EFFECT OF EXTRACTION METHODS ON ANTIFUNGAL ACTIVITY OF SEA CUCUMBER (*Stichopus japonicus)*

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ABSTRACT

The objective of this study was to investigate the antifungal activity of the soluble matter (SM) and crude saponins (CS) extracted from *Stichopus japonicus* using pressurized solvent extraction (PSE) with water or aqueous ethanol as a solvent, in comparison with traditional heat reflux extraction (HRE). The extraction yields were also determined for the SM and CS and compared for each extraction process and solvent. The antifungal activity of the SM and CS, extracted from the body wall of *Stichopus japonicus* using PSE or HRE with water or 70% aqueous ethanol, were investigated. Both SM and CS exhibited their highest antifungal activity when extracted by HRE with 70% ethanol and by HRE with water, respectively, while their highest yields were obtained when extracted by PSE with water. SM has more antifungal than potassium sorbate but weaker than propyl paraben, while CS has more antifungal than the two antifungal agents.

Keywords: Antifungal, heat reflux extraction, pressurized solvent extraction, *Stichopus japonicus*

INTRODUCTION

Sea cucumber, a cylindrical marine invertebrate, has been used not only as an outstanding tonic food in the Indo-Pacific region but also as a traditional medicine in East Asia due to its high nutraceutical value (Zhong et al., 2007; Husni et al., 2011). Antifungal activity is one of the most beneficial biological functions that can be obtained from sea cucumber. The strong antifungal activity reported for organic and aqueous extracts of various sea cucumber species, including *Stichopus japonicus*, *Holothuria pervicax*, *Hemoiedema spectabilis*, *Psolus patagonicus*, and *Actinopyga lecanora*, was found to be due to the presence of triterpene glycosides, commonly known as sea cucumber saponins or holothurins, which include holotoxins (A, B, A1, and B1), desulfated pervicosides (A, B, and C), hemoiedemosides (A and B), desulfated hemoiedemoside A, patagonicoside A, desulfated patagonicoside A, triterpene glycoside-(1), and holothurins (A and B) (Dong et al., 2008; Kitagawa et al., 1989; Chludil et al., 2002; Murray et al., 2001; Kumar et al., 2007).

For safe and economic delivery of the antifungal activity to food or cosmetic industry, the extract of sea cucumber needs to be prepared through an environmentally friendly and efficient extraction process that uses relatively inexpensive GRAS solvents such as water and aqueous ethanol. Pressurized solvent extraction (PSE) has been considered as one of the promising processes that can be used for such purposes, although it is not yet a routine technique in natural product extraction (Kaufmann and Christen, 2002; Denery et al., 2004; Herrero et al., 2005;Wang and Weller, 2006). The process is automated and usually performed at temperatures between 50 and 200°C and at pressures between 10 and 15 MPa under an oxygen-free and light-free environment with less solvent within a shorter extraction time, compared to traditional solvent extraction (ex. heat reflux extraction) processes (Denery et al., 2004;Wang and Weller, 2006). To the best of our knowledge, however, there is no published report on the antifungal activity of water or aqueous ethanol extract of sea cucumber, prepared by a standard extraction process such as PSE. *Aspergillus niger***,** *Candida albicans*, *Trichophyton mentagrophytes*, *T. rubrum*, *Epidermophyton floccosum***,** and *Microsporum canis* have been found associated with the degradation of food and/or skin problems.

The objective of this study was to investigate the antifungal activity of the soluble matter and crude saponins extracted from *Stichopus japonicus* using PSE with water or

aqueous ethanol as a solvent, in comparison with traditional heat reflux extraction (HRE). The extraction yields were also determined for the soluble matter and crude saponins and compared for each extraction process and solvent.

MATERIALS AND METHODS

Materials

Live specimens of sea cucumber *Stichopus japonicus* (average body weight 167 g) were purchased from a fishery market (Mokpo, Korea). RPMI 1640 medium, MOPS (morpholinepropanesulfonic acid), and propyl paraben were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sabouroud dextrose agar (SDA) and potassium sorbate were purchased from Difco (Detroit, MI, USA) and Junsei (Saitama, Japan), respectively. Ethanol, methanol, and diethyl ether were obtained from Fisher Scientific (ON, Canada). All the chemicals used in the study were of either analytical grade or HPLC grade.

Sample Preparation

The visceral organs and body fluid of fresh sea cucumber specimens were taken out and the body wall was washed with tap water. The body wall was cut into small pieces, freezed at –80°C for at least 12 h, and vacuum dried at 30°C for 72 h. The dried sample was ground into powder and sieved with a ≤ 600 μm sieve. The powder was stored in a closed dark bottle at 2°C before experiments.

Extraction Procedures

Two types of extraction, pressurized solvent extraction (PSE) and heat reflux extraction (HRE), were performed with two solvents, distilled water and 70% aqueous ethanol. PSE was performed using a fully automated pressurized solvent extraction system (ASE 300, Dionex Co., Sunnyvale, CA, USA) equipped with a solvent controller unit. The powder sample (5 g) was filled in a 100 mL extraction cell, and the extraction was conducted under the following conditions: pressure, 10.34 MPa; temperature, 80°C; heating time, 5 min; extraction time, 5 min; extraction cycles, 3 times; flush volume, 60%; purge time, 60 s. The final extract volumes were measured to be 150 and 162 mL for the extractions with distilled water and 70% ethanol, respectively. HRE was performed using an apparatus equipped with a cooling condenser, a rotating 1000 mL round-bottom flask, and a temperature-controlled water bath. The conditions for HRE were set based on those for PSE. A volume of 150 mL of distilled water or 162 mL of 70% ethanol was poured into the rotating round-bottom flask and heated until the temperature reached 80°C, followed by dispersing 5 g of the powder sample in the solvent. After 20 min extraction, the extract was filtered by suction filtration using a No.2 filter paper (Advantech Toyo Roshi International, Inc., Dublin, CA, USA). Finally, four types of extracts, PSE-W, PSE-Et, HRE-W, and HRE-Et, were obtained from PSE with water, PSE with 70% ethanol, HRE with water, and HRE with 70% ethanol, respectively. All the extracts were stored at 2°C until further analyses.

Isolation of Soluble Matter and Crude Saponins

The soluble matter (SM) and crude saponins (CS) of each of the four extracts were isolated to be tested for their antifungal activity. SM was isolated by evaporating the solvent of extract under reduced vacuum at 40°C using a rotary evaporator (Rotavor R-210, Flawi, Switzerland). CS was isolated according to the method of Kwon et al. (2003) with slight modifications. A volume of 250 mL of each of the four extracts was transferred to a round-bottom flask of 1000 mL and the solvent was evaporated under reduced vacuum at 55°C using the rotary evaporator. A volume of 125 mL of distilled water was poured into the flask to dissolve the residue. The aqueous solution was transferred to a separating funnel, treated twice with 125 mL of diethyl ether to remove lipid components, and then extracted four times with 125 mL of water-saturated *n*-butanol. The butanol fractions were collected and treated twice with 75 mL of distilled water to remove impurities, and CS was obtained by evaporating butanol under reduced vacuum at 55°C. The procedure was repeated several times to obtain sufficient amounts of CS for further analysis.

Yields of Soluble Matter and Crude Saponins

SM and CS were isolated from 5 and 20 mL of each extract, respectively, dried at 105°C for 6 h, and weighed after cooling in a desiccator. The yield of SM was calculated as follows: Yield (%) = $100W_{sm}/W_s$, where W_{sm} is the weight of SM, W_s is the weight of the powder sample used for extraction (= 5 g × (5/V)), and V is 150 mL for PSE-W and HRE-W and 162 mL for PSE-Et and HRE-Et. The yield of CS was calculated as follows: Yield (%) = $100W_{cs}/W_s$, where W_{cs} is the weight of CS, W_s is the weight of the powder sample used for extraction (= 5 g × (20/V)), and V is the same as above.

Antifungal Activity Assay

Minimum inhibitory concentrations (MICs) of SM and CS obtained from the four extracts were determined against *Aspergillus niger* KCTC 6985**,** *Candida albicans* KCTC 7752, *Trichophyton mentagrophytes* KCTC 6345, *Trichophyton rubrum* KCTC 6085, *Epidermophyton floccosum* KCTC 6921**,** and *Microsporum canis* KCTC 6591 according to the

microdilution method described by Rukayadi and Hwang (2007) and Rukayadi et al. (2006) with slight modifications. The stock solutions of SM and CS were prepared at concentrations of 20000 and 10000 µg/mL in sterile distilled water, respectively. *A. niger,T. mentagrophytes*, *T. rubrum*, *E. floccosum,*and *M. canis* were cultured on SDA at 35°C for 7 days, and then the culture was covered with 1 mL of 0.85% sterile saline. The colonies were gently probed with the tip of a Pasteur pipette, and the resulting suspension containing conidia and hyphal fragments was transferred to a sterile tube and allowed to stand for 5 min to settle heavy particles. The supernatant was vortexed for 15 s, and its transmittance was adjusted to 80 to 82% (for *A. niger*) and 90% (for *T. mentagrophytes*, *T. rubrum*, *E. floccosum,*and *M. canis*) at 530 nm using a V-530 UV/VIS Spectrophotometer (Jasco Co., Tokyo, Japan) to obtain an inoculum density of about 1 × 10^6 colony-forming units (CFU)/mL (Espinel-Ingroff and Kerkering, 1999,Falahati et al., 2005).*C. albicans* was grown on SDA at 37°C for 24 h, and then several colonies were collected and suspended in 2 mL of 0.85% sterile saline. The resulting suspension was adjusted to 70% transmittance at 530 nm to obtain an inoculum density of about 1 × 10^6 CFU/mL (Mahmoudabadi et al., 2007). Each of the two adjusted suspensions was diluted 1:50 in RPMI 1640 medium buffered with MOPS so that the final inoculum size corresponded to about 2 × 10^4 CFU/mL. Each of the stock solution was serially twofold diluted so that the concentrations of SM and CS ranged from 156.25 to 20000 µg/mL and from 7.81 to 1000 µg/mL, respectively. Serial twofold dilutions of potassium sorbate and propyl paraben were also prepared as controls in the concentration ranges from 156.25 to 20000 µg/mL and from 7.81 to 1000 µg/mL, respectively. Aliquots of 100 µL of each serial dilution were dispensed into the wells of 96-well microtiter plates, and then each microdilution well was inoculated with 100 µL of the diluted suspension of the fungal. For each microtiter plate, two antifungal-free control wells were included; one containing only 200 µL of microbial medium (sterility control) and the other containing 100 µL of microbial medium inoculated with 100 µL of the microbial suspensions (growth control). The plates were incubated at 35°C for 3 days for *A. niger,T. mentagrophytes*, *T. rubrum*, *E. floccosum,*and *M. canis* and for 2 days for *C. albicans.* The MICs of SM, CS, potassium sorbate, and propyl paraben were determined as the lowest concentrations that completely inhibited visible growth of the fungi. The whole experiment was conducted in duplicate.

HPLC Analysis

For all experiments, analysis were performed on an Agilent 1100 series (Agilent Technologies, CA, USA) equipped with a binary gradient pump, autosampler, column oven, diode array detector and mass spectra detector was used. The gradient elution consists of mobile phase of (A) water and (B) methanol. The parameters of the HPLC method was: solvent gradient (%B): 0 at 0 min, 10 at 5 min, 40 at 10 min, 50 at 20 min, 90 at 30 min, 100 at 35 min, 100 at 40 min; run time = 45 min, solvent flow rate = 1 mL/min, column temperature 35 °C, injection volume = 10 µL. The column used for separation was Agilent Eclipse XDB-C18, 4.6 x 150 mm, 5 µm. Peaks were detected at 254 nm (Endale et al., 2005).

Statistical Analysis

SPSS for Windows (version 11.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data were expressed as mean value ± standard deviation (SD). The significance of differences ($p< 0.05$) among the corresponding mean values was determined by using one-way analysis of variance (ANOVA) followed by Duncan's new multiple-range test.

RESULTS AND DISCUSSION

Yields of Soluble Matter (SM) and Crude Saponins (CS)

The yields of SM and CS of the four sea cucumber extracts ranged from 40.87 to 43.54% and from 1.14 to 3.08%, respectively, and the content of CS in SM varied between 2.63 and 7.06% (Table 1). PSE was more efficient than HRE in extracting SM. This is because of the pressure can increase the rate of mass transfer and significantly enhance the diffusion of solvent (Shouqin et al., 2007). The type of solvent (water or 70% ethanol), however, did not significantly influence the yield of SM in both PSE and HRE. The yield (3.08%) of CS for PSE-W was about 1.7-fold higher than that (1.85%) for HRE-W, while the yields were not considerably different between the two 70% ethanol extracts (PSE-Et and HRE-Et) (Table 1). This indicates that PSE was more efficient than HRE in extracting CS when using water as a solvent. Water was found to be more effective in both PSE and HRE for CS extraction, suggesting that the CS of *S. japonicas* body wall was more soluble in water than in 70% ethanol.

Antifungal Activity

The antifungal activity of SM and CS from the four sea cucumber extracts was evaluated by determining their minimum inhibitory concentrations (MICs) against *A. niger, C. albicans, T. mentagrophytes, T. rubrum, E. floccosum,* and *M. canis* (Table 2 and 3**)**. All types of SM and CS showed antifungal activity against the six fungal species, and the activity varied depending on the extraction conditions. The

MICs of SM (78-5000) were about 5- to 20-fold higher than those of CS (8-250 µg/mL), indicating that CS possesses much stronger antifungal activity than SM. It seems that CS was mainly responsible for the antifungal activity of SM, although its content in SM was only 2.63 to 7.06%. However, the four types of CS did not show the same antifungal activity, and no correlation was observed between the CS content in SM and the antifungal activity of SM. This is probably because different types of saponins were obtained from different extraction methods, and/or other components in addition to saponins also contributed to the antifungal activity (Denery et al., 2004).

Table 1. Yields of soluble matter (SM) and crude saponins (CS) of *Stichopus japonicus* body wall extracts (Mean ± SD, n = 3)[a]

Extract	SM (%)	CS (%)	CS/SM (%)
PSE-W	43.54 ± 0.40 a	3.08 ± 0.60 a	7.06 ± 1.33 a
PSE-Et	43.43 ± 0.50 a	1.14 ± 0.19 c	2.63 ± 0.47 c
HRE-W	41.50 ± 0.58 b	1.85 ± 0.07 b	4.46 ± 0.16 b
HRE-Et	40.87 ± 0.23 b	1.54 ± 0.20 bc	3.78 ± 0.48 bc

[a]Values in the same column with different letters are significantly different at $p < 0.05$.

PSE-W: extract from pressurized solvent extraction with water; PSE-Et: extract from pressurized solvent extraction with 70% ethanol; HRE-W: extract from heat reflux extraction with water; HRE-Et: extract from heat reflux extraction with 70% aqueous ethanol.

Table 2. Minimum inhibitory concentrations (MICs) of soluble matter (SM) extracted from body wall of *Stichopus japonicus* against *Aspergillus niger* (A.n.), *Candida albicans* (C.a.), *Trichophyton mentagrophytes* (T.m.), *Trichophyton rubrum* (T.r.), *Epidermophyton floccosum* (E.f.), and *Microsporum canis* (M.c.)

Extract/standard	MIC (µg/mL)					
	A.n.	C.a.	T.m.	T.r.	E.f.	M.c.
PSE-W	3750	5000	2500	5000	3750	5000
PSE-Et	469	1875	1250	1250	156	625
HRE-W	938	1250	1250	1250	1250	1250
HRE-Et	469	1875	625	1250	78	156
Potassium sorbate	5000	10000	2500	2500	5000	5000
Propyl paraben	250	500	125	125	125	125

Abbreviations: those for extracts are the same as in Table 1.

When water was used as a solvent, the SM from PSE showed higher (p<0.05)MICs (3750-5000 µg/mL) than that from HRE (MICs = 78-1875 µg/mL), while when 70% ethanol was used, the two types of SM from PSE and HRE had the similar trend MICs against each fungus (Table 2). A very similar trend was also observed for CS (Table 3). The results indicate that the SM and CS extracted by HRE were more antifungal than those obtained by PSE especially when water was used as a solvent, although their yields were lower in HRE (Table 1). Considering the higher antifungal activity of the CS from HRE-W compared to that from PSE-W, it is suggested that more active saponins and/or other strong antifungal substances were extracted by HRE rather than by PSE especially when using water as a solvent. A reason of the weak antifungal activity of the CS from PSE-W may come from the possible pressure-induced degradation of saponin molecules in a water-dominated environment (Denery et al., 2004).

Table 3. Minimum inhibitory concentrations (MICs) of crude saponins (CS) extracted from body wall of *Stichopus japonicus* against *Aspergillus niger* (A.n.), *Candida albicans* (C.a.), *Trichophyton mentagrophytes* (T.m.), *Trichophyton rubrum* (T.r.), *Epidermophyton floccosum* (E.f.), and *Microsporum canis* (M.c.).

Extract/standard	MIC (µg/mL)					
	A.n.	C.a.	T.m.	T.r.	E.f.	M.c.
PSE-W	125	250	250	250	125	250
PSE-Et	31	125	31	63	31	63
HRE-W	63	125	250	125	63	125
HRE-Et	16	63	31	31	8	31
Potassium sorbate	5000	10000	2500	2500	5000	5000
Propyl paraben	250	500	125	125	125	125

Abbreviations: those for extracts are the same as in Table1.

The MICs of all the tested SM and CS were lower than those of potassium sorbate, a widely used food-grade fungicide, which were determined to be 2500-10000 µg/mL (Table 2 and 3). This indicates that the SM and CS obtained in the current study were more effective than potassium sorbate in inhibiting the fungal growth. The MICs of propyl paraben, a strong fungicide widely used in foods, pharmaceuticals, and cosmetics, were 125-500 µg/mL. The values were lower than those of SM (MICs = 78–5000 µg/mL) but higher than those of CS (MICs = 8–250 µg/mL), which indicates that SM was less effective but CS was more effective than propyl paraben against the fungal growth.

Antifungal activity of the aqueous ethanol extract (both in SM and CS) from PSE is comparable with the aqueous ethanol extract from HRE. However, SM of water extract from PSE exhibited lower antifungal activity than SM of water extract from HRE. Figure 1andFigure 2 show the chromatogram of SM and CS from sea cucumber exracts, respectively, of sea cucumber extracts. Base on the chromatogram of SM of aqueous ethanol extracts (Figure 1(C) and **(D)**, there is one major peak (t_R = 2.0 min) may contribute to antifungal

activity of aqueous ethanol extracts. On other hand, based on the chromatogram of SM of water extract from HRE (Figure 1(B)) there are 3 major peaks (t_R = 1.3 min, t_R = 2.6 min, and t_R = 3.9 min) and SM of water extract from PSE (Figure 1(A)) there are also display 3 major peaks but have a little difference (t_R = 1.2 min, t_R = 2.6 min, and t_R = 3.8 min). Based on the chromatogram of CS of aqueous ethanol extracts (Figure 2(C) and (D), there is also one major peak (t_R = 2.0 min) may contribute to antifungal activity of CS of aqueous ethanol extracts. On other hand, based on the chromatogram of CS of water extract from HRE (Figure 2(B)) there are 2 major peaks (t_R = 2.6 min, and t_R = 3.9 min) and CS of water extract from PSE (Figure 2(A)) there are also display 2 major peaks but have a little difference (t_R = 2.6 min, and t_R = 3.8 min)Dong et al. (2008) reported that there are four triterpene glycosides have been isolated from sea cucumber *S. japonicas* including holotoxins A, holotoxins B, holotoxins A1 and holotoxins B1.

Only limited information is available on the MICs of SM and CS extracted from sea cucumber species. Kumar et al. (2007) reported that the MIC of SM from the methanol extract of *Actinopyga lecanora* was higher than 500 µg/mL against *C. albicans*. The author also showed that the CS obtained from the methanol extract had an MIC of 125 µg/mL against *C. albicans*, which is comparable to the MICs of CS obtained in the current study (63–250 µg/mL against *C. albicans*) (Table 3). Some pure triterpene glycosides isolated from sea cucumber showed a little higher antifungal activities than the CS obtained in this study (MICs of desulfated pervicosides = 1.56–12.5 µg/mL against *A. niger* and 6.25–100 µg/mL against *C. albicans*; MICs of holothurins 12.5–50 µg/mL against *C. albicans*) (Kitagawa et al., 1989; Kumar et al., 2007).

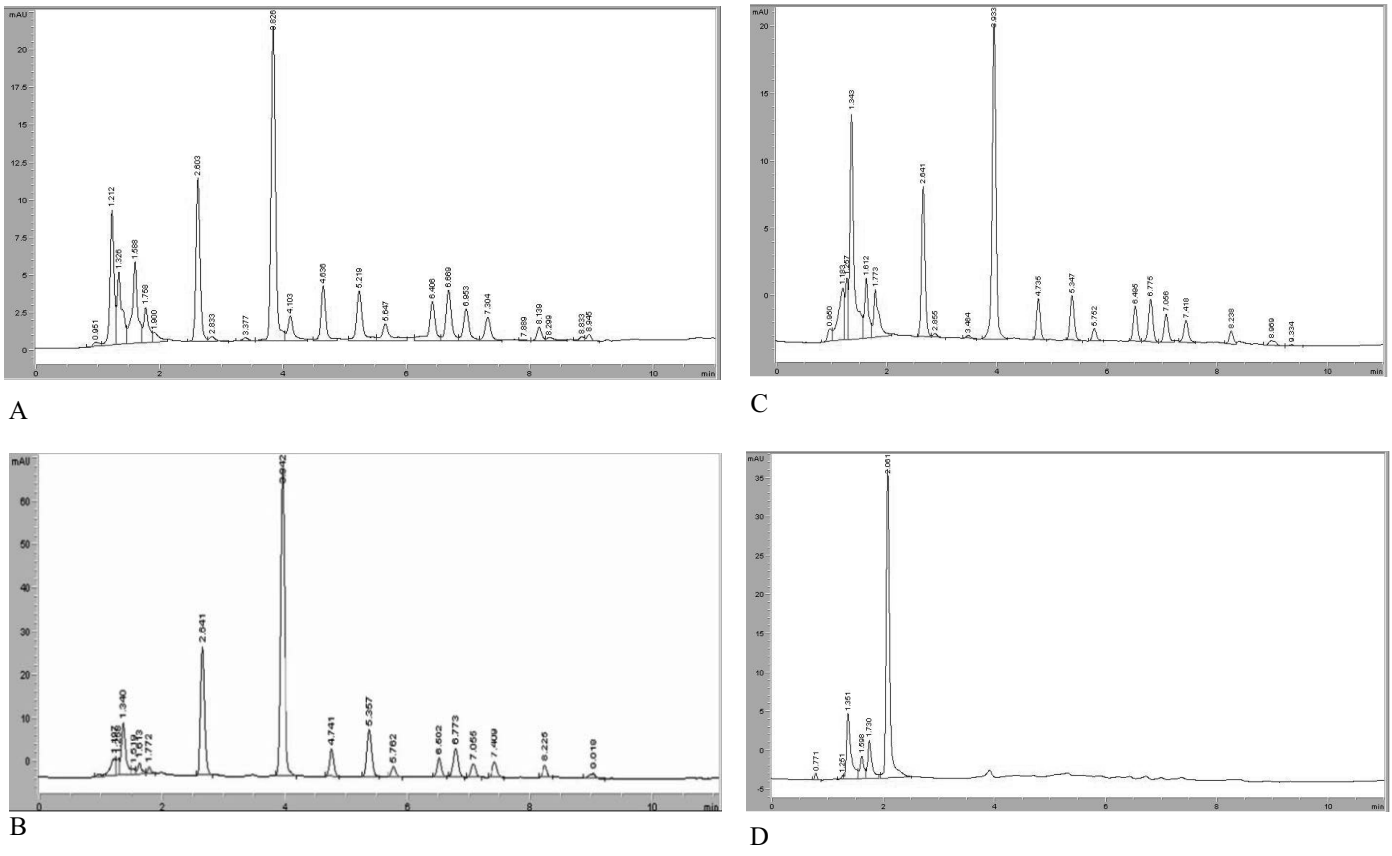

Figure 1. Chromatogram of SM from PSE-W (A), HRE-W (B), PSE-Et (C), and HRE-Et (D). Abbreviations: those for extracts are the same as in Table 1.

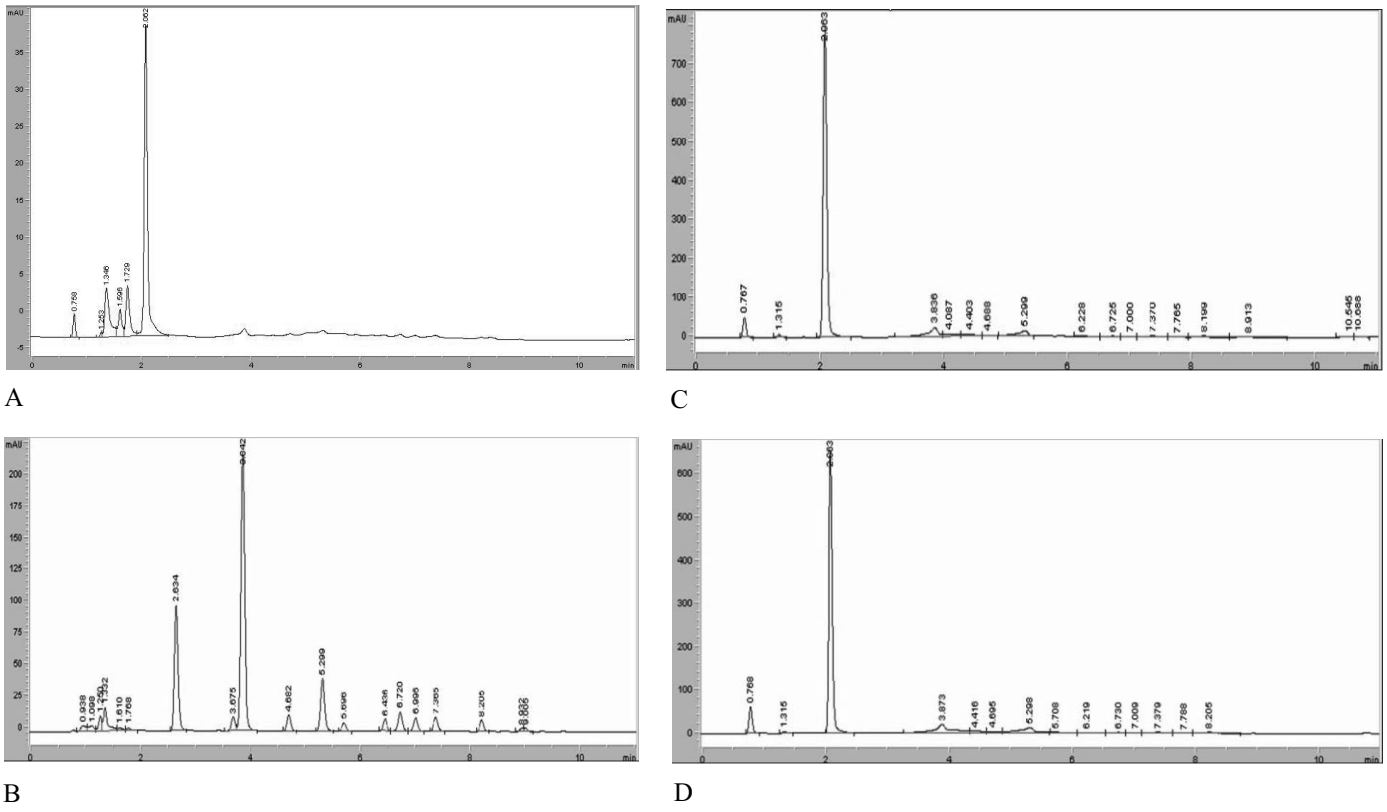

Figure 2. Chromatogram of CS from PSE-W (A), HRE-W (B), PSE-Et (C), and. HRE-Et (D). Abbreviations: those for extracts are the same as in Table 1.

CONCLUSSION

All types of SM and CS showed antifungal activity against the six fungal species, and the activity varied depending on the extraction conditions. The CS possesses much stronger antifungal activity than the SM. It seems that CS was mainly responsible for the antifungal activity of SM. However, the four types of CS did not show the same antifungal activity, and no correlation was observed between the CS content in SM and the antifungal activity of SM.

The SM and CS obtained in the current study were more effective than potassium sorbate in inhibiting the fungal growth. On the other hand the SM was less effective but CS was more effective than propyl paraben against the fungal growth.

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