Thesis for the Degree of Master of Engineering

In vitro Antiviral Substances Isolated from *Eisenia bicyclis* against Murine Norovirus as a Norovirus Surrogate

by

Sun-Young Moon

Department of Interdisciplinary Program of Biomedical Engineering, the Graduate School

Pukyong National University

February 2014

Eisenia bicyclis 추출물의 Norovirus Surrogate 인 Murine Norovirus 에 대한 항바이러스 활성

Advisor: Prof. Myunggi Yi

by

Sun-Young Moon

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Engineering

in Department of Interdisciplinary Program of Biomedical Engineering,
the Graduate School,
Pukyong National University

February 2014

In vitro Antiviral Substances Isolated from *Eisenia bicyclis* against Murine Norovirus as a Norovirus Surrogate

A dissertation

by

Sun-Young Moon

Approved as to style and content by:

(Chairman) Myung-Suk Lee

(Member) Young-Mog Kim

(Member) Myunggi Yi

February, 2014

Eisenia bicyclis 추출물의 Norovirus Surrogate 인 Murine Norovirus 에 대한 항바이러스 활성

문 선 영

부경대학교 대학원 의생명융합공학협동과정

요약

노로바이러스 (Norovirus)는 과거에 winter vomiting disease 라고 불렸던 만큼 낮은 온도에서도 활성을 유지하며 염소와 같은 소독제에도 내성이 있다. 또, 면역성이 없어 백신개발에 어려움을 겪는 대표적인 식중독 원인 바이러스이다. 노로바이러스 감염에 의한 식중독은 대규모로 발생하는 경우가 많아 사회경제적으로 중대한 영향을 미치고 있다. 그러나 유전자 변이가 심하고 *in vitro* 상태에서 배양의 어려움을 이유로 다양한 연구가 부족한 편이다,

해조류는 일부 아시아 국가에서 소비되는 1 차 가공식품으로써 섭취를 제외하고 다양한 분야에 적용된 사례가 적은 편이지만 근래, 육상생물보다 해양생물 중에 생리활성물질이 풍부하다는 연구결과가 보고되고 있다. 그

중 *Eisenia bicyclis* 추출물의 항균효과, 항염증 및 항노화효과가 있다는 보고가 있었다. 그러나, *E. bicyclis* 추출 분획물의 노로바이러스 억제효과에 대한 연구는 아직 보고된 바 없다.

이에 본 연구에서는 대황추출물의 노로바이러스에 대한 항바이러스 효과를 연구하였다. 노로바이러스의 경우 세포배양이 불가능하기 때문에 노로바이러스의 대체바이러스인 Murine norivirus 를 이용하여 항바이러스 활성을 조사하였다. *E. bicylis* 의 methanol 추출물에서 5 개의 분획물을 얻었고, 그 중 Murine norovirus 에 대하여 가장 뛰어난 항바이러스 활성을 보였던 ethyl acetate 층에서 2 개의 단일물질을 분리 및 정제하였다. 5 개의 유기용매 분획물과 ethyl acetate 층에서 분리한 2 개의 단일추출물을 처리하고 murine norovirus 를 감염시켜 RAW 264.7 세포에서의 MNV 에 대한 감수성을 검토하였다. 또, MNV 의 숙주세포가 되는 RAW 264.7 세포에 대한 *E. bicyclis* 추출물의 독성을 평가하여 Selectivity Index 값을 구하였을 때, 2.2~125 를 나타내어 E. bicylis 에는 노로바이러스를 불활화 시키는데 유효한 성분을 지니는 것을 확인하였다. 그리고 *E. bicyclis* 분획물의 각 페놀함량을 측정하였을 때, 항바이러스 활성을 보였던 분획물에 페놀함량이 비례적으로 나타나 페놀계통의 물질이 바이러스의 저해에 영향을 미치는 물질로 추정된다.

Table of Contents

List of Figures

List of Tables

Introduction

Norovirus is genetically diverse group of non-enveloped RNA virus belonging to genus within the family Caliciviridae (King *et al*., 2011), and well known acute implication of over 90% of nonbacterial gastroenteritis among worldwide (Inouye *et al*., 2000; Fankhauser *et al*., 2002; Lopman *et al*., 2004). According to Center for Disease and Prevention (2012) over 70% of water-related gastroenteritis patients and over 50% of food-consumption causative of them resulted from norovirus infection in USA. Epidemic food poisoning occurred in 2006, Korean high school approximately 2,872 students were hospitalized and the norovirus was detected from each patients' fecal (Korea Center for Disease and Prevention, 2012). In recent years, norovirus causative issues have been great concern over world. Despite numerous norovirus induce food poisoning and awareness of its danger such as low infectious dose and various gene mutations, norovirus research has been very limited due to absence of virus cultivation method *in vitro* (Caul, 1996; Clarke *et al*., 1998; Green *et al*., 2000; Lopman *et al*., 2004; Duizer *et al*., 2004; Dubois *et al*., 2002; Straub *et al*., 2007). Consequently, most norovirus studies are performed using its surrogate virus for instance, felince calicivirus and murine norovirus (Lee *et al*., 2011;

Su and D'souza, 2013). Recent animal model study reveals that structure of murine norovirus, its genetic system and replication cycle have similarity to human norovirus (Moreno-Espinosa *et al*., 2003; Lee *et al*., 2011) Also, The genetic relatedness of MNV to human norovirus combined with its ability to survive under gastric pH levels makes this virus a promising and relevant surrogate for studying environmental survivability of human norovirus (Cannon *et al*., 2006; Zhang *et al*., 2012).

There has been great interest in natural derived substances as effective antioxidant that inhibiting aging induced illness (Su and D'souza, 2013). Various extracts and purified compounds that fractioned from marine source algae have been studied to possess antibacterial activities, inflammation activities and radical scavenging activities (Eom *et al*., 2012; Kang *et al*., 2013). According to Choi *et al*. (2013) *Eisenia bicyclis* methanolic extract has potential to be an anti-norovirus agent exhibiting inhibition of feline calicivirus infection on CrFK cells by 50% at concentration of 80 μg.

In respect to this, this study consisted to estimate antiviral activity of *E. bicyclis* on MNV as a norovirus surrogate. Also, it is investigate the implications of *E. bicyclis* for virus infectivity and its cytotoxicity. Furthermore, it is discussed that these observations could contribute on

future studies and potential application as pharmaceutical source to control norovirus.

Materials and Methods

1. Preparation of raw material and extraction

In the late September 2010, *E. bicyclis* was purchased from Ulleung Trading Co. A voucher specimen was refrigerated at -80°C. Fresh *E. bicyclis* was desalinizated by washing then dried at 60°C. Dried *E. bicyclis* was triturated into powder with electronic grinder (HMF-1000A; Hanil Electronics, Seoul, Korea). The dried *E. bicyclis* powder (1.0 kg) was educted with methanol (MeOH; 10 L × 3) at 70°C for 3 hr (3 times) and the solvent was evaporated in vacuo. A liquid/liquid solvent fractionation procedure was performed to fraction an antiviral substance according to relative polarity. The methanolic extract of *E. bicyclis* was hydrated with distilled water and the solution was fractionated by the same volume of organic solvent. The combined crude MeOH extract (164.3 g) was suspended in 10% MeOH (1.0 L) and then fractionated in turn with *n*-hexane (Hexane; 1.0 L × 3), dichloromethane (DCM; 1.0 L × 3), ethyl acetate (EtOAc; 1.0 L × 3), and *n*-butanol (BuOH; 1.0 L × 3) solution. Each extract was evaporated using rotary evaporator (Eyela Co., Tokyo, Japan) under vacuum at 45°C.

2. Cell and virus culture

Raw 264.7 cells were grown in Delbecco's modified Eagle's medium (DMEM; GIBCO/BRL, NY, USA) with 1% penicillin (100 U/mL; Gibco BRL)-streptomycin (100 μg/mL; Gibco BRL) and heat inactivated 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 atmosphere. MNV was propagated in raw 264.7 cells and harvested by freeze- thawing method of three times. The virus stock was stored at -80°C until use throughout the experiments.

3. Cytotoxicity assay (MTT assay)

The cell cytotoxicity was determined by quantifying raw 264.7 cells viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Fig. 1). The medium containing RAW 264.7 cells were cultured into a 96-well plate at a density of 3 x 10^5 cells/ml. CC_{50} value was examined following 24 hrs of raw 264.7 cell monolayers exposure to different concentration of extracts and equal volume of medium was a control and incubated at 37°C, 5% CO_2 atmosphere. Formazan solubilizing solution was added after incubation. Then, the absorbance of each well was

measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, USA). The optical density of formazan formed by untreated cells was taken as 100% of viability.

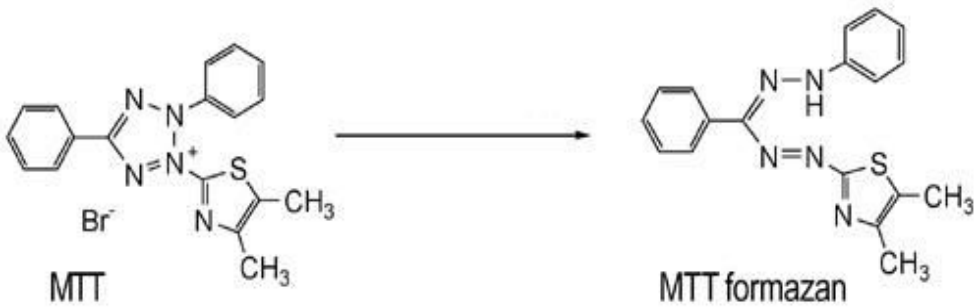


Fig. 1. Molecular structure of MTT and its corresponding reaction product.

4. Antiviral activity assay

The antiviral activity of *E. bicyclis* extracts against MNV was evaluated by tissue culture infectivity dose 50 ($TCID_{50}$), as described by Kim *et al*. (2010). The antiviral effective concentration was expressed as the EC_{50} value, defined as the concentration of the sample required to exhibit only 50% of cytopathogenic effects (CPEs) caused by the virus (Armond *et al*., 1996; Goodfellow *et al*., 2005, Choi *et al*., 2013). Briefly, the concentration of Log 5 $TCID_{50}$ per mL MNV suspension was treated with identical volumes of serially diluted extracts at room temperature for 24 hrs. The mixtures were added to a monolayer of raw 264.7 cells in a 96 well plate. After incubating at 37°C, 5% of CO_2 for 72 hrs, the cells were fixed with formaldehyde and stained with crystal violet solution. Untreated cells were suspended with maintenance medium containing 2% of FBS instead of test extract as the control. The relative effectiveness of the investigational extract in inhibiting viral infectivity compared to inducing cell death is defined as the selectivity index (i.e., CC_{50} value/EC_{50} value). It is desirable to have a high selectivity index giving maximum antiviral activity with minimal cell toxicity as well as higher possibility of application (Oh *et al*., 2013).

5. Quantification of total phenolic contents

The contents of total phenolic (TP) compounds evaluation in the fractionated *E. bicyclis* extracts were accordance to modified Folin-Ciocalteu assay (Kim *et al*., 2006; Lee *et al*., 2009) and the results were estimated by phloroglucinol equivalents (PGE). An aliquot (0.1 mL) of diluted sample was mixed in a 1.5 mL microtube with 0.5 mL of 1 N Folin-Ciocalteu reagent. After adding 0.4 mL of 20% Na_2CO_3, the mixture was allowed to stand for 3 mins. Samples were then incubated at room temperature for 45 mins in the dark and centrifuged (1600 × g, 8 min). The optical density of the supernatant was measured at 765 nm using a microplate reader (VERSAmax, US). The concentration of TP compounds in each extract was calculated using the linear equation based on a calibration curve. The following equation was $y = 19.961\ x + 0.1596$ and the regression coefficient of the equation was 0.9976.

6. Isolation and purification of phlorotannins from *E. bicyclis*

Structure was determined by correlations based on scalar coupling between protons and carbons separated by two or three chemical bonds of

organic molecules. The ^{1}H and ^{13}C NMR spectra were measured on a Hitachi U-2000 spectrophotometer (Varian, Inc., Walnut Creek, USA) at 600 and 150 MHz, respectively. The chemical shifts are indicated by δ (ppm) values comparative of the solvent DMSO-d_6 (δH 2.49; δC 39.7) on a tetramethylsilane (TMS) scale. The internal standard pulse sequences programmed into the instruments were used for each 2D measurement. The JCH value was set at 8 Hz in the Heteronuclear Multiple Bond Correlation (HMBC) spectra. Fast Atom Bombardment (FAB-MS) using 3-nitrobenzyl alcohol as the matrix agent was performed on a Micro Mass Auto Spec OA-TOF spectrometer. High-performance liquid chromatography (HPLC) analysis was conducted on a YMC-Pack ODS A-302 column (4.6 mm i.d. × 150 mm; YMC Co., Ltd., Kyoto, Japan) and developed at 40℃ with 1% formic acid (HCOOH : MeCN = 8 : 2; Detection : 280 nm). LiChroprep RP-18 gel (Merck, Darmstadt, Germany) and Sephadex LH-20 (Merck, Darmstadt, Germany) were used in order to column chromatography. The Kieselgel 60 F254 plates (0.25 mm layer thickness, Merck, Darmstadt, Germany) and UV irradiation (254, 365 nm) with 10% H_2SO_4 reagent were used for detecting.

Results and Discussion

1. Antiviral activity of *E. bicyclis* extracts

The methanolic extract of *Eisenia bicyclis* showed an antiviral activity against MNV, norovirus surrogate, proposing that the extract contains an antiviral compound against norovirus. Also, the methanolic extract of *E. bicyclis* exhibited similar antiviral activity against FCV (Choi *et al*., 2013). In order to identify an antiviral compound against MNV, the methanolic extract was further fractionated using organic solvents (Fig. 2). A lyophilized powder (1.0 kg) of *E. bicyclis* was percolated in methanol (3 times × 1.0 L), followed by partitioning with several organic solvents to yield Hexane-soluble extract (42.3 g), DCM-soluble extract (2.5 g), EtOAc-soluble extract (23.8 g), BuOH-soluble extract (26.5g) and H_2O-soluble extract (69.1 g) in extract..

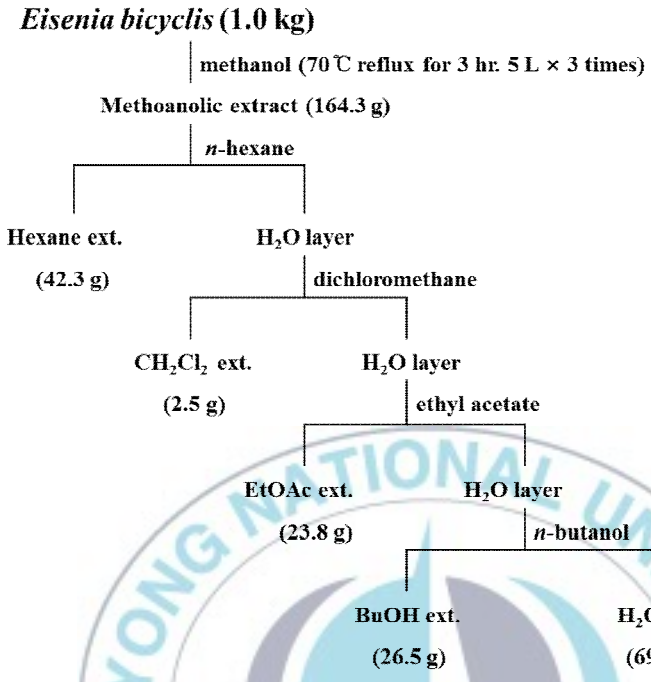


Fig. 2. Scheme of extraction and liquid-liquid partition. *n*-hexane soluble extract (Hexane ext.); dichloromethane-soluble extract (DCM ext.); ethyl acetate-soluble extract (EtOAc ext.); *n*-butanol extract (BuOH ext); water-soluble extract (H_2O ext.).

2. Total phenolic (TP) contents of the methanol extract and its solvent soluble extracts

Phenolic compounds in plants have been widely reported to have various biological effects such as antioxidative, antimicrobial, antivirial, antimutagenic, anticarcinogenic, antiproliferative activities and vasodiliatory action (Lee and Jeon, 2013). Similarly, several polyphenols in marine algae have potent antioxidant properties (Rösch *et al*., 2003; Danesi *et al*., 2013). On the contrary of terrestrial plants polyphenols, algae polyphenols are called phlorotannins (McClintock and Baker, 2010). The amounts of the total phenolic contents (TP) in the MeOH extract and its other organic solvent soluble extracts of *E. bicyclis* are shown in Table 1.

In addition, TP contents of the extracts were also determined to clarify the relationship between their antimicrobial activities and phlorotannins contents. Among *E. bicyclis* extracts, the TP content of EtOAC-soluble extract was the highest (126.4 mg PGEs/g of dry basis) followed by DCM-soluble extract (25.1 mg PGEs/g of dry basis), Hexane-soluble extract (44.9 mg PGEs/g of dry basis) and H_2O-soluble extract (4.0 mg PGEs/g of dry basis).

These results revealed that the order of the amounts of TP content was

similar to that of their antiviral activities (Table 2). Several studies have been shown close relationship between antimicrobial activities and the amount of TP (Silvia *et al*., 2011). Moreover, Van Hoof *et al*. (1989) suggest phenols are the major classes with antiviral activity against diverse virus families. Therefore, it was considered that antivirial actvities of *E. bicyclis* extracts also correlated with their TP contents.

Table 1. Total polyphenolic content expressed as phloroglucinol equivalents in *Eisenia bicyclis* extracts

Samples†	Total phenolics* (mg PGE/g, dry basis)
MeOH	29.5 ± 2.1
Hexane	44.9 ± 1.5
DCM	25.1 ± 10.4
EtOAc	126.4 ± 8.3
BuOH	45.9 ± 10.7
H_2O	4.0 ± 4.1

† MeOH, methanolic extract; DCM, dichloromethane-soluble extract; EtOAc, ethyl acetate-soluble extract; BuOH, butanol-soluble extract; H_2O, water-soluble extract.

3. Cytotoxicity of *Eisenia bicyclis* fractions

The cytotoxicity of *E. bicyclis* extract fractions and isolated compounds was evaluated by determining 50% Cytotoxicity concentration (CC_{50}) values using MTT assay. Confluent cells in DMEM were incubated in the absence or presence of twofold diluted samples (0.25-2400 μg/mL or μM/mL) for 72 hrs, after which MTT reagents were added to the cells. The CC_{50} value of the Water fraction was 2150 μg/mL, the lightest cytotoxicity and DCM fraction showed mild cytotoxicity following by 1100 μg/mL. The EtOAc fraction exerted strong influence to the cells by 320 μg/mL among 5 different fractions (Table 2).

4. Anti-viral activity of *Eisenia bicyclis* extracts

The anti-viral activities of Hexane-, DCM-, EtOAc-, and H_2O-soluble extracts were evaluated by measuring EC_{50} value. Among them, the EtOAc-soluble extract showed the strongest anti-viral activity and followed by DCM-, BuOH- and Hexane-soluble extract in the order water-soluble extract hardly shows antiviral activity (Table 2), suggesting that the extract contains an antiviral substance against MNV. Also, the methanolic extract of *E. bicyclis* exhibited antiviral activity against fish rhabdovirus (Kamei

and Aoki, 2007). In order to identify an antiviral compound exert anti-MNV activity, further classified organic solvent soluble fractions were exposed. As a result, each extracts from *E. bicyclis* showed different arrest virus infection by *in vitro* assay. The EtOAc soluble extract exhibited extent disinfection of MNV by 50% at 4 μg/mL correspond with selectivity index (SI) value of 125. The *n*-BuOH soluble extract was followed at 20 μg/mL but as its cytotoxicity effect high at 320 resulted in SI value of 16. Along with water soluble extract showed low cytotoxicity at 2150 μg/mL and reasonably high virucidal activity at 44 μg/mL, account for 48.86 of SI value. In order to identify a single antiviral substance against MNV of the *E. bicyclis*, EtOAc fration was further fractionated and antiviral activity experiments were performed.

Table 2. *In vitro* anti norovirus activities of *Eisenia bicyclis* extract solvent soluble fractions against Murine norovirus (MNV) on raw 264.7 cells using the mixed treatment assay

Extract of compounds	CC_{50}(μg) [b]	EC_{50}(μg) [c]	SI [d]
MeOH[a]	940	42	22.4
Hexane	1100	300	2.2
DCM	670	180	3.7
EtOAc	500	4	125.0
BuOH	320	20	16.0
H_2O	2150	44	48.86

[a]MeOH, methanolic extract; DCM, dichloromethane-soluble extract; EtOAc, ethyl acetate-soluble extract; BuOH, butanol-soluble extract; H_2O, water-soluble extract

[b]CC_{50} : mean (50%) value of cytotoxic concentration.

[c]EC_{50} : mean (50%) value of effective concentration.

[d]SI : selective index, CC_{50}/EC_{50}

5. Isolation of phlorotannins from *E. bicyclis*

Several reports suggest that marine brown algae has various biologically active components specifically, phenols and polyphenols as secondary metabolites and these are responsible their biological activities (Kim *et al*., 2006; Okada *et al*., 2004; Holdt and Kraan, 2011). The previous reports on *E. bicyclis* have revealed that it contains abundant phlorotannin derivatives with various bioactivities, such as antioxidation (Kwon *et al*., 2013), anti-inflammation (Jung *et al*., 2013), anti-diabetes (Broadhurst *et al*., 2000; Eom *et al*., 2012) and anti-cancer (Lee *et al*., 2013). However, there have been few researches regarding the antimicrobial activities of isolated phlorotannins from *E. bicyclis*. Also, only limited information is available concerning their antiviral activity. Hence, this study attempted to isolate new phlorotannins from *E. bicyclis* for evaluating its antiviral effects against MNV, the important public health matter. For the purpose, the EtOAc-soluble extract of *E. bicyclis* solvent was subjected to isolated active compounds.

The EtOAc-soluble extract (23.8 g) was chromatographed on a Sephadex LH-20 column using MeOH as solvent to yield a subfraction (Fig. 3). A portion (23.8 g) of the EtOAc extract was chromatographed on a Sephadex

LH-20 column (4.0 cm i.d. × 50 cm) with MeOH and fractioned into a subfraction. The Subfraction were subjected to column chromatography over a LiChroprep RP-18 column (1.1 cm i.d. × 37 cm) with aqueous MeOH to yield pure compounds **1** (3.6 mg) and **2** (32.9 mg).

6. Identification of compounds isolated from *E. bicyclis*

Successive column chromatographic purification of the EtOAc-soluble fraction of the methanolic extract of *E. bicyclis* led to isolation and characterization of two phenolic derivatives **1–2**. The isolated known compounds were phlorofucofuroeckol-A and dieckol by comparison of their physicochemical and spectroscopic data (^{1}H, ^{13}C-NMR, 2D NMR, and MS) with those of authentic samples and reference data (Kang *et al*., 2003, Okada *et al*., 2004).

7. Spectroscopic properties of compounds 1-2 isolated from the EtOAc-soluble extract (Table 3)

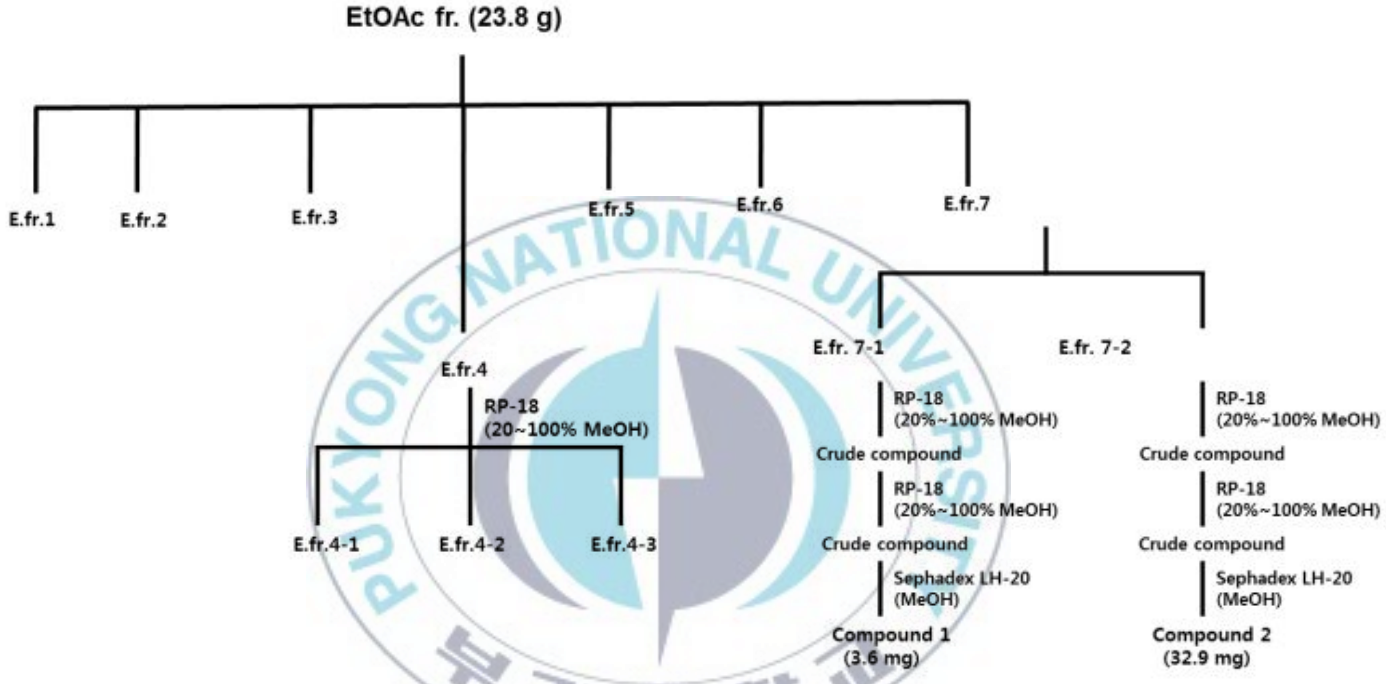
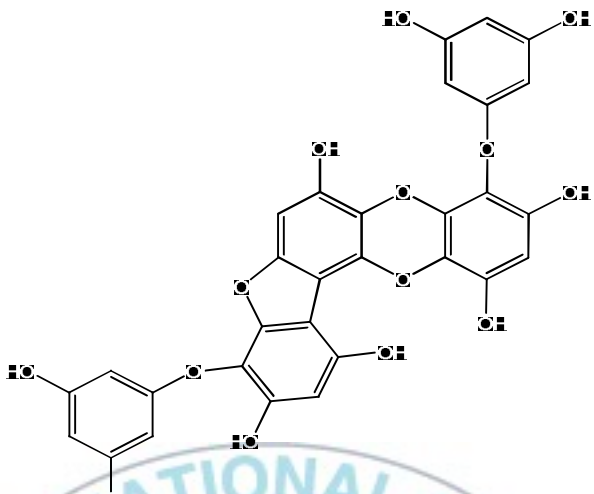


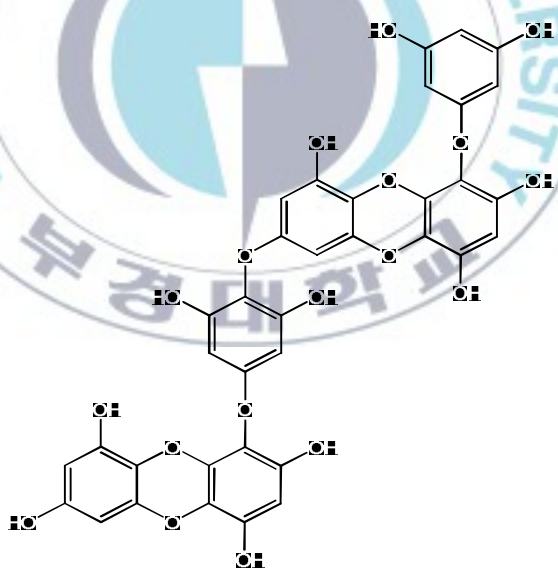
Fig. 3. Isolation of compounds **1-2** from the ethyl acetate-soluble extract of *Eisenia bicyclis.*

Table. 3. ^{13}C NMR (600MHz) data for isolated phlorotannins (**1**-**2**) in DMSO-d_6

Position	Compound 1	Compound 2
1	122.2	122.2
2	146.5	146.0
3	97.8	98.3
4	141.6	141.8
4a	122.2	123.2
5a	133.5	142.3
6	102.8	93.5
7	103.0	154.2
7a		
8	146.1	98.0
8a		
9	98.7	145.8
9a		124.0
10	150.4	
10a		137.2
11	119.7	
11a	149.1	
12		
12a	149.9	
13	94.4	
13a		
13b		
14	144.3	
14a	125.9	
15a	136.4	
1′	159.8	160.2
2′	93.3	93.6
3′	158.4	158.7
4′	95.9	96.1
5′	158.4	158.6
6′	93.3	93.6
1″	159.5	122.2
2″	93.1	145.8
3″	158.6	98.2
4″	96.1	141.9
4a″		123.1
5″	158.6	
5a″		142.5
6″	93.1	93.8
7″		153.0
8″		98.0
9″		146.0
9a″		122.6
10a″		137.0
1		155.8
2		94.4
3		151.1
4		124.2
5		151.1
6		94.4

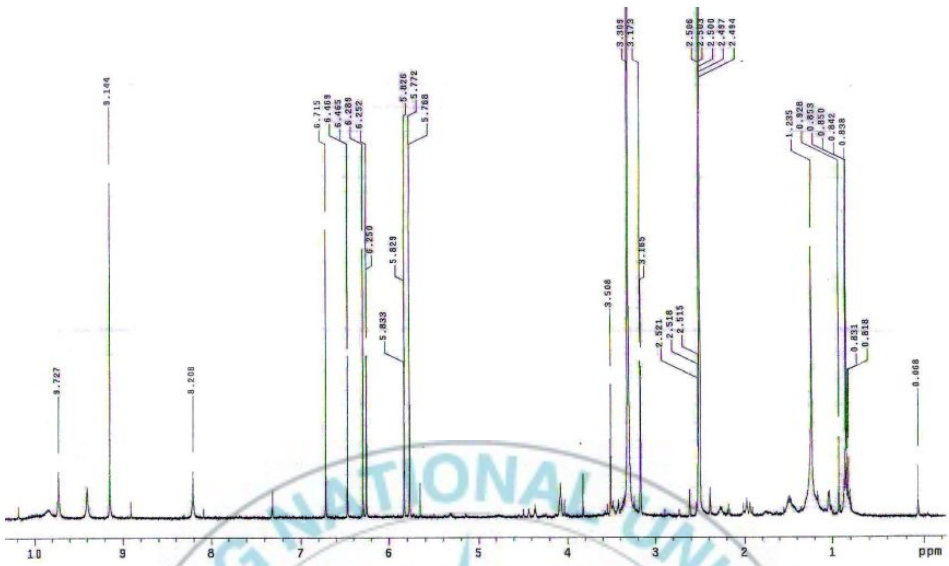


1

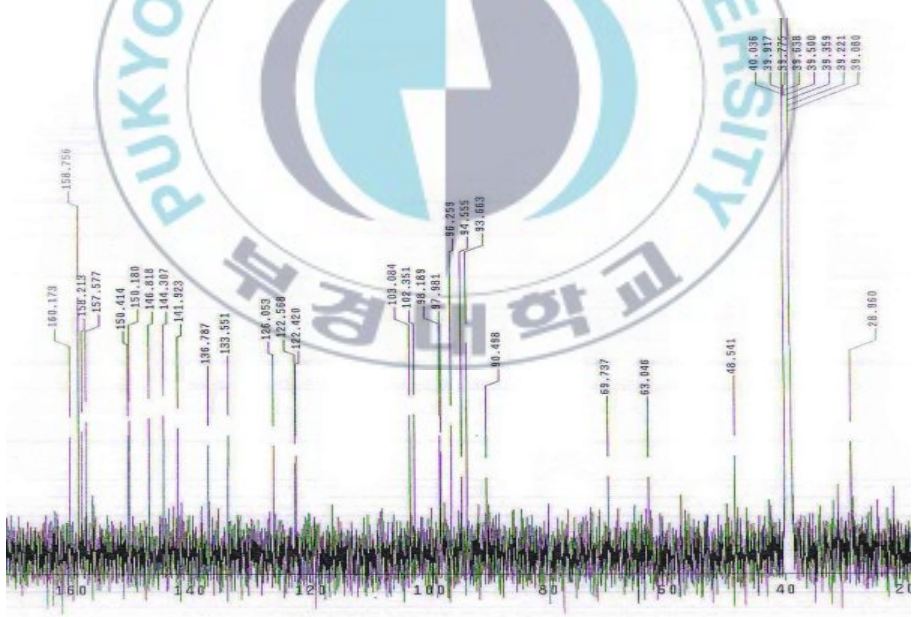


2

Fig. 4. Structures of the compounds **1-2** isolated from *Eisenia bicyclis* (Compound **1,** phlorofucofuroeckol-A; Compound **2**, dieckol)

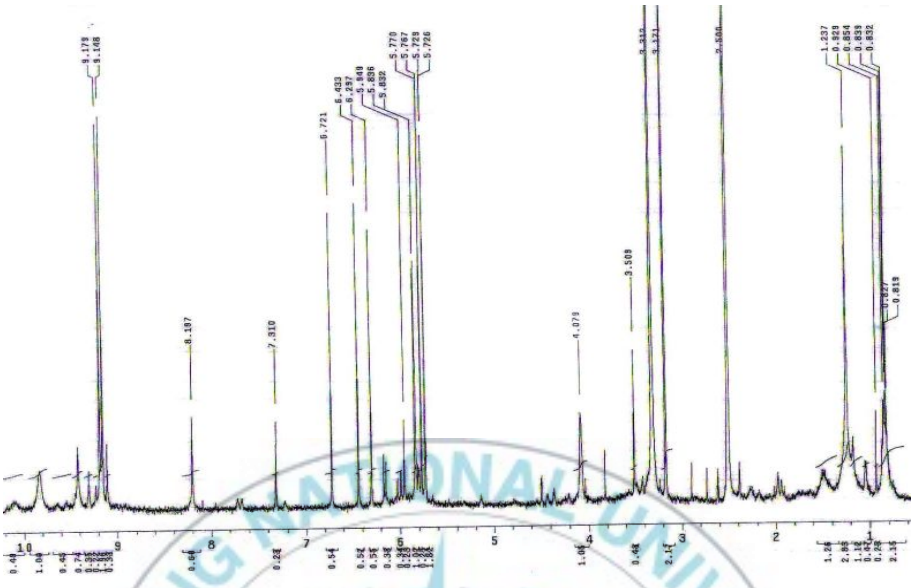


(a)

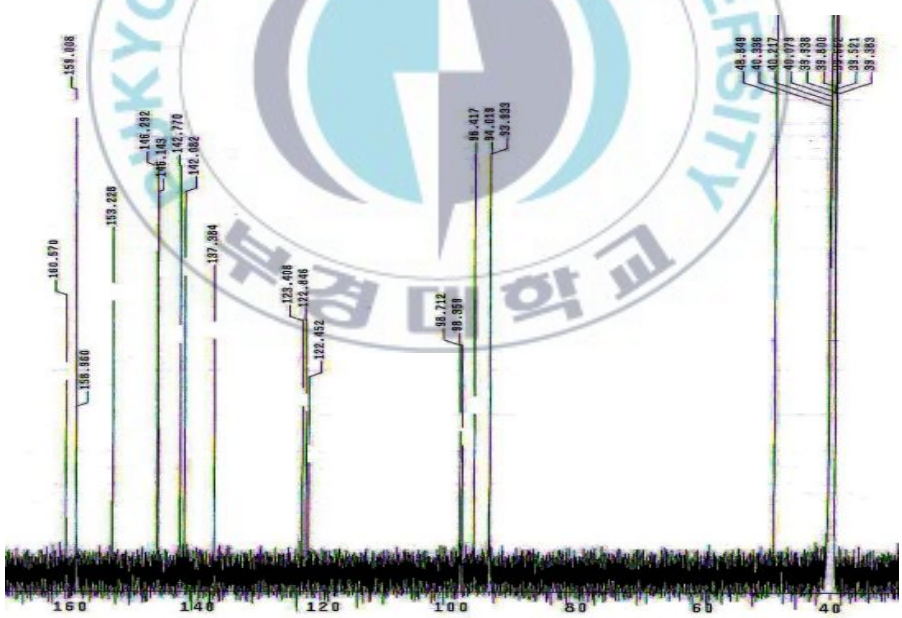


(b)

Fig. 5. ^{1}H-NMR spectrum (a) and ^{13}C-NMR spectrum (b) of compound **1** in DMSO-d_6.



(a)



(b)

Fig. 6. ^{1}H-NMR spectrum (a) and ^{13}C-NMR spectrum (b) of compound **2** in DMSO-d_6.

Compound **1** (phlorofucofuroeckol A, PFF): light-brown powder, FAB-MS *m/z* 603 $[M + H]^+$. $C_{30}H_{18}O_{14}$. ^{1}H-NMR (DMSO-d6, 600 MHz) δ: 10.12 (1H, s, OH-14), 9.85 (1H, s, OH-4), 9.42 (1H, s, OH-10), 9.25 (1H, s, OH-2), 9.18 (2H, s, OH-3″,5″), 9.15 (2H, s, OH-3′, 5′), 8.20 (1H, s, OH-8), 6.72 (1H, s, H-13), 6.42 (1H, s, H-9), 6.30 (1H, s, H-3), 5.83 (2H, t, *J* = 2.4 Hz, H-4′, 4″), 5.77 (2H, d, *J* = 1.8 Hz, H-2′ , 6′), 5.73 (2H, d, *J* = 1.8 Hz, H-2″, 6″). ^{13}C- NMR (DMSO-d_6, 100 MHz) δ: 159.8 (C-1′), 159.5 (C-1″), 158.6 (C-3″, 5″), 158.4 (C-3′, 5′), 150.4 (C-10), 149.9 (C-12a), 149.1 (C-11a), 146.5 (C-2), 146.1(C-8), 144.3 (C-14), 141.6 (C-4), 136.4 (C-15a), 133.5 (C-5a), 125.9 (C-14a), 122.2 (C-1, 4a), 119.7 (C-11), 103.0 (C-7), 102.8 (C-6), 98.7 (C-9), 97.8 (C-3), 96.1 (C-4″), 95.9 (C-4′), 94.4 (C-13), 93.3 (C-2′), 93.3 (C- 6′), 93.1 (C-2″, 6″) ; see Table 3 and Fig. 5.

Compound **2** (dieckol, DE): pale brown powder, FAB-MS *m/z* 743 $[M + H]^+$. $C_{36}H_{22}O_{18}$. ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 9.65 (1H, s, OH-9), 9.55 (1H, s, OH-9″), 9.45 (1H, s, OH-4″), 9.40 (1H, s, OH-4), 9.31 (2H, s, OH -3 , 5), 9.23 (1H, s, OH-2″), 9.18 (1H, s, OH-2), 9.17 (1H, s, OH-7″), 9.10 (2H, s, OH-3′, 5′), 6.16(1H, s, H-3″), 6.14(1H, s, H-3), 6.02 (1H, d, *J* = 3.0 Hz, H-8), 5.99 (1H, d, *J* = 3.0 Hz, H-8″), 5.95 (2H, s, H-2 , 6), 5.82 (1H, d, *J* = 3.0 Hz, H-6), 5.81 (1H, d, *J* = 3.0 Hz, H-6″), 5.80 (1H, d, *J* = 1.8 Hz, H-4′), 5.72 (2H, d, *J* = 1.8 Hz, H-2′, 6′). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 160.2 (C-1′), 158.7 (C-3′) 158.6 (C-5′), 155.8 (C-1), 154.2 (C-7), 153.0 (C-7″), 151.1 (C-3 , 5), 146.0 (C-2, 9″), 145.8 (C-2″, 9), 142.5 (C-5a″), 142.3 (C-5a), 141.9 (C-4″), 141.8 (C-4), 137.2 (C-10a), 137.0

(C-10a″), 124.2 (C-4), 124.0 (C-9a), 123.2 (C-4a), 123.1 (C-4a″), 122.6 (C-9a″), 122.2 (C-1, 1″), 98.3 (C-3), 98.2 (C-3″), 98.0 (C-8, 8″), 96.1 (C-4′), 94.4 (C-2 , 6), 93.8 (C-6″), 93.6 (C-2′, 6′), 93.5 (C-6); see Table 3 and Fig. 6.

8. Cytotoxicity of compounds isolated from EtOAc- soluble extracts

Partitioned *E. bicyclis* metanolic extract EtOAc fraction resulted in significant enhancement in cytotoxic effect on the host cells compared to EtOAc fraction. An isolated compound **2**, dieckol showed cytotoxicity of 50% at a concentration of 500 μM and compound **1**, phlorofucofuroeckol-A was found to be lower cytotoxicity than dieckol at 600 μM (Table 4).

9. Anti-viral activity of compounds isolated from EtOAc- soluble extracts

The finding shows phlorofucofuroeckol-A and dieckol treated cells were considerably inhibit MNV infection at concentration of 0.9 μM, 0.9 μM, respectively. Consequently, SI value for phlorofucofuroeckol-A is 666.67 and for dieckol is 888.9 (Table 4).

Also, Fukuyama *et al*. (1990), elucidated the mechanism of virus replication hindrance by phlorofucofuroeckol-A affecting on carbon-proton coupling at a concentration of 1.0 μg/mL against α_2-macroglobulin and 0.3 μg/mL against α_2-plasmin inhibitor. Moreover, Ahn *et al*. (2004) revealed dieckol from *Ecklonia cava* showed great inhibitory of HIV-1 by 50% at a

concentration of 5.3 μM.

Several of phytochemicals mechanisms studies have proven phenolic derivatives are widely distribute in plants and class of its secondary metabolites that are characterized by the presence of one or more hydroxyl (-OH) groups are responsible for various biological benefits not only antiviral activity (Dubois *et al*., 2002; Wang, 2011; Zandi *et al*., 2012). Accordingly, it is assumed that aromatic rings with complex hydroxyl group contribute to prevent viral infection as shown at Fig. 4 and Table 4.

Table 4. *In vitro* anti norovirus activities of isolated compounds from *E. bicyclis* EtOAc-soluble extracts against Murine norovirus (MNV) on raw 264.7 cells using the mixed treatment assay

Isolated compounds	CC_{50}(μM) [a]	EC_{50}(μM) [b]	SI [c]
phlorofucofuroeckol-A	600 ± 0.3	0.9	666.7
dieckol	500 ± 0.4	0.9	888.9

[a]CC_{50} : mean (50%) value of cytotoxic concentration.

[b]EC_{50} : mean (50%) value of effective concentration.

[c]SI : selectivity index, CC_{50}/EC_{50}.

Conclusion

In recent years, norovirus has gained a lot of attention concerning the management of food safety since 50% of all outbreaks of food-poising caused by norovirus across the nations.

Although various biological activities of marine organism have been reported, there are many potential to be utilized still unknown. Therefore, it was investigated the anti norovirus activity of blown alga *E. bicyclis* among edible and safe marine organisms.

To demonstrate anti norovirus activity of *E. bicycils*, the *E. bicyclis* methanolic extract was fractionated with organic solvents. Among them, an ethyl acetate soluble extract showed a highest antiviral activity among other soluble fractions according to MTT assay and $TCID_{50}$ assay. Furthermore, the total phenolic contents of the each fraction were evaluated the relationship between antiviral activity and phlorotannin contents. As the results, the ethyl acetate soluble fraction from *E. bicyclis* methanol extract exhibited the most impressive reduction of murine norovirus infectivity at 4 μg/mL concentration. Also, the virucidal effectiveness (SI 125) was highly correlated with their total phenolic contents (126.4 mg PGE/g). To elucidate the most effective agent from *E. bicyclis*, phlorofucoeckol-A and dieckol were

isolated from the ethyl acetate fraction of *E. bicyclis* methanol extract and these compounds (**1**-**2**) showed potent antiviral activity at 0.9 μg/mL.

In conclusion, *E. bicyclis* is a valuable marine source could be introduced for the food addictive and pharmaceutical ingredient as a good approach for the treatment and or prevention of norovirus infectious disease. Thus, further studies are needed to delineate the mechanism of anti norovirus activity with isolated compounds.

Acknowledgement

이 논문이 완성되기까지 용기와 힘이 되어주신 분들 덕분에 대학원 석사과정 2 년의 시간을 성실히 마칠 수 있었기에 고마운 마음을 전하고자 합니다.

때로는 엄격하게 또는 부드럽게 사랑으로 지도해주시며 연구자로서 방향을 제시해주신 이명숙 교수님과 김영목 교수님께 감사의 말씀을 전하고 싶습니다.

또한 저에게 연구실생활의 즐거움과 학문적 기초를 갈고 닦을 수 있도록 물심양면으로 지원해주신 엄성환선배님, 배향남선배님, 이윤경선배님, 강민승선배님, 김태완선배님, 대학생활을 함께하며 따뜻한 힘이 되어주는 송원선배님, 임근식선배님, 황혜진선배님, 유대웅선배님, 신동원선배님, 김태영선배님, 주광희선배님, 박명철선배님, 박재홍선배님, 이광덕선배님, 윤해원선배님, 조현아선배님, 도형훈선배님, 정연중선배님, 김유랑선배님, 노호준선배님, 박은영선배님, 이은주선배님, 손현주선배님, 미생물학과 생활에 적응하는데 너무 큰 도움이 되어준 윤한성선배님, 김정배선배님, 정태혁선배님, 현철이, 유리, 태경이, 정균이, 옥금이, 형화, 이하 일미방을 거쳐간 애증의 후배님들 지산이, 성현이, 희애, 정현이, 동우, 승태, 은지, 영준이, 수민이, 윤정이, 민석이, 주원이, 인혜, 다솔이, 가희 그리고 김미진선생님, 손수경선생님께도 감사의 말씀을 전하고 싶습니다.

학교생활을 이제 시작하거나 아직 끝이 보이지 않을 승용이, 예지, 은혜, 효정이, 신국이, 해나, 지훈이, 덕훈이와 열거하지 못한 식미방 후배들에게는 응원의 마음을 전합니다.

마지막으로 딸의 고집을 무한한 사랑으로 지지해주신 부모님과 언니에게 감사의 인사를 전하면서 이 논문을 마칩니다.

References

Ahn, M. J., Yoon, K. D., Min, S. Y., Lee, J. S., Kim, J. H., Kim, T. G., ... and Kim, J. 2004. Inhibition of HIV-1 reverse transcriptase and protease by phlorotannins from the brown alga *Ecklonia cava*. Biological and Pharmaceutical Bulletin, 27(4), 544-547.

Armond, S.G. and Bellur, S.P. 1996. Medical Microbiology. Baron S., ed. University of Texas Medical Branch at Galveston, retrieved on 26 Oct. 2013, http://www.ncbi.nlm.nih.gov/books/NBK7795/#mmed_ch1.

Broadhurst, C. L., Polansky, M. M., & Anderson, R. A. 2000. Insulin-like biological activity of culinary and medicinal plant aqueous extracts *in vitro*. Journal of agricultural and food chemistry, 48(3), 849-852.

Centers for Disease Control and Prevention. 2012. Updated norovirus Outbreak Management and Disease Prevention Guidelines. Retrieved on 26 Oct. 2013, http://www.cdc.gov/mmwr/preview/mmwrhtml/rr6003a1.htm.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L. A., and Vinje, J. 2006. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. Journal of Food Protection®, 69(11), 2761-2765.

Caul, E. O. 1996. Viral gastroenteritis: small round structured viruses, caliciviruses and astroviruses. Part I. The clinical and diagnostic perspective. Journal of clinical pathology, 49(11), 874-880.

Choi, Y., Kim, E., Moon, S., Choi, J.D., Kim, Y.M. and Lee M.S. 2013. Phaeophyta Extracts Exhibit Antiviral Activity against Feline Calicivirus. Journal of Fisheris Science and Technology. in press.

Clarke, I.N., Lambden, P.R. and Caul, E.O. 1998. Human enteric RNA viruses: caliciviruses and astroviruses. In: Mahy BWJ, Collier L, editors. Topley & Wilson's Microbiology and Microbial Infections. London, 511–535.

Danesi, F., Pasini, F., Caboni, M. F., D'Antuono, L. F., and Bordoni on behalf of the BaSeFood Consortium. 2013. Traditional foods for health: screening of the antioxidant capacity and phenolic content of selected Black Sea area local foods. Journal of the Science of Food and Agriculture, 93(14), 3595-3603.

Dubois, E., Agier, C., Traore, O., Hennechart, C., Merle, G., Cruciere, C., & Laveran, H. 2002. Modified concentration method for the detection of enteric viruses on fruits and vegetables by reverse transcriptase-polymerase chain reaction or cell culture. Journal of Food Protection®, 65(12), 1962-1969.

Duizer, E., Schwab, K. J., Neill, F. H., Atmar, R. L., Koopmans, M. P., and Estes, M. K. 2004. Laboratory efforts to cultivate noroviruses. Journal of General Virology, 85(1), 79-87.

Eom, S. H., Kim, D. H., Lee, S. H., Yoon, N. Y., Kim, J. H., Kim, T. H., Chung, Y. H., Kim, S. B., Kim Y. M., Kim H. W., Lee, M. S. and Kim, Y. M. 2012. *In vitro* antibacterial activity and synergistic antibiotic effects of phlorotannins isolated from *Eisenia bicyclis* against methicillin-resistant Staphylococcus aureus. Phytotherapy Research. 27, 1260-1264.

Fankhauser, R. L., Monroe, S. S., Noel, J. S., Humphrey, C. D., Bresee, J. S., Parashar, U.D., ... and Glass, R. I. 2002. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. Journal of Infectious Diseases, 186(1), 1-7.

Fukuyama, Y., Kodama, M., Miura, I., KINZYO, K., Mori, H., Nakayama, Y., and Takahashi, M. 1990. Anti-plasmin inhibitor. VI: Structure of phlorofucofuroeckol A, a novel phlorotannin with both dibenzo-1, 4-dioxin and dibenzofuran elements, from *Eklonia kurome* OKAMURA. Chemical and Pharmaceutical Bulletin, 38(1), 133-135.

Goodfellow, I. G., Evans, D. J., Blom, A. M., Kerrigan, D., Miners, J. S., Morgan, B. P., and Spiller, O. B. 2005. Inhibition of coxsackie B virus infection by soluble forms of its receptors: binding affinities, altered particle formation, and competition with cellular receptors. Journal of virology, 79(18), 12016-12024.

Green J., Vinje J., Gallimore C. I., Koopmans M., Hale A. and Brown D. G. 2000. Capsid protein diversity among Norwalk-like viruses. Virus Genes, 20, 227–236.

Holdt, S. L., and Kraan, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. Journal of applied phycology, 23(3), 543-597.

Inouye, S., Yamashita, K., Yamadera, S., Yoshikawa, M., Kato, N., and Okabe, N. 2000. Surveillance of viral gastroenteritis in Japan: pediatric cases and outbreak incidents. Journal of Infectious Diseases, 181(Supplement 2), S270-S274.

Jung, H. A., Jin, S. E., Ahn, B. R., Lee, C. M., and Choi, J. S. 2013. Anti-inflammatory activity of edible brown alga *Eisenia bicyclis* and its constituents fucosterol and phlorotannins in LPS-stimulated RAW264. 7 macrophages. Food and Chemical Toxicology. 59, 199-206.

Kamei, Y., and Aoki, M. 2007. A chlorophyll c2 analogue from the marine brown alga *Eisenia bicyclis* inactivates the infectious hematopoietic necrosis virus, a fish rhabdovirus. Archives of virology, 152(5), 861-869.

Kang, H. S., Chung, H. Y., Jung, J. H., Son, B. W., and Choi, J. S. 2003. A new phlorotannin from the brown alga *Ecklonia stolonifera*. Chemical and pharmaceutical bulletin, 51(8), 1012-1014.

Kang Y.M., Eom S.H. and Kim Y.M., 2013. Protective effect of phlorotannins from *Eisenia bicyclis* against lipopolysaccharide-stimulated inflammation in HepG2 cells. Environ toxicol pharmacol, 35, 395-401.

King, A. M., Adams, M. J., Lefkowitz, E. J., and Carstens, E. B. (Eds.). 2011. Virus taxonomy: IXth report of the International Committee on Taxonomy of Viruses. 9.

Kim, K. L., Park, M. S., Lim, K. S., Lee, D. H., and Kim, Y. M. 2010. Antiviral activity of seaweed extracts against feline calicivirus. Fisheries and Aquatic Sciences, 13(2), 96-101.

Kim, M. M., Ta, Q. V., Mendis, E., Rajapakse, N., Jung, W. K., Byun, H. G., Jeon, Y. J. and Kim, S. K. 2006. Phlorotannins in *Ecklonia cava* extract inhibit matrix metalloproteinase activity. Life Sciences, 79(15),

1436-1443.

Korea Centers for Disease Control & Prevention. 2012. Laboratory Surveillance of Viral Acute Gastroenteritidis, 2005-2008. Retrieved on 26 Oct. 2013, http://www.cdc.go.kr/CDC/notice/CdcKrInfo0301.jsp?menuIds=HOME001-MNU0004-MNU0036-MNU0037&cid=12417.

Kwon, T. H., Kim, T. W., Kim, C. G., and Park, N. H. 2013. Antioxidant Activity of Various Solvent Fractions from Edible Brown Alga, *Eisenia bicyclis* and Its Active Compounds. Journal of food science.

Lee, D. S., Eom, S. H., Je, J. Y., Kim, Y. M., Lee, M. S., and Kim, Y. M. 2013. Antibacterial Activity of an Ethyl Acetate Extract of Pseudomonas sp. UJ-6 against Methicillin-Resistant Staphylococcus aureus. Fisheries and Aquatic Sciences, 16(2), 79-84.

Lee, J. Y., Kim, S. M., Jung, W. S., Song, D. G., Um, B. H., Son, J. K., and Pan, C. H. 2012. Phlorofucofuroeckol-A, a potent inhibitor of aldo-keto reductase family 1 member B10, from the edible brown alga *Eisenia bicyclis*. Journal of the Korean Society for Applied Biological Chemistry, 55(6), 721-727.

Lee, M. H., Lee, B. H., Jung, J. Y., Cheon, D. S., Kim, K. T., and Choi, C. 2011. Antiviral effect of Korean red ginseng extract and ginsenosides

on murine norovirus and feline calicivirus as surrogates for human norovirus. Journal of ginseng research, 35(4), 429.

Lee, S. H., and Jeon, Y. J. 2013. Anti-diabetic effects of brown algae derived phlorotannins, marine polyphenols through diverse mechanisms. Fitoterapia.

Lee, S. H., Karadeniz, F., Kim, M. M., and Kim, S. K. 2009. α-Glucosidase and α-amylase inhibitory activities of phloroglucinal derivatives from edible marine brown alga, *Ecklonia cava*. Journal of the Science of Food and Agriculture, 89(9), 1552-1558.

Lopman, B., Vennema, H., Kohli, E., Pothier, P., Sanchez, A., Negredo, A., ... and Koopmans, M. 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. The lancet, 363(9410), 682-688.

McClintock, J. B., and Baker, B. J. (Eds.). 2010. Marine chemical ecology. CRC press. 168-170.

Moreno-Espinosa, S., Farkas, T., and Jiang, X. 2004. Human caliciviruses and pediatric gastroenteritis. In Seminars in pediatric infectious diseases. 15(4), 237-245.

Oh, E. G., Kim, K. L., Shin, S. B., Son, K. T., Lee, H. J., Kim, T. H., Kim Y. M., Cho E. J., Kim D. K., Lee E. W., Lee M. S., Shin I.S. and Kim

J. H. Kim, J. H. 2013. Antiviral activity of green tea catechins against feline calicivirus as a surrogate for norovirus. Food Science and Biotechnology, 22(2), 593-598.

Okada, Y., Ishimaru, A., Suzuki, R., and Okuyama, T. 2004. A new phloroglucinol derivative from the brown alga *Eisenia bicyclis*: potential for the effective treatment of diabetic complications. Journal of natural products, 67(1), 103-105.

Rösch, D., Bergmann, M., Knorr, D., and Kroh, L. W. 2003. Structure-antioxidant efficiency relationships of phenolic compounds and their contribution to the antioxidant activity of sea buckthorn juice. Journal of Agricultural and Food Chemistry, 51(15), 4233-4239.

Silva, A., Morais, S. M., Marques, M. M. M., Lima, D. M., Santos, S. C. C., Almeida, R. R., Vieira I. G. P. and Guedes, M. I. F. 2011. Antiviral activities of extracts and phenolic components of two Spondias species against dengue virus. Journal of Venomous Animals and Toxins including Tropical Diseases, 17(4), 406-413.

Straub, T. M., zu Bentrup, K. H., Coghlan, P. O., Dohnalkova, A., Mayer, B. K., Bartholomew, R. A., Valdez C. O., Bruckner-Lea C. J., Gerba C. P., Abbaszadegan M., and Nickerson, C. A. 2007. *In vitro* cell culture

infectivity assay for human noroviruses. Emerging infectious diseases, 13(3), 396.

Su, X., and D'Souza, D. H. 2013. Naturally Occurring Flavonoids Against Human Norovirus Surrogates. Food and environmental virology, 1-6.

Van Hoof, L., Totte, J., Corthout, J., Pieters, L. A., Mertens, F., Vanden Berghe, D. A., ... and Esmans, E. 1989. Plant Antiviral Agents, VI. Isolation of Antiviral Phenolic Glucosides from Populus Cultivar Beaupre by Droplet Counter-Current Chromatography. Journal of natural products, 52(4), 875-878.

Wang, B. 2011. HIV-1 integrase: mechanism and inhibitor design(9). N. Neamati (Ed.). John Wiley & Sons.

Zandi, K., Teoh, B. T., Sam, S. S., Wong, P. F., Mustafa, M. R., and AbuBakar, S. 2012. Novel antiviral activity of baicalein against dengue virus. BMC complementary and alternative medicine, 12(1), 1-9.

Zhang, X. F., Dai, Y. C., Zhong, W., Tan, M., Lv, Z. P., Zhou, Y. C., and Jiang, X. 2012. Tannic acid inhibited norovirus binding to HBGA receptors, a study of 50 Chinese medicinal herbs. Bioorganic & medicinal chemistry, 20(4), 1616-1623.