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RESEARCH ARTICLE

EXAMINATION OF BIOACTIVITIES ACCORDING TO THE EXTRACTION CONDITIONS OF *CIRSIUM JAPONICUM* VAR. USSURIENSE.

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Abstract

This study was carried out to obtain a basic data for application in meat products via examination of bioactive materials from *Cirsium japonicum* var. Ussuriense tuber. Hot water extract showed high activities against BC (*Bacillus cereus*) and ST (*Salmonella* Typhimurium) as well as inhibited nitric oxide (NO) production. The supernatant of methanol fractionation (MS)50 and MS70 exhibited antibacterial activity against BC, whereas the precipitated pellet of methanol fractionation (MP)30 and MS50 showed the activity against ST. DPPH radical scavenging activity showed the highest value in MP50, but exhibited higher value in the supernatant than in the precipitated pellet. NO production in BV2 cells treated with LPS was highly inhibited in MS50 and MS70, but the activity in RAW 264.7 cells showed a high inhibition rate in MS70. As a result of ethyl acetate partition, antimicrobial activity showed high values in organic and water phases of ethyl acetate partition (MS70EAO and MS70EAW) against BC, but presented high activity in MS70EAW against ST. MS70EAO showed the highest NO production inhibition rate in BV2 and RAW 264.7 cells. In purification of Sep-Pak R18, the NO activity was showed by higher values by MS70EAO.FT (flow through), MS70EAW.FT, and MP70EAW.FT in BV2 cells, whereas observed by broad fractions in RAW264.7 cells. In summary, since the partially purified bioactive materials from *C. japonicum* have variously biological activities, we suggest that it is possibility of application to functional meat products.

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Introduction:-

Cirsium japonicum is a multi-year-old plant belonging to the Asteraceae family and is mainly grown in temperate regions of the Northern Hemisphere such as Korea, China, and Japan. In Korea there are 13 species, 6 strains and 1

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variant, which known as spiny herb (Cho et al., 2016). The thistle plants contain various flavonoid components such as apigenin, luteolin, myricetin, kaemferol, and pectolinoin which are excellent in physiological activity (Kim and Kim, 2003). Especially, among their bioactive materials, apigenin has cancer-preventing and neuroprotective effects (Fang et al., 2005). In addition, thistles have been used for a number of physiological functions including antibiotics, pain and inflammation, anticancer, hemostasis, liver protection and improvement of memory as well as nervous system (Ishida et al., 1987; Martine-Vazquez et al., 1998; Perez et al., 2001; Lee et al., 2002; Yamazaki et al., 2001; Loizzo et al., 2004; Park et al., 2004).

Recently, it has been reported that thistle extract has antimutagenic, anticancer activity, immunity enhancement and antidepressant action (Lee et al., 2002; Lee et al., 2003; Lui et al., 2006; Park et al., 2006). It is highly possible to develop food and medicine materials with excellent efficacy. As the cultivation area increases recently, it is necessary to study not only standardization as food but also various application methods.

Therefore, this study was done for a basic study to perform functional analysis via column purification after extracting with hot water of thistle and fractionating with various organic solvents, and for the utilization of health functional foods and medicinal materials

Materials and Methods:-

Hot water extraction of *Cirsium japonicum:-*

C. japonicum purchased from the general market was mixed with distilled water at a ratio of 1:9. Hot extraction was carried out three times at 100℃ for 90 min, and each extract was combined and concentrated four times. The concentrated thistle extract was used as a sample for further studies.

Fractionation by organic solvent:-

The concentrated thistle extract obtained from hot water extract were done by organic solvent precipitation with 30, 50 and 70% methanol. After adjusting to each concentration of methanol, the treated solution was reacted at 4℃ for 1 h and then separated into supernatant and precipitated pellet by centrifugation at 2,000 rpm for 20 min. The precipitate was resuspended by distilled water into the same volume as the supernatant, and then concentrated 10 times with respect to the original start volume.

Organic solvent partition:-

The 70% methanol precipitate to have high activity was mixed with the same volume of ethyl acetate and chloroform. The mixed samples were separated into each phase by centrifugation at 2,000 rpm for 20 min. Each separated phase was collected, concentrated and resuspended by distilled water into the same volume with the started volume.

Purification by Sep-Pak C18 catridge column:-

The ethyl acetate partition solution was loaded on a Sep-Pak® plus C18 catridge (Waters). After loading in a catridge and spontaneously eluting, the loaded samples were eluted by 20 ml of hexane, hexane: ethylacetate (8: 2, 6: 4, 5: 5, 4: 6 and 2: 8), and ethyl acetate (water: methanl saturated). The eluted sample was concentrated and resuspended into 10-fold concentrated volume by distilled water.

Pretreatment of samples and induction of antiimflammatory reaction:-

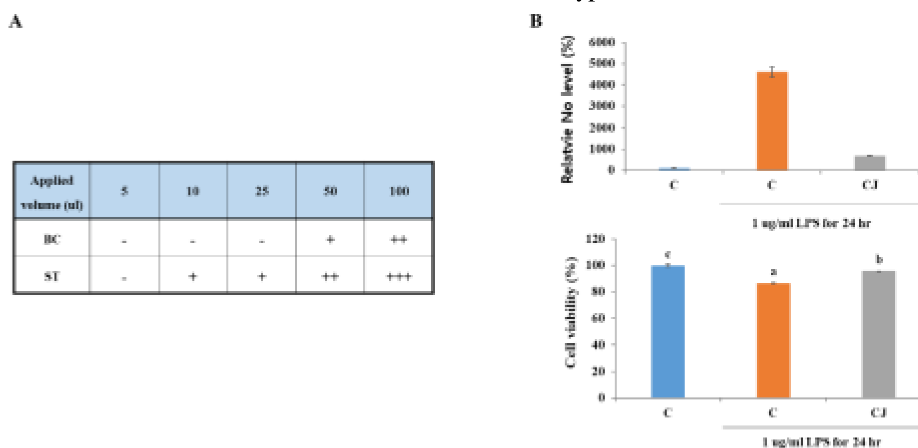
The medium for cell culture was prepared by DMEM-low glucose medium (Sigma-Aldrich Korea, Seoul, South Korea), glucose 4 g/l, sodium bicarbonate 3.7 g/l, 10% fetal bovine serum (FBS) 100 units/ml penicillin and 100 μg/ml streptomycin). The final pH was adjusted into 7.2-7.4, and then sterilization was done by a filter of 0.22 μm.

In this study, samples were pretreated with cells prior to induction of inflammation in order to examine preventive effects of the samples to be analyzed. For this purpose, DMSO concentration used to dissolve the sample to be analyzed was treated with the final 0.5% and used as a negative (-) control. After pretreatment of the assay samples, lipopolysaccharide (LPS; Sigma-Aldrich Korea, Seoul, South Korea) was used to induce inflammatory responses in the cell line. Thus, cell culture medium was treated with LPS at a concentration of 1 μg/ml and reacted for 19 or 24 h.

Antiimflammatory assay in inflammatory-induced cells:-
Cells were seeded into each well of a 96-well plate at 0.5×10^5 cells, and were used for analysis after fixation on the bottom of each well. Cytotoxicity was assessed using the MTS assay kit (Promega, Madison, Wis., USA) after pretreatment for 5 h and LPS treatment for 19 or 24 h. As MTS assay, cells were seeded in each well of a 96-well plate, and when the sample to be analyzed was pretreated for 5 h and treated for 19 or 24 h, the supernatant of the medium was measured for nitric oxide. In order to compare the amount of nitric oxide secreted by cells induced by inflammation, the obtained medium samples were mixed with Griess reagent (Enzo Life Sciences, Inc., Farmingdale, NY, USA) at a ratio of 1: 1, and then OD value at 540 nm was measured using a Microplate reader (Synergy HT).

Statistical analysis:-
The collected data were analyzed by GLM (General linear model) of SAS program (V. 9.2, Cary, NC, USA). Mean values was verified by 5% significant level in Duncan's multiple range test (DMRT).

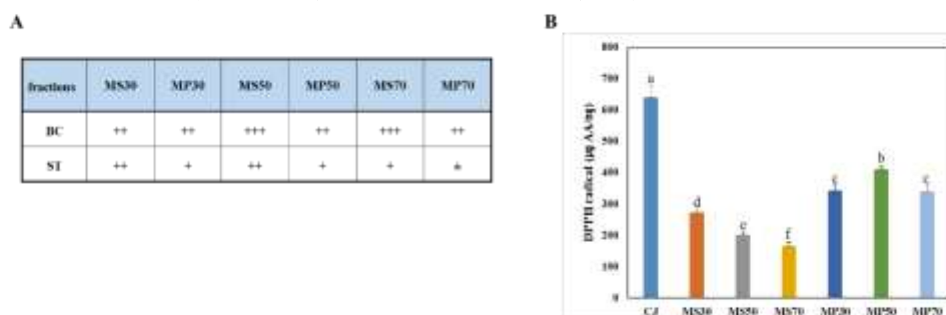
Fig. 1:-Antibacterial and antioxidative activities by bioactive materials extracted with hot water from *Cirsium japonicum* var. ussuriense Kitamura. A and B; antibacterial and antiinflammatory assay results. The dried sample was extracted by hot water (100℃ 180 min * 3 times), CJ; *Cirsium japonicum* var. ussuriense Kitamura, BC; *acillus cereus*, ST; *Salmonella* Typhimurium.



A

Applied volume (ul)	5	10	25	50	100
BC	-	-	-	+	++
ST	-	+	+	++	+++

Fig. 2:-Bioactivities for fractions derived from methanol fractionation of Cirsium japonicum var. ussuriense Kitamura. A; antibacterial assay results, B; antioxidative assay results, C and D; BV2 and RAW264.7 cell assay results, respectively. The dried sample was extracted by hot water (100℃ 180 min * 3 times), and then 30%, 50% and 70% methanol fractionation. CJ; *Cirsium japonicum* var. ussuriense Kitamura, BC; *Bacillus cereus*, ST; *Salmonella* Typhimurium, S; supernatant of methanol fractionation, PP; precipitated pellet of methanol fractionation, 30, 50 and 70; methanol concentration, AA; ascorbic acid.



A

fractions	MS30	MP30	MS50	MP50	MS70	MP70
BC	++	++	+++	++	+++	++
ST	++	+	++	+	+	+

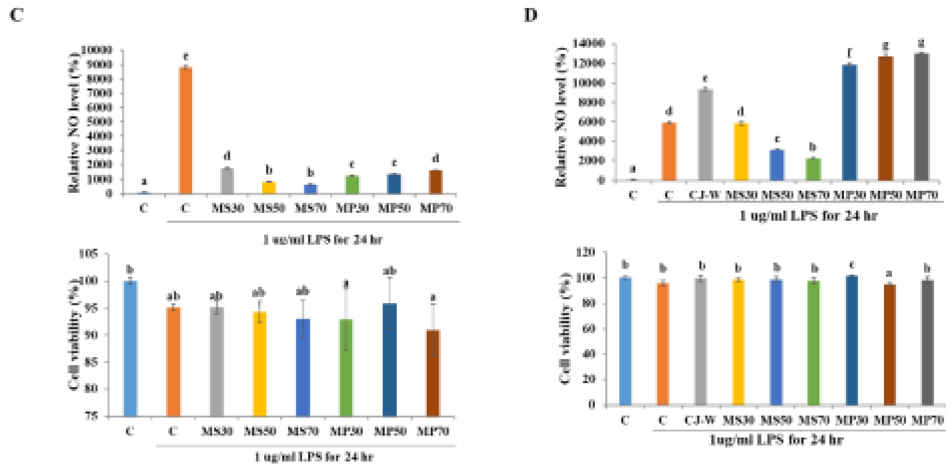
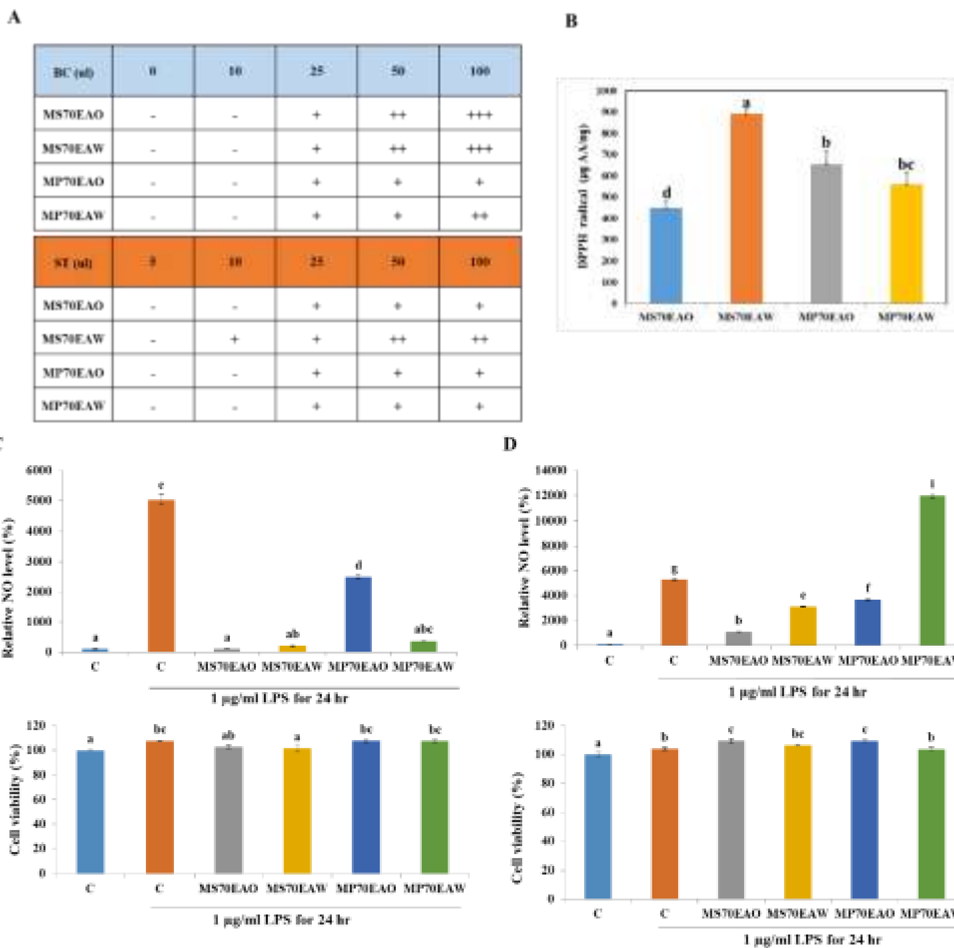


Fig. 3:-Bioactivities for fractions derived from organic solvent partition of *C. japonicum* var. ussuriense Kitamura. A and B; antibacterial and antioxidative assay results. C and D; BV2 and RAW264.7 cell assay results, respectively. The dried sample was purified by hot water (100℃ 180 min * 3 times), 70% methanol fractionation and then ethyl acetate partition BC; *Bacillus cereus*, ST; *Salmonella* Typhimurium, MS70; supernatant of 70% methanol fractionation, MP70; precipitated pellet of 70% methanol fractionation, EAO and EAW; organic and water phases of ethyl acetate partition, AA; ascorbic acid.



A

BC (ul)	0	10	25	50	100
MS70EAO	-	-	+	++	+++
MS70EAW	-	-	+	++	+++
MP70EAO	-	-	+	+	+
MP70EAW	-	-	+	+	++
ST (ul)	**0**	**10**	**25**	**50**	**100**
MS70EAO	-	-	+	+	+
MS70EAW	-	+	+	++	++
MP70EAO	-	-	+	+	+
MP70EAW	-	-	+	+	+

Fig. 4:-Antibacterial and radical scavenging activities for Sep-Pak R18 eluents from *C. japonicum* var. ussuriense Kitamura. A and B; antibacterial and antioxidative assay results. The dried sample was purified by hot water (100℃ 180 min * 3 times), 70% methanol fractionation, ethyl acetate partition, and then Sep-Pak R18 catridge. BC; *Bacillus cereus*, ST; *Salmonella* Typhimurium, MS70; supernatant of 70% methanol fractionation, MP70; precipitated pellet of 70% methanol fractionation, EAO and EAW; organic and water phases of ethyl acetate partition, AA; ascorbic acid. FT; flow through, WT; wash with hexane, H80, H60, H50, H40 and H20; hexane: ethyl acetate (8:2, 6:4, 5:5, 4:6, 2:8), W.MeOH; ethyl acetate saturated with water/methanol.

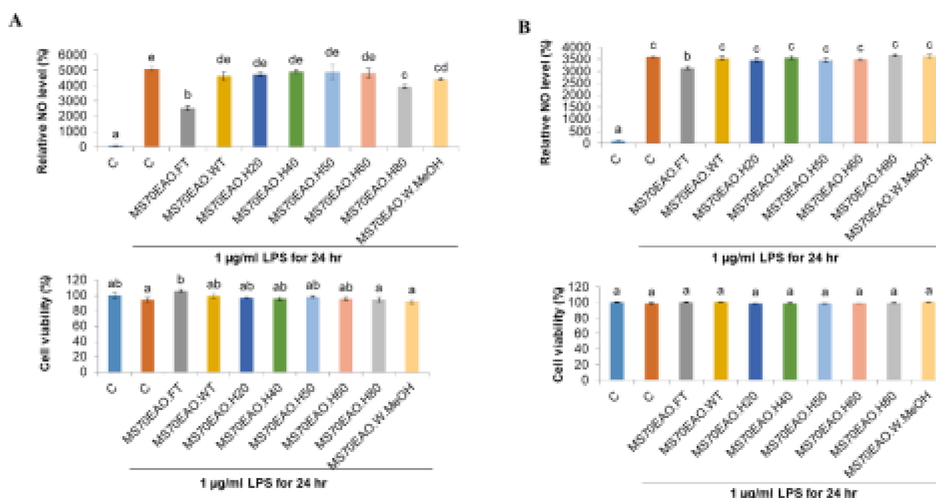
A

ST	FT	WT	H80	H60	H50	H40	H20	W.MeOH
MS70EAO	+	-	+	-	-	-	-	-
MS70EAW	-	-	-	-	-	-	-	-
MP70EAO	-	-	-	-	-	-	-	-
MP70EAW	-	-	-	-	-	-	-	-
BC	**FT**	**WT**	**H80**	**H60**	**H50**	**H40**	**H20**	**W.MeOH**
MS70EAO	-	-	-	-	-	-	-	-
MS70EAW	-	-	-	-	-	-	-	-
MP70EAO	-	-	-	-	-	-	-	-
MP70EAW	-	-	-	-	-	-	-	-

B

ug AA/mg	FT	WT	H80	H60	H50	H40	H20	W.MeOH
MS70EAO	-	-	-	-	-	-	-	-
MS70EAW	200.6	-	-	-	-	-	-	19.6
MP70EAO	-	-	-	-	-	-	-	-
MP70EAW	157.8	-	-	-	-	-	-	5.4

Fig. 5:-Antiinflammatory activities for soluble fraction purified by Sep-Pak R18 from *C. japonicum* var. ussuriense Kitamura. A and B; BV2 and RAW264.7 cell assay results by MS70EAO, and C and D; BV2 and RAW264.7 cell assay results by MS70EAW, respectively. The upper and lower panels indicate NO and MTS assays, respectively. The dried sample was purified by hot water (100℃ 180 min * 3 times), 70% methanol fractionation, ethyl acetate partition, and then Sep-Pak R18 catridge. MS70; supernatant of 70% methanol fractionation, EAO; organic phase of ethyl acetate partition, AA; ascorbic acid. FT; flow through, WT; wash with hexane, H80, H60, H50, H40 and H20; hexane: ethyl acetate (8:2, 6:4, 5:5, 4:6, 2:8), W.MeOH; ethyl acetate saturated with water/methanol.



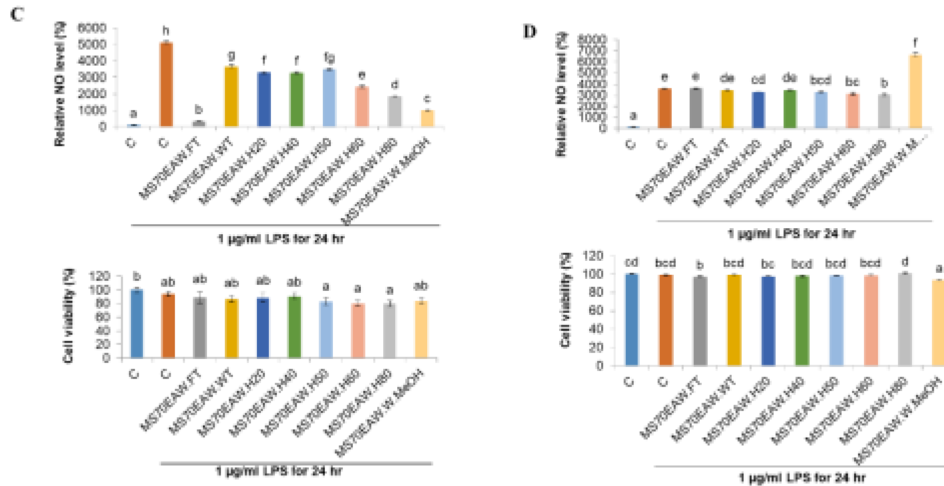
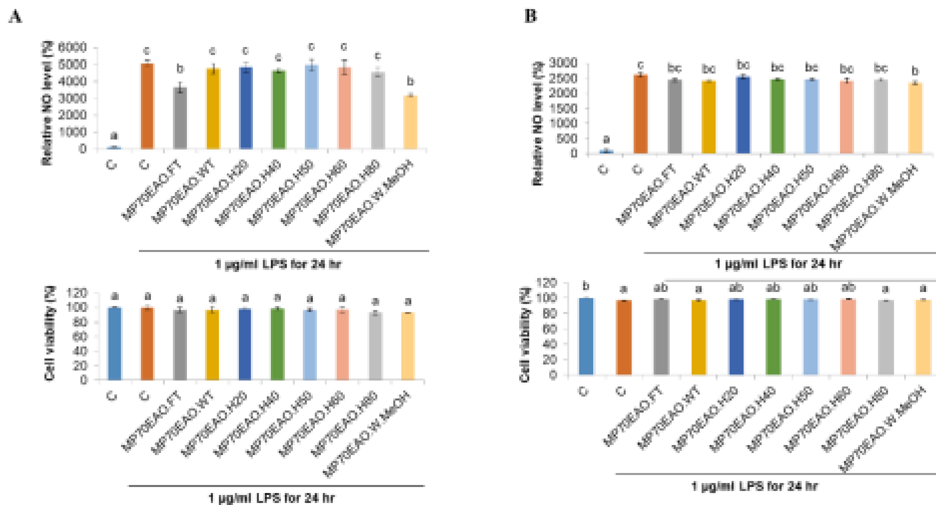
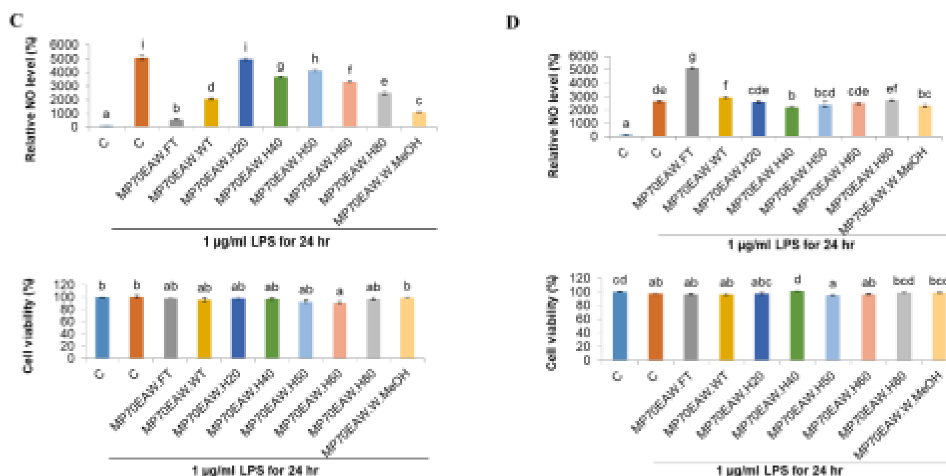


Fig. 6;-Antiinflammatory activities for partially soluble fraction purified by Sep-Pak R18 from Cirsium japonicum var. ussuriense Kitamura. A and B; BV2 and RAW264.7 cell assay results byMP70EAO, and C and D; BV2 and RAW264.7 cell assay results by MP70EAO, respectively. The upper and lower panels indicate NO and MTS assays, respectively. The dried sample was purified by hot water (100℃ 180 min * 3 times), 70% methanol fractionation, ethyl acetate partition, and then Sep-Pak R18 catridge. MP70; precipitated pellet of 70% methanol fractionation, EAO; organic phase of ethyl acetate partition, AA; ascorbic acid. FT; flow through, WT; wash with hexane, H80, H60, H50, H40 and H20; hexane: ethyl acetate (8:2, 6:4, 5:5, 4:6, 2:8), W.MeOH; ethyl acetate saturated with water/methanol.





Results and Discussion:-

Bioactivity of hot water extract:-

An antimicrobial and anti-inflammatory assays were done for hot-water extract of *Cirsium japonicum*. As a result of the antibacterial assay, the extract showed high activities against BC (Bacillus cereus) and ST (Salmonella Typhimurium), especially in ST (Fig. 1A). Since these results are similar to those of 70% ethanol extracts (Jang et al., 2014), it is assumed that the extract is an effect of antibacterial activity.

The hot water extract was no cytotoxicity, but inhibited NO production (Fig. 1B). This result is similar to the previous results of the inhibition of NO production in thistle extract (Mok, et al., 2011; Tae, 2013).

Bioactivities of methanol fractionation products:-

In order to analyze the functionality according to the properties of bioactive materials, we examine antioxidative, antimicrobial and antiinflammatory activities of substances in the supernatant and the precipitate according to methanol concentration. As a result of antibacterial activity for fractionated product of 30, 50, and 70% methanol, MS50 (50% methanol supernatant) and MS70 (70% methanol supernatant) showed strong antimicrobial activities against BC, whereas MS30 (30% methanol supernatant) and MS50 exhibited strong activities against ST (Fig. 2A).

The DPPH radical scavenging activity showed the highest activity of 411 ug AA / ml in MP50 (50% methanol precipitated pellet) (Fig. 2B). In addition, the activity in the precipitate was higher than the supernatant. High DPPH radical scavenging activity is known to have an effect to inhibit the aging of the human body by increasing the free radical scavenging activity such as antioxidant activity and active oxygen due to high free radical reduction or offset ability (Aoshima et al., 2004; Kim et al., 1995).

RAW264.7 and BV2 microglial cells were used to investigate the antiinflammatory effect of thistle. NO production in BV2 cells treated with LPS was highly inhibited in MS50 and MS70, whereas NO production in RAW 264.7 macrophages cells showed a high inhibition rate in only MS70 (Fig. 2C and D). As a result similar to this study, the previous study shows no cytotoxicity in BV2 microglial and RAW 264.7 macrophage cells by thistle extract, but inhibits NO production (Tae, 2013).

Activities of bioactive materials obtained from organic solvent partition:-

Among the methanol fractions of this study, since the 70% methanol precipitate showed the highest antibacterial, antioxidative and anti-inflammatory activities, this fraction was applied for the organic solvent partition. Ethyl acetate was used as an organic solvent and each phase was separated to measure antioxidative, antimicrobial and antiinflammatory activities.

In antimicrobial activity, MS70EAO (70% methanol supernatant and ethyl acetate organic phase) and MS70EAW (70% methanol supernatant and ethyl acetate water phase) showed strong antimicrobial activity in BC, but MS70EAW in ST (Fig. 3A). Antioxidative radical scavenging activity also showed the highest value in MS70EAW (Fig. 3B). NO productions in BV2 and RAW 264.7 cells treated with LPS was highly inhibited in MS70EAO (Fig.

3C and D).

Functional analysis of Sep-Pak catridge eluents:-

In the results of antibacterial activity for Sep-Pak R18 eluents, Antibacterial activity was not observed for BC and ST in this analysis condition, but DPPH radical scavenging activity showed the highest value at MS70EAW.FT (Fig. 4A and B). In addition, MS70EAO.FT, MS70EAW.FT and MP70EAW.FT presented the most inhibition in antiinflammatory BV2 cells, whereas RAW264.7 cells showed its activity in broad fractions (Fig. 5 and 6).

In summary, since the partially purified products of *C. japonicum* has been found to have antimicrobial, antioxidant and anti-inflammatory activities, we suggest that it can apply as a physiologically active substance for the development of functional foods and medicinal materials in the future.

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