with vigorous shaking. After keeping these mixtures at room temperature for 30 min, the remaining amounts of DPPH were determined by the absorbance at 517 nm. The radical scavenging activity of each compound was expressed by the ratio of the reduced absorption of DPPH (%) relative to that (100%) of the DPPH in

the absence of compounds.

Antifungal and antimicrobial activity assay

To evaluate tolerance against plant disease and potency of antimicrobial activity in transgenic *R. glutinosa* producing stilbene compounds (resveratrol, resveratrol-3-O-β-D-glucoside), a paper disk method was used (Gker *et al.*, 1998). The antimicrobial activities of the purified resveratrol and resveratrol-3-O-β-D-glucoside against two yeast, *Pichia jadinii* (KCTC 7293), *Candida albicans* (KCTC 7965), and five bacteria, *Staphylococus aureus* (KCTC 1916), *Bacillus subtilis* (KCTC 3728), *Klebsiella pneumonia* (KCTC 2001), *Escherichia coli* (KCTC 1924), *Salmonella typhimurium* (KCTC 1925) were tested. The fungi, yeast, and bacteria were grown on PDA (potato dextrose agar, Difco), while nutrient agar or Miicrococcus medium were used for *B. subtilis, K. pneumonia, S. typhimurium, E. coli, S. aureus* and YM (Yeast-Malt, Difco) was used for *Candida albicans, P. jadinii*. The fungus, *Fusarium oxysporum* R-10 isolated from field-infected roots was grown on PDA. Assays were performed by using a cork borer to cut mycelial disks (diameter, 5 ㎜) from the margins of mycelial mats grown in petridishes (diameter, 90 ㎜) on PDA for 7 to 10 days. The disks were then inoculated onto the centers of new PDA plates (10 ㎖; diameter, 90 ㎜). Sterilized paper disks (diameter, 8 ㎜) were charged with 1 ㎖ of a resveratrol or resveratrol-3-O-β-D-glucoside solution at each concentrations (20000, 10000, 1000, 500, 250, 10 ppm in H_2O-MeOH [1:4, v/v]), and the solvent was evaporated at 40℃ under a vacuum. The paper disks charged with test compounds were then placed around the inocula at a distance of 30 ㎜, and the plates were incubated at 28℃ for 2 to 7 days. Resveratrol and resveratrol-3-O-β-D-glucoside were evaluated for antimicrobial activity against yeast and bacteria *in vitro*. Cells of the other test microorganisms were suspended in each other growth medium and incubated at a optimal temperatures and periods. The 100 ㎕ of suspension was poured into petridishes and spreaded by smearer. Multiple plates of serial dilution were overlaid with target strains and incubated under anaerobic condition for 24 hrs at 30, 37, and 25℃. Antimicrobial assay was performed to similar methods of antifungal activity. After incubation for 24 hr, the antimicrobial activity of each strains was evaluated based on the formation of a clear zone (㎜) around the paper disk.

RESULTS AND DISCUSSIONS

Isolation of resveratrol glucoside from transgenic *R. glutinosa* L.

The transgenic plants of *Rehmannia glutinosa*, used in this study synthesized many polyphenolic compounds which were extracted by aqueous acetone. Thin layer chromatography (TLC) showed that this compound predominantly in the EtOAc extract of the initial aqueous residue, a white crystalline powder, produced a violet colouration on cellulose 2D-TLC, and appeared spot of dark brown under the UV lamp (Rf, 0.29/solvent A, 0.28/solvent B).

Individual control and RS3 transgenic lines of *Rehmannia glutinosa* were analysed by HPLC for the accumulation of resveratrol. HPLC profiles from extracts of leaves in RS3 transgenic plants revealed the presence of an unknown peak that did not appear in extracts of control leaves (Fig. 1). The retention time (62.1 min) of the unknown peak was shorter than the one expected for resveratrol (75.5 min), but the UV spectra of the former and of the resveratrol standard were nearly identical (data not shown). The leaves extract of 0.1N-HCl:acetonitrile in control plants were not appeared resveratrol contents while transgenic plants of *R. glutinosa* contained little amounts of resveratrol. In this study, spectroscopic data were consistent with those previously given in the literature for resveratrol-3-O-β-glucoside (Mattivi *et al.*, 1995).

This compound has also been discovered in leaves of *Eucalyptus* (Hasagawa & Hillis, 1966) and in roots of *Polygonum cuspidatum* which were used in Asia as a treatment for atherosclerosis (Yuchi & Kimura, 1986). It has also been described as a stress inducible compound produced by *Veratrum grandiforlium* leaves when treated with cupric chloride. This compound has also been discovered in leaves of *Eucalyptus* (Hasagawa & Hillis, 1966) and in roots of *Polygonum cuspidatum* which were used in Asia as a treatment

for atherosclerosis (Yuchi and Kimura, 1986). It has also been described as a stress inducible compound produced by *Veratrum grandiforlium* leaves when treated with cupric chloride (Hanawa *et al.*, 1992). Also, the physiological effect of this compound has been reported on tumour growth and lung metastasis in mice bearing highly metastastic Lewis lung carcinoma (LLC) tumors and on angiogenesis, differentiation of human umbilical vein endothelial cells (HUVECs) to form a capillary network (Kimura & Okuda, 2003).

In plants, the conjugation of glucose to small hydrophobic molecules and compound can lead to the formation of glucose esters or glucosides. The former are high energy compounds and have long been regarded as biosynthetic intermediates (Mock & Strack, 1993), whereas glucosides are generally considered to represent the storage forms of the aglycons (Hostel, 1981). Both transfer reactions are suggested to occur in the cytoplasm of cells (Pflugmacher & Sandermann, 1998), with the attachment of glucose providing access to membrane transport systems and passage into either the vacuole or the extracellular space.

This presented result could indicate the possible modes of fluctuation of products by gene transformation.

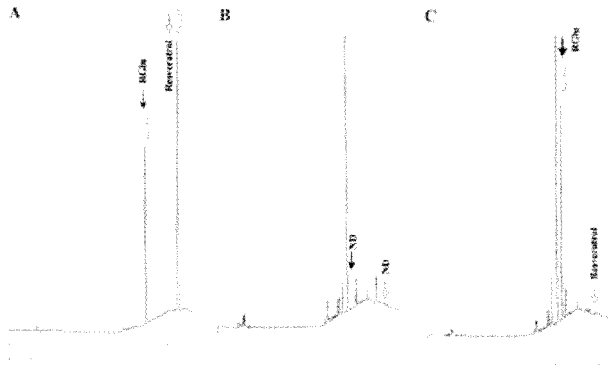

Fig. 1. Detection of resveratrol and R-gluc by HPLC in the leaves of RS3 transgenic plants. A. R-gluc and resveratrol standard compounds were separated as the peaks with the retention time of 62.3 (closed vertical arrow) and 75.6 min (open vertical arrow), respectively. B and C. HPLC chromatograms of the leaf from the control (B) and transgenic plants (C)

One of possible explanation is that some of enzyme exist for adhering glucose to resveratrol and then glycosidically-bound resveratrol derivatives produced. Alternatively, the change in product formed by gene transformation could be explained by an indirect mechanism in which metabolic condition of medicinal plant as *R. glutinosa* provided other substrate for adhering glucose from more polar cytosolic condition. Another possible explanation suggested that the existence of β-glucosidase could hydrolyze glycosidically-bound derivatives and release the corresponding resveratrol isomer in *A. hypogaea*, but this enzyme not activate in transgenic plant which only target gene integrated. The other possible explanation considered that transgenic plant contained more resveratrol-3-O-β-glucoside than resveratrol as if resveratrol-3-O-β-glucoside more abundant than resveratrol in grape skin (Roggero & Archier, 1994) because grape skin harbor more abundant sugar contents than other tissue.

Antioxidant potency of resveratrol and resveratrol-3-O-β-D-glucoside

Isolation and structural determination of stilbene derivatives, together with known resveratrol from an ethylacetate fraction of the transgenic plants of *Rehmannia glutinosa* were conducted. Further investigation of more polar constituents of an extract than resveratrol, O-glucosyl stilbene oligomers, resveratrol-3-O-β-D-glucoside were isolated as a major compound. In nature, the occurrence of stilbene oligomer glycosides is very rare, especially as O-glycosides. Therefore, the structure of the compounds drew attention in view of biological activities (Jang *et al.*, 1997). Many stilbene derivatives have been isolated from various plants, and their biological effects, not only chemopreventive activity (Jang *et al.*, 1997) but also anti-inflammatory activity (Kitanaka *et al.*, 1990), inhibition of histamine release (Inamori *et al.*, 1991) and gastric ATPase (Murakami *et al.*, 1992) have been reported. There is continuing interest in resveratrol glucoside (piceid, astringin etc.) among stilbene derivatives since the aglycon has been found to inhibit the copper-catalysed oxidation of human low density lipoprotein (Frankel *et al.*, 1995) and the aggregation of platelet. The

resveratrol glucoside can also be hydrolysed by glucosidase in human gastro-intestinal tract. Although, the exact molecular mechanisms involved in the anticarcinogenic effects of these compounds are not fully understand, scavenging ROS (reactive oxygen species) is believed to be responsible, at least partially, for their anticarcinogenic effects. The antioxidant potency of these compounds was investigated by measuring the inhibition of lipid peroxidation induced by Cu^{2+} (IC_{50}= concentration at which one-half of the induced peroxidation in inhibited) which has been reported as a constituent of *Vitis vinifera* cell (Norren *et al.*, 1997). Norren *et al.*, (1997) have shown that the isomer of resveratrol forming stilbene having other side chain appeared different activity through structure characteristic, its nonplanar conformation and catechol structure.

The catechol structure is essential for the antioxidant activity of stilbenes. The glycosylation of stilbenes reduces their activity when compared to the corresponding aglycons, but glucoside may be hydrolyzed by glucosidase in the human gastrointestinal tract (Goldberg, 1995).

In this study, inhibition of the lipid peroxidation of mouse LDL by stilbenes derivatives newly extracted from transgenic plants of *R. glutinosa*. Astringin (3'-OH-*trans*-piceid) was six fold more potent than resveratrol-3-O-β-D-glucoside (3'-H-piceid), from transgenic plants of *R. glutinosa*. which confirm the major importance of catechol structure in antioxidant effect. On the other hand, the conjugation of an OH group with a sugar decreased the antioxidant potential, according to the IC_{50} values of resveratrol vs resveratrol-3-O-β-D-glucoside in transgenic plants of *R. glutinosa*. This result suggested that induced new stilbene derivatives could be represent high level of biological activity more than known compound in production of stilbene by STS gene transformation therefore, useful lead compounds for drug development. The case of resveratrol-3-O-β-D-glucoside which demonstrated decrease of antioxidant effects. This particular behavior can be easily explained by the assumption that the resveratrol possesses glucose units distributed in opposite sides of the aglycon moiety, which considerably reduces its hydrophobicity, resulting in the loss of the antioxidative features (Table 1).

Table 1. IC_{50} values for the antioxidant activities of stilbenes isolated from transgenic *Rehmannia glutinosa* and trolox.

Compounds		IC_{50} values (μM)†	
		DPPH	LDL
Standard	*trans*-Resveratrol	68±4.5	2.6±0.4
	Astringin	67±3.7	3.3±1.0
Stilbenes compound isolated from transgenic plant	Resveratrol-3-O-β-D-glucoside	198±16.8	19.1±3.0
	trans-Resveratrol	72±4.5	2.4±0.2
Trolox		10±0.5	4.7±0.4

†Inhibitory concentration 50%, Each value is the mean of at least three independent experiments ± SD. Statistical analysis was performed by using Student's t-test.

Antifungal and antimicrobial activity of resveratrol and resveratrol-3-O-β-D-glucoside

Resveratrol and resveratrol-3-O-β-D-glucoside from transgenic Rehmannia glutinosa were evaluated for antimicrobial activity against two yeast, *Pichia jadinii* (KCTC 7293), *Candida albicans* (KCTC 7965), and five bacteria, *Staphylococus aureus* (KCTC 1916), *Bacillus subtilis* (KCTC 3728), *Klebsiella pneumonia* (KCTC 2001), *Escherichia coli* (KCTC 1924), *Salmonella typhimurium* (KCTC 1925) *in vitro* test. Table 3 showed the results of *in vitro* activity determination by paper disk method. The prepared compounds at concentrations of 20000 ppm, antimirobial activity appeared on all strain respectively. Typically resveratrol was more active than resveratrol-3-O-β-D-glucoside but resveratrol-3-O-β-D-glucoside seems to enhance

Table 2. Clear zone of stilbenes compound (resveratrol, resveratrol-3-O-β-D-glucoside) isolated from transgenic *Rehmannia glutinosa* against fungi, yeast, and bacteria

Compounds	Conc. (ppm)	Clear zone (㎜)†						
		P. jadinii	*C. albicans*	*S. aureus*	*B. subtilis*	*K. pneumonia*	*E. coli*	*S. typhimurium*
Resveratrol-3-O-β-D-glucoside	20,000	11.0	10.8	10.6	10.1	9.8	12.4	12.7
	10,000	-‡	-	-	-	-	11.3	10.8
	1,000	-	-	-	-	-	10.1	-
	500	-	-	-	-	-	-	-
	250	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Resveratrol	20,000	13.7	12.8	12.9	14.2	11.5	19.8	18.6
	10,000	11.5	11.7	11.8	12.4	-	13.2	14.5
	1,000	-	-	-	-	-	10.1	12.0
	500	-	-	-	-	-	-	10.0
	250	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-

† Clear zone diameter: ‡No inhibition.

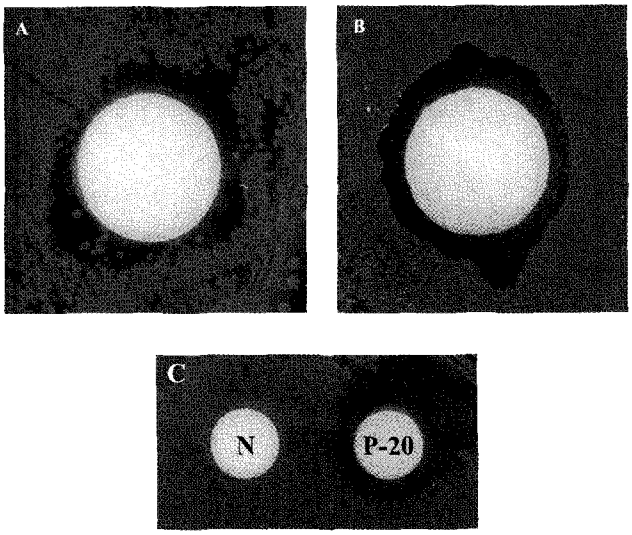

Fig. 2. Antimicrobial and antifungal activity of stibenes (resverarol-3-O-β-D-glucoside) isolated from transgenic *Rehmannia glutinosa* using paper disk method, Photograph were taken after 24h of incubation of a *Salmonella typhimurium* in presence of 1000 ppm resveratrol-3-O-β-D-glucoside (A); 1,000 ppm resveratrol (B); Clear zones of P-20 (2000 ppm of resveratrol-3-O-β-D-glucoside) compared to N (non-treated) in *Escherichia coli* plate (C).

disease resistance in transgenic plants with antimicrobial and antifungal activity.

Exception were *E. coli* and *S. typhimurium* on which resveratrol-3-O-β-D-glucoside did not have any appreciable effect at 10,000 ppm below (Table 2). Both of stilbene compounds, resveratrol and resveratrol-3-O-β-D-glucoside showed the growth inhibition zone against E. coli and *S. typhimurium* significantly (Table 2, Fig. 2A). The clear zone of resveartol (Figure 2B) and resveratrol-3-O-β-D-glucoside (Figure 2A) was appeared 10.8 and 14.5 ㎜ clear zone diameter at concentration of 10,000 ppm in plate of *S. typhimurium* respectively. The highest antimicrobial activity was observed in 20,000 ppm of resveratrol against *E. coli* with 19.8 ㎜ clear zone diameter (Figure 2C).

ACKNOWLEDGEMENTS

This research was conducted by the financial support of the Rural Development Administration made in the BioGreen 21 project.

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