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Antibacterial characteristics of grape seed extract and nano-grape seed extract in *in vitro* and *in vivo* assays

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Abstract

This study was done to examine the antimicrobial activity of grape seed extract (GSE) and nano grape seed extract (NGSE) in *in vivo* and *in vitro* assays. For the i*n vitro* assay, the antimicrobial activity of grape seed extract and nano-grape seed extract against *Escherichiacoli* and *Salmonella typhi* was examined by modified agar-well diffusion method. For the *in vivo* assay, the microbial population of ileum of the broiler chickens fed diet supplemented with GSE and NGSE was evaluated. Treatments included control diet, and control diet supplemented with 3 levels (150, 300, 450 ppm) of GSE and NGSE. Results showed that GSE and NGSE had bactericidal activity against *E. coli* and bacteriostatic activity against *S. typhi*. GSE at the level of 450 ppm caused the largest inhibition zone against *E. coli* (32 mm). Nanoliposomes of grape seed extract could not improve the antimicrobial effect of GSE in vitro situation. In vivo assay, NGSE at the level of 450 ppm caused the lowest ileal coliforms and *E. coli* populations. However, there was no significant difference between population of lactobacillus in birds of different groups. Further study needs to make clear the potential of using grape seed extract nano-liposomes in human and animal drugs as a natural antimicrobial compound.

Key words: anti-bacterial, nano-liposome, grape seed extract, in vivo

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Introduction

The increasing antimicrobial resistance of pathogens isolated from humans and animals combined with the ban of the use of antibiotics as feed additives has accelerated the investigations of alternative options for organic compounds with antimicrobial activity. Application of plant extracts as an alternative to chemical or synthetic antimicrobials and antioxidants to combat the food borne pathogens, inhibiting lipid oxidation and thus extending the shelf life is an increasing trend nowadays (Perumalla and Hettiarachchy, 2011).

Grape, one of the world's largest fruit crops, with more than 60 million tons is cultivated mainly as *Vitis vinifera* for wine production (Sanda Chedea et al., 2011). It is estimated that around 13% of the total weight of grapes used for the wine making results in grape pomace, which is a by-product in this process (Sanda Chedea et al., 2011). Grape seeds are considered rich sources of polyphenolic compounds that show antioxidant or antimicrobial effects (Furiga et al., 2008). Grape seed extract sold commercially as dietary supplement listed on the "everything added to Food in the United States (EAFUS)" and also has generally recognized as safe (GRAS) status approved by the food and drug administration. GSE is a rich source of polymers of flavan-3-ols like catechin and epicatechin, thus, its antimicrobial properties can be referred to the general mechanisms of phenolics. Phenolic compounds of grape have shown an inhibitory effect on bacteria (Perumalla and Hettiarachchy, 2011). The increasing order of the antimicrobial activity reported in grape plant was flesh, whole fruit, grape extracts, fermented pomace, skin, leaves and seeds (Xia et al., 2010).

Today, nanotechnology has rapid progress and it affects different aspects of human and animal life in addition to environmental effects. Researchers stated that silver nano-particles have different physical and chemical characteristics compared to their larger equivalents because of a very high surface to volume ratio, physical activity and chemical stability (Singh et al., 2009; Rawat et al., 2006). Little study has been done to examine the efficacy of the nano organic particles. Thus, the objective of this study was to evaluate the anti-bacterial effect of grape seed extract and nano grape seed extract in *in vitro* and *in vivo* assays.

Material and Methods

Grape Seed Analysis

Black grape samples were collected from Sari, Iran. After harvest, berries were snipped from the cluster. The seeds from berries were manually separated from pulp, washed with distilled water and air dried. The composition of grape seeds was measured by AOAC procedures (AOAC, 1990).

Preparation of Grape Seed Extract

Grape seeds were grounded, and extracted with acetone: methanol: water (60:30:10v/v/v) for 12h with shaker incubator. Solvents were removed by rotary evaporator. Then, the extract was dried in vacuumed oven and kept in freezer under -20°C (Salari et al., 2009).

Grape Seed Extract Analysis

The chromatographic analysis was carried out on a Knauer HPLC system (Berlin, Germany) equipped with a Triathlon auto sampler, a K-1001 pump and a UV–visdetector (K-2600). A reversed-phase C18 Nucleosil 100 (12.5 cm × 5.0mm × 5.0 µm) column was used for the separation of sample components. Analysis of catechein, epicatechin, procyanidin B1, B2, C1 performed according to the method of Iacopini et al. (2008) with some modifications. Standards of catechein, epicatechin, procyanidin B1, B2, C1 were purchased from Sigma-Aldrich (St. Louis, USA). Before injection, each sample was centrifuged in an Eppendorf tube (4 min at 5000 rpm) and the centrifuged supernatant was allowed to pass through a 0.45 µm pore size membrane filter. Injection volume was 20 µL and the flow rate was 0.8 mL /min. The HPLC grade solvents used were formic acid/water (5:95 v/v) as solvent A, and acetonitrile/ formic acid/water (80:5:15 v/v) as solvent B. The elution gradient was linear as follows: from 0 to 10 min, 0.0% B, from 10 to 28 min, 10.0% B, from 28 to 35 min, 25% B, from 35 to 40 min, 50% B, from 40 to 45 min, 80% B, and for last 10 min again 0% B. The different polyphenolic compounds were identified by comparing their retention times and spectral characteristics with data of original reference standard compounds. All analyses were done in duplicate at least.

Preparing nanoliposomes entrapping of grape seed extract

Nanoliposomes entrapping of grape seed extract was prepared by the method of Yang et al. (2012) with some modification at the development research center of Sari agricultural and natural resources university, Sari, Iran. The average diameter of nano-liposome grape seed extract was about 65 nm.

Preparing microorganisms

Microorganisms (*Escherichia coli* NCTC10418, *Salmonella typhi* NCTC 52311) were obtained from the culture collections of the department of Microbiology at the fisheries research organization of North of Iran. Inocula were prepared by diluting over night cultures in saline to approximately 10^8 colony-forming units (cfu) /mL for each bacterium.

In vitro assay of antibacterial activity of GSE and NGSE

The extracts were tested for activity against *E. coli* and *S. typhi* bacteria using modified agar-well diffusion method procedures described by Clinical and laboratory standard institute (2006). Five hour broth cultures of the test bacteria adjusted to 10^8 CFU^{-1} respectively were applied on the surface of Nutrient agar. A sterile flamed cork borer of 8 mm diameter size was used to punch wells into each of the plates and 0.5 mL of 150, 300, 450 ppm GSE and NGSE were dispensed in each well. The plates were then incubated at

Table 1. Ingredients and nutrient composition of experimental diets.

Ingredient (%)	0-10 d	11-22 d
Corn, ground	56.20	59.90
Soybean meal	37.11	32.55
Soybean oil	2.26	3.30
Dicalcium phosphate	1.92	1.86
Oyster shell	1.16	1.12
Common salt	0.30	0.30
Minerals mix[1]	0.25	0.25
Vitamins mix[2]	0.25	0.25
DL-Methionine	0.31	0.26
L-Lysine hydrochloride	0.24	0.21
Calculated composition		
ME (kcal/kg)	3000	3105
CP (%)	21.23	19.46
Ca (%)	1.00	0.96
AP (%)	0.50	0.48
Lysine (%)	1.32	1.19
Methionine+Cystine (%)	0.98	0.89

[1]Mineral mix supplied the followings per kg of diet: Cu, 20 mg; Fe, 100 mg; Mn, 100 mg; Se, 0.4; Zn, 169.4 mg.

35ºC for 24h. The experiments were done in triplicate and the means of the diameters of the inhibition zones were calculated.

In vivo assay of antibacterial activity of GSE and NGSE

This experiment was carried out using a total of 420 *Cobb*-500 male broiler chicks. One-day-old chicks (with initial weights of 36.28 ± 0.38) were obtained from a local hatchery and divided into 35 groups of 12 birds each. All procedures for the use and the care of animals were conducted after approval by the Ferdowsi University of Mashhad. There were 7 experimental diets including control, 150, 300, 450 mg GSE/kg diet, and 150, 300, 450 mg NGSE/kg diet diet. The feeding program consisted of a starter (1 to 10 d), grower (11 to 22 d). The basal diet was fed in mash form and prepared with the same batch of ingredients for starter, grower, and finisher periods and was formulated to meet the nutrient requirements according to *Cobb*-500 rearing guidelines (Cobb-Vantress, 2012). All birds had free access to feed and water during whole rearing period. The ingredients and chemical composition of the basal diets are shown in Table 1. Each desired level of grape seed extract and nano liposome grape seed extract was added to 100 mL water, well mixed and sprayed on the basal diet. The feed was prepared weekly and stored in airtight containers. The temperature was initially set at 32°C on d 1 and decreased linearly by 0.5°C per day up to 22 d. At 22 d, two birds from each pen slaughtered and the ileum, which was assigned from Meckel's diverticulum to ileo-caecal junction, were excised and contents were collected by gently fingers into tubes. Digesta were pooled with a replicate, put on ice until they were transported to the laboratory for enumeration of microbial populations. One gram of ileal contents was homogenized in 9 mL sterile water. Each sample was serially diluted. Using these diluted subsamples, *Lactobacillus* spp was enumerated on De Man-Rogosa-Sharpe (MRS) agar after incubation at 37°C in an anaerobic chamber for 48 h (Guban et al., 2006), and coliforms and *E. coli* were

counted on CHROM agar ECC (EF322- Paris France) after incubation at 37°C in an in an aerobic chamber for 48 h (Sallam, 2007).

Statistical Analysis

Data of the in vitro were expressed as mean ± standard deviation. The data were analyzed using one way analysis of variance (ANOVA) using SPSS version 17. Data of *in vivo* assay were analyzed by analysis of variance using GLM procedures (SAS Institute Inc., 2001). Means were compared using Duncan's new multiple ranges test (Duncan, 1955). The level of significance was reported at $P < 0.05$.

Results and Discussion

Grape Seed Composition and Grape Seed Extract Analysis

The chemical composition (crude fat, crude protein, nitrogen free extract, fiber, calcium, total phosphorus and ash) of the grape seed and the content of catechin, epicatechin and procyanidins of grape seed extract is shown in Table 2.

***In vitro* assay of antibacterial activity of GSE and NGSE**

Results of the inhibition zone (mm) are shown in Table 3. GSE at all concentrations was effective on *E. coli* with inhibition zones ranging from 30 to 32 mm. Grape seed extract showed potent antibacterial effects with minimum growth inhibitory effect at 300 ppm against *S. typhi* and minimum bactericidal effect at 150 ppm against *E. coli*. NGSE showed a bacteriostatic effect against both *E. coli* and *S. typhi* bacteria, however, it did not show bactericidal effect against these bacteria. Grape seed extract and nano grape seed extract showed antibacterial potent at the present study. *E. coli* was more susceptible to the antibacterial effect of grape seed extract than *S. typhi* bacterium. GSE at the level of 450 ppm caused the largest inhibition zone (32 mm) against *E. coli*. Previous studies showed that the outer cell membrane or cytoplasmic membrane of a bacterium is essentially composed of a phospholipid bi-layer and proteins and is the major site of interaction with antimicrobial compounds.

Damage to this vital membrane result in the death of the bacterium and can occur in the following ways: (i) physical disruption of the membrane (Shimamura et al., 2007); (ii) dissipation of the proton motive force (Juven et al., 1994) and (iii) inhibition of membrane-associated enzyme activity. It is stated that functional hydroxyl groups and conjugated double bonds in the reactive groups of natural plant extracts may be involved in their binding to the cell wall components (Mason and Wasserman, 1987). Catechins have deteriorating effect on the lipid bi-layer membrane that results in the loss of cell structure and function eventually leading to bacterial death (Cox et al., 2001). Also, presences of gallic acid esters in epicatechin, epigallocatechin gallate are responsible for their high affinity for lipid bi-layers, and affect the membrane structure (Hashimoto et al., 1999). On the other hand, major phenolic constituents like epicatechin, caffeic

Table 2. Composition of the grape seed analyzed by AOAC methods and analysis of the grape seed

Grape seed		Grape seed extract	
Dry Matter (%)	90.93	Catechin (mg/100 g)	1420
Gross Energy (Kcal/kg)	3292	Epicatechin (mg/100 g)	1080
Crude Fat (%)	24.83	Procyanidine B1(mg/100 g)	830
Crude Protein (%)	10.17	Procyanidine B2 (mg/100 g)	770
Nitrogen Free Extract	17.86	Procyanidine C (mg/100 g)	530
Crude Fiber (%)	35.39		
Calcium (%)	0.56		
Phosphorus (%)	0.31		
Ash (%)	2.68		

Table 3. Antimicrobial activity of different levels of grape seed extract and nanoliposomes of grape seed extract in vitro

	Zone of inhibition (mm)		
	150 ppm	300 ppm	450 ppm
GSE			
E. Coli	30 ± 0.58	30 ± 0.58	32 ± 0.61
S. Typhi	-	-	-
NGSE			
E. Coli	-	-	-
S. Typhi	-	-	-

No inhibition zone

Table 4. Effects of grape seed extract and nanoliposomes of grape seed extract on ileal microbial population (log CFU/g of digesta) of broilers at d 22.

	Coliforms	E.Coli	Lactobacillus
Control group	5.642^{a}	5.865^{a}	5.807
GSE			
150 PPM	4.852^{b}	5.533^{ab}	5.781
300 PPM	4.777^{bc}	5.386^{bc}	5.680
450 PPM	4.685^{bcd}	5.271^{bcd}	5.653
NGSE			
150 PPM	4.361^{dce}	5.112^{cde}	5.861
300 PPM	4.249^{de}	4.976^{de}	5.775
450 PPM	4.168^{e}	4.859^{e}	5.732
SEM	0.117	0.103	0.116
Pr>F	<0.001	<0.001	0.9629

Means within the same row with uncommon superscript differ significantly ($P < 0.05$).

acid, benzoic acid and syringic acid may alter the cell morphology by influencing the osmotic pressure of the cell, thus disrupting the cytoplasmic membrane and causing leakage of cell constituents (Sivarooban et al., 2008a). Ignat et al. (2013) stated that Gram-positive bacteria are more sensitive in general than gram-negative species to the phenolic compounds of plant extracts. This can be due to the lipophilic nature of phenolics, so they fail to diffuse across the outer membrane. NGSE (150, 300, 450 ppm) had bacteriostatic effect of *E. coli* and *S. typhi*, but it had not bactericidal effect so it did not cause inhibition zone. It can hypothesize that nano-liposome encapsulating of grape seed extract may help to human and animal health by reducing enzymatic digestion of grape seed materials in the upper part of the gastrointestinal tract, so higher bioavailability of antimicrobial compounds of grape seed such as polyphenols in the hind gut.

***In vivo* assay of antibacterial activity of GSE and NGSE**

Different levels of GSE and NGSE decreased ileal coliforms and *E. coli* populations of birds' intestinal microflora ($P < 0.05$). A diet supplemented with NGSE more decreased ileal coliforms and *E. coli* population of broiler chicken compare to GSE supplemented diets. NGSE supplementation at the level of 450 ppm caused the lowest ileal coliforms and *E. coli* populations compared to the control group (Table 4).

Jamroz and Kamel (2002) reported that the dietary herbal treatment results in lower *E. coli* count compared to the control group. Also, it was reported that a mixture of thymol and carvacrol increases the population of *Lactobacillus* spp in the ileum (Akyurek and Yel, 2011). Polyphenols could have bacteriostatic or bactericidal actions or act to inhibit adhesion of infection-causing bacteria within cells of the intestinal tract (Viveros et al., 2011). As the pH in the gastrointestinal tract may be decreased by the active components of the herbal derivatives, thus the components can prevent the growth of pathogenic bacteria and promote the population of non-pathogenic ones like *Lactobacillus* spp and *Bifidobacterium* spp (Khaksar et al., 2012). Also, preparing nano-liposomes of grape seed extract improve its antibacterial effect on birds' intestinal tract. This might be due to preservation of grape seed extract by liposomes from upper intestinal degrading or might be due to increased interactive surface of grape seed extract. The results of this study showed that there was no significant difference between populations of *Lactobacillus* spp in broilers. This is in contrast with the findings of Viveros et al. (2011), who reported that in the ileal content, birds fed control and GSE diets had the highest populations of *Lactobacillus* spp. They also stated that animals fed grape pomace and GSE diets showed a higher biodiversity degree than those fed control diets, and the frequency of detection of several potential phenol-degrading bacteria as well as unidentified and uncultured organisms was increased in animals fed GPC and GSE diets. Viveros et al. (2011) reported that some microorganisms are able to use these compounds as nutritional substrates. In the particular case of lactobacilli, these bacteria have the ability to metabolize phenolic compounds supplying energy to cells and positively affecting the bacterial metabolism (García-Ruíz et al., 2008). Isolation of tannin-degrading lactobacilli from humans capable of degrading hydrolyzable tannins in human gut microflora has been reported by Osawa et al. (2000).

Conclusions

In conclusion, grape seed extract (150, 300, 450 ppm) had antimicrobial activity against *E. coli* and *S. typhi* under *in vitro* conditions. However, nano-emulsification of grape seed extract could not improve the antimicrobial effect of GSE under *in vitro* conditions but it did improve GSE antibacterial effect against coliforms and *E. coli* populations in the intestinal tract of broiler chickens. Since there are no data about the evaluation of NGSE in *in vitro* and *in vivo* assays, further studies should be done to examine the optimum level of NGSE as a supplemental nutrient to improve human and poultry health or as a pharmacological supplement to treat infectious diseases.

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