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Antibacterial and Antioxidant Potential of Methanol Extract of *Viburnum sargentii* Seeds

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Antibacterial and antioxidant activities of plant sources have attracted a wide range of interest across the world over the last decade. This is due to the growing concern for safe and alternative sources of antibacterial and antioxidant agents. In this study, we focused on the antibacterial and antioxidant activities and the chemical composition of a methanol extract from *Viburnum sargentii* seeds. The chemical composition was determined by gas chromatography-mass spectroscopy (GC-MS), and the antibacterial activity was screened by a disc diffusion assay. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the microbroth dilution and spread plate method, respectively. The *V. sargentii* extract showed growth inhibition activity on all tested Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*, and *Staphylococcus saprophyticus*) and Gram-negative (*Escherichia coli*, *Pseudomonas putida*, and *Proteus vulgaris*) pathogenic bacteria. The MIC and MBC ranged from 0.156~1.25 mg/ml for Gram-positive and 0.625~5.0 mg/ml for Gram-negative tested bacteria. The GC-MS results revealed the presence of several phytochemicals such as β-sitosterol and vitamin E, which are known for their pharmacological applications. The antioxidant activities of *V. sargentii* extract were investigated by three different methods: the 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay, the reducing power assay, and the total antioxidant capacity assay. The results showed a concentration-dependent antioxidant potential for all three used methods. In sum, our findings suggest that the methanol extract of *V. sargentii* seeds has the potential to inhibit the growth of pathogenic bacteria and provide antioxidant compounds, making it therefore worthy of further investigation.

Key words : Antibacterial, antioxidant, crude extract, GC-MS, *Viburnum sargentii*

Introduction

Medicinal plants have been used since ancient time for the management of various human and animal ailments. Many peoples, worldwide depends partially on the traditional medicine for its primary healthcare needs. At the present time, need more than one antibiotic for controlling bacterial infection, and multidrug resistant Gram-negative bacterial infection is a challenge for clinicians in 21st century [25]. The natural product contains secondary metabolites for protecting themselves from foreign infection [6]; secondary metabolites can be a good option for application in therapeutics, and it is more attracting to researchers due to its abundance availability in the nature and does not shows significant toxicity. A plant product includes terpenoids, alkaloids, and phenolic compounds are the good antibacterial agents against bacteria expressing multidrug resistance [12]. Several studies have been reported that continuous treatment with conventional antibiotics leads to the development of bacterial resistance [16, 20], these problems encourage researchers to find new antibacterial substance from various sources, such as medicinal plants. Medicinal plants are known for their effectiveness and widely used in the formulation of cosmetics, herbal drugs, in medicines and as a food [13, 14]. The search for novel natural compounds for therapeutics and food preservation with antioxidative activity is a very active domain of research. Oxygen derived free radicals or reactive oxygen species (ROS) are formed during normal cel-

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lular metabolism and energy production in body, but when present in high concentration they act as toxic [4]. The intracellular enzymes such as catalase or glutathione-peroxidase, and superoxide dismutase play a defensive role in cells; in addition, external sources such as vitamins (E, A, Beta-carotene), minerals and proteins can provide additional protection [18]. Protein denaturation, DNA and membrane damage in cells are the major causes of oxidative stress [9]. The lipid oxidation in food cause denaturation of nutritional value, changes texture and color loss, formation off-flavors and accumulation of compounds which may be detrimental to consumers [26].

Viburnum sargentii is a flowering plant that grow up to 10 ft. and broad and belong to family Caprifoliacea. It is an important medicinal plant and has been reported for pharmacological properties. For example, 9′-*O*-methyl-vibsanol isolated from stem bark shows strong concentration dependent cytotoxic effects on MCF-7 and A549 cancer cell lines [2], 5,7,4′-trihydroxy-flavonoid-8-C-β-D-glucopyranoside and quercetin-3-O-rutinoside, phenolic compounds isolated from the fruits and show free radical scavenging activity [27]. The ethanol extract of *V. sargentii* fruit was reported for antibacterial activity [28]. The methanol extract from *V. sargentii* aerial part was reported for anti-inflammatory, analgesic and hepatoprotective effect [11]. The chemical composition of *V. sargentii* includes, phenolic compounds [2, 27], and valeriana-type iridoid glucosides [24]. In this study, we report the chemical composition by gas chromatography-mass spectroscopy (GC-MS), antibacterial activity against Gram-positive and Gram-negative pathogenic bacteria and antioxidant activity by three different methods, DPPH free radical scavenging assay, reducing power assay and total antioxidant capacity of the methanol extract of *V. sargentii* seeds.

Materials and Methods

Materials

Iodonitrotetrazolium chloride (INT), Dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid, Ampicillin and Kanamycin were purchased from Sigma Aldrich, USA. Potassium ferricyanide, Tri-chloroacetic acid, Ferric chloride and Ammonium molybdate were procured from Daejung Chemicals and Metals, Korea. Mueller hinton agar (MHA), Mueller hinton broth (MHB), Tryptic soy agar (TSA), and Tryptic soy broth (TSB) were obtained from BD Diagnostic, France.

Bacterial strains

The pathogenic bacteria used in the present study was purchased from Korean Culture Center of Microorganisms (KCCM), Republic of Korea. Specific strains were used; Gram-positive bacteria *Staphylococcus aureus* ATCC6538p, *Staphylococcus saprophyticus* KCTC3345, *Listeria monocytogenes* ATCC7644 and Gram-negative bacteria *Escherichia coli* ATCC 25922, *Proteus vulgaris* KCTC2512, and *Pseudomonas putida* ATCC49128. These strains were maintained on TSA plates. An isolated colony was inoculated on TSB and incubated for 24 hr at 37℃. For antibacterial test, the turbidity of bacteria was adjusted to 0.08-0.1 at 600 nm (i.e. 0.5 McFarland standards) by spectrophotometry (Libra S22, Biochrom Ltd., Cambridge, England).

Plant material

The methanol extract of *Viburnum sargentii* seeds (KPEB ID: PB4642.6) was provided by Plant Extract Bank (KPEB) in Daejeon, Republic of Korea. The extract prepared as per following procedure; seeds were deep in 99.9% HPLC grade methyl alcohol at 45℃ followed by 15-minute sonication (SD-ULTRASONIC CLEANER, SDN-900H). Sonication repeated in interval of every 2 hr, 10 times in a day and same procedure repeated for 3 days. After 3 days, extract was filtered and concentrated by rotary evaporator (N-1000SWD, EYELA) at 45℃. Drying of extract by lyophilization (Biotron Corporation, Modul spin 40) at temperature 45℃ and cold trap at -70℃ for 24 hr. and prepared extracts stored at -4℃ until required. Working stock were prepared in DMSO with final concentration of 100 mg/ml and used for antibacterial, and antioxidant activities. Samples were stored in refrigerator until required. Methanol was used to dissolve the extract for phytochemicals composition detection by GC-MS analysis.

Antibacterial activity

The disc diffusion assay was performed to check antibacterial activity of the *V. sargentii* seeds (methanol-extract) on selected bacterial strains as per reported method [5, 19] with minor modifications. Briefly, MHA plates were spreaded with 100 µl (0.5 McFarland standards) of each bacterial suspension separately and placed 8 mm paper disc containing 50 µl of *V. sargentii* extract (20 mg/ml) and incubated at 37℃ for 24 hr. Likewise, 50 µl of ampicillin (0.2 mg/ml) and kanamycin (0.2 mg/ml) were used as a positive control for Gram-positive and Gram-negative bacterial, respectively.

All experiment performed in triplicate and the zone of inhibition (ZOI) was measured in mm.

Minimum inhibitory concentration and minimum bactericidal concentration

The MIC determination was performed by INT colorimetric method [7, 21] with minor modification. In brief, test samples were serial diluted in MHB with different concentrations (5.0, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.020, 0.010 mg/ml) and 0.5 McFarland standards bacterial suspension also prepared in MHB. 96-well plate containing 100 µl of bacterial suspension and 100 µl of diluted test samples (each well) were incubated at 37℃ for 24 hr. After incubation, 40 µl (0.2 mg/ml) of INT solution were added to each wells and further incubated for 30 min. The formation of pink color due to interaction of INT with viable bacteria only [7]. MIC was indicated as the test sample concentrations prevent color change. MBC was performed by spread plate method; 100 µl aliquot (bacteria with test sample) were spread on MHA plate with the help of sterile glass rod and incubated at 37℃ for 24 hr; the concentration of test sample shows complete inhibition of bacterial growth indicated as MBC.

Antioxidant activity

DPPH free radical scavenging assay

The DPPH free radical scavenging assay was performed as per method described by Tian *et al*. [23]. In brief, 1.0 ml of *V. sargentii* extract (5, 10, 15, 20, and 25 µg/ml in methanol) was added to 4.0 ml of DPPH solution (0.07 mM in methanol), reaction mixture was shaken for 2 min and incubated at room temperature in dark for 30 min. Reaction mixture without sample prepared as a control, and ascorbic acid was used as a standard for comparison. The absorbance at 517 nm (Libra S22) was recorded after incubation. The decrease of absorbance indicates an increased activity of free radical scavenging [18, 23]. The percentage of free radical scavenging activity or inhibition was calculated by employing the formula:

$$\% \text{ inhibition} = ((\text{Control } A_{517} - \text{Sample } A_{517}) / \text{Control } A_{517}) \times 100$$

The IC_{50} is the concentration required to inhibit 50% of the initial DPPH free radical. All the reactions were performed in triplicates and the mean ± standard deviation (SD) represents values of each measurement.

Reducing power assay

The reducing power assay was performed according to the method reported previously [8]. Briefly, 1 ml of *V. sargentii* extract (50, 100, 150, 200, and 250 µg/ml) were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of potassium ferricyanide (1% v/w). The reaction mixture was mixed properly and incubated for 20 min in water bath (50 ℃). To this mixture, 2 ml of Tri-chloroacetic acid (10%) was added, which was then centrifuged at 3,000 rpm for 10 min. After centrifugation, 2 ml of supernatant solution diluted with 2 ml of distilled water and addition of 0.5 ml of 0.1% $FeCl_3$. The absorbance was recorded at 700 nm (Libra S22), ascorbic acid and phosphate buffer were used as a standard and blank, respectively. Stronger reducing power indicated by increasing absorbance of reaction mixture. All experiment was repeated in triplicate and results presented as mean ± SD.

Total antioxidant capacity

Total antioxidant capacity of *V. sargentii* seeds extract was performed by the phosphomolibdenum method [1, 19]. Briefly, 0.3 ml of *V. sargentii* extract (50, 100, 150, 200, and 250 µg/ml) was mixed with 3 ml of reagent solution (4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 mM sulfuric acid; mixed in a 1:1:1 ratio) in the screw capped tube and incubated in water bath at 90℃ for 90 min. After cooling the reaction mixtures at room temperature, absorbance was recorded at 700 nm (Libra S22). Ascorbic acid was used as a standard and reaction solution without sample used as blank. The total antioxidant capacity was expressed as equivalent of ascorbic acid. All experiment was repeated in triplicate and results presented as mean ± SD.

Gas chromatography-mass spectroscopy

Different phytochemical constituents (volatile or semi-volatile) of *V. sargentii* seeds methanol-extract was performed using the GCMS (GCMS-QP2010 Ultra, Shimadzu, Japan) instrument with an Agilent DB-5 ms column (30 m × 0.25 mm × 0.25 µm). In the GC-MS, 1 µl of the sample (prepared in methanol) was injected in split mode with the injector temperature at 280℃. The oven temperature was programmed from 60℃(hold time 2 min), with an increasing of 10℃/min to 200℃, then 5℃/min to 325℃(hold time 10 min). Helium gas was used as a carrier with a constant flow at 1.0 ml/min. The other programmed conditions including: purge flow 3.0 ml/min, split ration 50.0, ion-source temperature 200℃, solvent cut time 5.0 min, and total flow 54.0 ml/min. Total

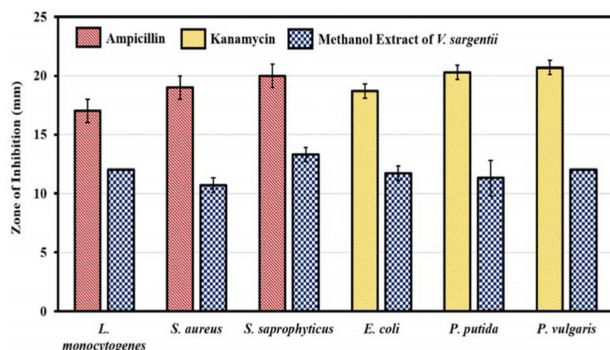

Fig. 1. Antibacterial activity of *Viburnum sargentii* seeds methanol-extract, ampicillin and kanamycin against pathogenic bacteria measured on agar medium by disc diffusion method. All values presented as mean of triplicates and error bars indicates ± standard deviation.

GC-MS running time from 5.0 min to 51.0 min and fragments from 25 to 600 Da were analyzed. Mass spectra were taken at 70 eV; scan interval 0.30 sec and GCMS real time analysis software were used to record results. The resulting phytochemical constituents were identified by comparing retention time and mass spectra with those standards or retention indices (RI) with published data and their mass spectra with the Wiley library, and National Institute of Standards and Technology (NIST) library.

Results and Discussion

Antibacterial studies

The antibacterial activity of *V. sargentii* seeds (methanol) extract was initially screened by disc diffusion assay. The variations in ZOI were observed with respect to the type of bacterium, which might be due to difference in the bacterial outer cell membrane characteristic. The result indicated that the *S. saprophyticus* having large ZOI (13.3±0.6 mm) while, *S. aureus* showed smaller ZOI (10.7±0.6 mm) among all tested organisms (Fig. 1). In the second step, qualitative analysis for bacterial growth inhibitory concentration and bactericidal concentrations were determined by MIC and MBC, respectively. Microbroth dilution and spread plate method were used for MIC and MBC respectively, and all tested bacteria showed susceptibility to the *V. sargentii* extract. The MIC and MBC results for test sample and antibiotics that are presented in Table 1; indicate that *S. saprophyticus* (MIC and MBC is 0.156, 0.625 mg/ml respectively) is most sensitive while, *P. vulgaris* (MIC and MBC is 1.25, 5.0 mg/ml respectively) is a strongest bacterium among the treated organisms. The susceptibility of *V. sargentii* seeds extract show more effective to tested Gram-positive bacteria compared to Gram-negative bacteria; this may be due to Gram-negative bacteria having lipopolysaccharides in their outer cell membrane which protect them from the binding of foreign material [3]. According to literature investigation, only one report was found on antibacterial activity of *V. sargentii* fruit extracts (water and ethanol), and the MIC and MBC is too high compared to present study [28].

Antioxidant activity

DPPH free radical scavenging assay

Assay based upon the use of DPPH radical is among the most popular spectrophotometric methods for determination of antioxidant capacity of plan extracts because the radical compounds can directly react with antioxidants. The reducing power of *V. sargentii* extract and ascorbic acid was analyzed by spectrophotometric and 50% inhibition of radicals was expressed as IC_{50}. Ascorbic acid was used as a positive control for comparison due to its stability at higher temperature [10]. Results presented in Fig. 2A. IC_{50} for ascorbic acid and *V. sargentii* extract were observed at 10.0±1 μg/ml and 15.33±0.58 μg/ml, respectively. *V. sargentii* extract shows concentration dependent DPPH radical scavenging

Table 1. Antibacterial activity of *Viburnum sargentii* seeds methanol extract

Bacteria name	Methanol extract		Ampicillin		Kanamycin	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
L. monocytogenes ATCC7644	0.625	1.25	0.039	0.078	ND	ND
S. aureus ATCC6538p	0.312	1.25	0.039	0.156	ND	ND
S. saprophyticus KCTC3345	0.156	0.625	0.039	0.078	ND	ND
E. coli ATCC25922	0.312	0.625	ND	ND	0.039	0.078
P. putida ATCC49128	0.312	1.25	ND	ND	0.039	0.156
P. vulgaris KCTC2512	1.25	5.0	ND	ND	0.039	0.156

MIC- Minimum inhibitory concentration, MBC- Minimum bactericidal concentration, ND- Not determined.

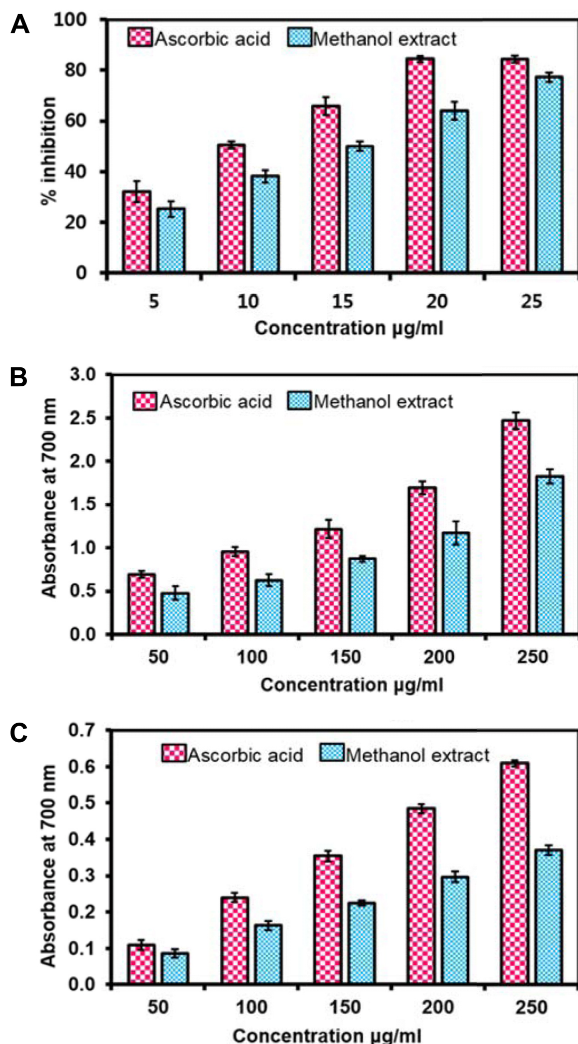

Fig. 2. Histograms indicates antioxidant activity of methanol extract from seeds of *Viburnum sargentii* by (A) DPPH free radical scavenging assay, (B) Reducing power assay, and (C) Total antioxidant capacity. All results are presented as a mean ± Standard deviation (n=3).

activity; the increasing activity from 5 to 25 µg/ml concentration was observed. Similar observations were observed by Patil *et al*. [19] and Ferreira *et al*. [8]. When DPPH radicals encounter a proton donating substance such as an antioxidant, the radical is scavenged and absorbance is decreased by changing color from purple to yellow [15] and efficiency of an antioxidant substance to reduce DPPH depends on hydrogen donating ability, which is totally related to phenolic hydroxyl moieties [23]. The literature review revealed that the antioxidant activity of *V. sargentii* seeds methanol-extract has not been reported previously.

Reducing power assay

It was suggested that the electron donating capacity, reflecting the reducing power of bioactive compounds, is associated with antioxidant activity. Fig. 2B indicates the concentration dependent reducing power of *V. sargentii* seeds methanol extract in comparison with ascorbic acid as a standard. In this method, the initial color of reaction mixture is yellow and in presence of antioxidant compounds it turns to blue or green due to reduction of the ferric (Fe^{3+}) to ferrous (Fe^{2+}) form [8]. The hydrogen-donating ability of antioxidant substance responsible for blocking radical chain reaction; more hydrogen-donation shows more reducing activity [8, 23]. Therefore, the concentration of ferrous form can be monitored at 700 nm. The *V. sargentii* seeds methanol extract shows concentration dependent reducing power and gradually an increase reducing power from lower to higher concentration were observed. The comparative study with standard indicates *V. sargentii* extract has less activity, but it's a crude extract and fractionation of the extract may improve reducing activity. Present study results are in good agreement with previously reported studies [8, 19, 23].

Total antioxidant capacity

Fig. 2C indicates the concentration dependent total antioxidant activity of *V. sargentii* seeds methanol extract in comparison with ascorbic acid as a standard. This method is applicable to evaluate antioxidant capacity (total) for fat-soluble and water-soluble antioxidants. This method based on reduction of Mo (VI) to Mo (V) by the antioxidant substances and formation of green complex (phosphate/Mo (V)) at acid pH. The phosphate/Mo (V) complex absorbance maxima can be measured at 700 nm [1]. The assay was used to analyze total antioxidant capacity of *V. sargentii* seeds methanol extract and ascorbic acid (standard). In present study, increasing of concentration dependent total antioxidant capacity from 50 to 250 µg/ml was observed. Our findings are compatible with previously reported studies [1, 19].

Gas chromatography-mass spectroscopy analysis

GC-MS is employed to separate the volatile and semi-volatile compounds from the methanol extract of *V. sargentii* seeds. Fig. 3 shows the GC-MS chromatogram; the peaks observed in methanol extract of *V. sargentii* seeds indicates that there are 5 known phytochemicals. On comparison of the mass spectra of the constituents with Wiley and NIST library, the 5 phytocompounds were characterized and identified and their retention time (RT), molecular formula, molecular weight, and peak area percentage are listed in Table 2. To the best of our knowledge this is the first study from

Table 2 GC-MS data for the methanol soluble fractions of *Viburnum sargentii* (seeds extract)

RT	Compounds name[a]	Formula	MW	Peak area (%)
13.653	Guanosine	$C_{10}H_{13}N_5O_5$	283	52.28
14.385	1,6-Anhydro-beta-D-glucopyranose (Levoglucosan)	$C_6H_{10}O_5$	162	3.72
38.369	Vitamin E	$C_{29}H_{50}O_2$	430	4.54
40.895	Stigmast-5-en-3-ol, (3.beta.)-	$C_{29}H_{50}O$	414	12.69
41.083	Stigmata-5,24(28)-dien-3-ol, (3.beta.,24E)-	$C_{29}H_{48}O$	412	4.14

RT: Retention time in minute and MW: Molecular formula; [a] Compounds where analyzed by comparing the peaks with the library (NIST and WILEY).

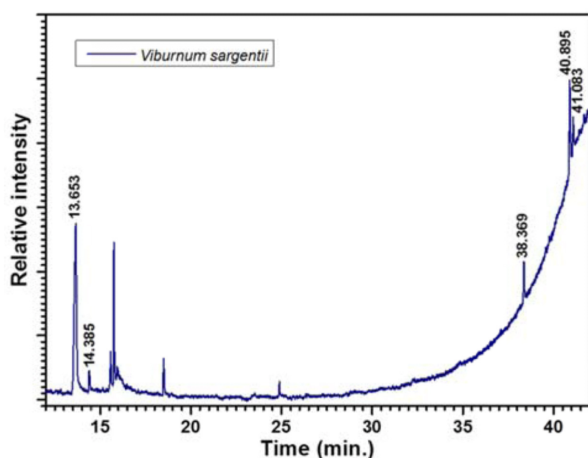

Fig. 3. GC-MS chromatogram of the methanol extract of *Viburnum sargentii* seeds.

V. sargentii seeds. From GC-MS it is found that Guanosine and Stigmast-5-en-3-ol,(3.beta.)- are the major component with 52.28 and 12.69 percentage, respectively. Vitamin E has antioxidant activity [18], and 4.54 percent peak area was observed in *V. sargentii* seeds extract, which might be involved in enhancing total antioxidant capacity. Stigmast-5-en-3-ol,(3.beta.)- is a phytosterol that has been reported for their antidiabetic activities by regulating glucose transport [22], G2/M phase growth arrest and apoptosis through the Bcl-2 and PI3K/Akt pathway [17]. Phenolic compounds and phenolic glycosides from fruits of *V. sargentii* Koehne were reported for antioxidant activity [27]. All these results indicate that *V. sargentii* seeds can be useful in other biomedical applications.

In the present study, antibacterial and antioxidant activities of *V. sargentii* seeds methanol extract was evaluated using different in-vitro methods. The results of this study indicate *V. sargentii* seeds methanol extract have good activity as an antibacterial agent against Gram-positive and Gram-negative bacteria. Antioxidant activity by different methods indicates that *V. sargentii* seeds methanol extract is a good antioxidant agent. The presence of vitamin E in extract which was revealed by GC-MS analysis might be responsible for enhancing antioxidant activity. Stigmast-5-en-3-ol,(3.beta.) - also known as β-Sitosterol and for its many pharmacological application, which makes more importance to the *V. sargentii* seeds. In order to improve activity or use of *V. sargentii* seeds as an antibacterial or antioxidant agent, further studies such as isolation, identification and characterization of chemical compounds is necessary. This study indicates the *V. sargentii* methanol extract of seeds can be useful in therapeutics as an antioxidant or antibacterial agent.

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초록 : *Viburnum sargentii* 종자 메탄올 추출물의 항균 및 항산화 활성에 대한 연구

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최근에 들어서 전세계적으로 식물 자원에서의 항균력 및 항산화력에 대한 관심이 광범위하게 증가하고 있다. 이는 사용되고 있는 항균제와 항산화제보다 안전한 대체 자원에 대한 관심이 높아졌기 때문으로 볼 수 있다. 따라서 본 연구에서는 *Viburnum sargentii* 씨앗의 메탄올 추출물의 항균력 및 항산화력과 화학적 구성에 대하여 연구를 진행하였다. 추출물의 화학 조성은 가스 크로마토 그래피-질량 분석법으로 측정하였고, 디스크 확산 분석법을 이용하여 항균 활성을 알아보았다. 액체 배지 감수성 실험을 통해 최소 억제 농도(MIC)를 측정하였고, 한천 희석법을 통해 최소 살균 농도(MBC)를 측정하였다. *V. sargentii* 메탄올 추출물은 실험에 사용된 그람 양성균(*Listeria monocytogenes, Staphylococcus aureus, Staphylococcus saprophyticus*) 및 그람 음성균(*Escherichia coli, Pseudomonas putida, Proteus vulgaris*)의 성장을 억제하였고, MIC와 MBC는 그람 양성균에서 0.156-1.25 mg/ml, 그람 음성균에서는 0.625-5.0 mg/ml의 범위를 나타내었다. 가스 크로마토 그래피-질량 분석법 결과에서는 *V. sargentii* 메탄올 추출물이 약리 활성을 가지는 β-sitosterol과 vitamin E와 같은 여러 파이토케미컬을 포함하고 있는 것을 확인하였다. *V. sargentii* 메탄올 추출물의 항산화 활성은 DPPH free radical scavenging assay, reducing power assay, total antioxidant capacity의 세 가지의 실험 방법을 통하여 확인하였으며, *V. sargentii* 메탄올 추출물의 농도가 증가함에 따라 항산화 활성도 증가하는 것을 세 가지 실험에서 모두 확인하였다. 이러한 결과를 통하여 *V. sargentii* 씨앗이 병원성 균의 성장을 억제할 수 있는 활성을 가질 뿐만 아니라, 항산화 활성을 가지는 구성물이 풍부함으로 추가적인 연구를 진행하기에도 충분한 가치가 있는 것을 확인하였다.