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ARTICLE

Citrus unshiu leaf extract containing phytol as a major compound induces autophagic cell death in human gastric adenocarcinoma AGS cells

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Abstract The pharmaceutical potential of the methanolic extract of *Citrus unshiu* leaves (MECL) was assessed through analysis of its inhibitory effect on cancer cells. The antiproliferative activities of the leaves were evaluated using several cancer cell lines and considerable cytotoxicity was observed in human gastric adenocarcinoma AGS cells. Inhibition of AGS cell viability was both time- and dose-dependent, and MECL induced non-apoptotic cell death. AGS cells treated with MECL increased the formation of acidic vesicular organelles and GFP-LC3 puncta. Pretreatment with an autophagy inhibitor, 3-methyladenine, inhibited MECL-induced cell death. These results indicated that the mechanism underlying the anticancer effects of MECL in AGS cells could be via the induction of autophagic cell death. The major compounds of MECL were identified as phytol, 4-ethenyl-2-methoxyphenol, hexadecanoic acid, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, and vitamin E using gas chromatography–mass spectrometry. These results indicate that *C. unshiu* leaves can be exploited for numerous pharmaceutical applications as a source of anticancer ingredients.

Keywords AGS human gastric adenocarcinoma cells · Autophagy · *Citrus unshiu* · Phytol

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Introduction

An analysis of chemotherapeutic agents and their sources indicated that over 74.8 % of approved drugs are derived from natural compounds (da Rocha et al. 2001; Newman and Cragg 2012). Some pharmaceuticals derived from natural sources have received a great deal of attention from both the scientific community and the public owing to their demonstrated ability to suppress cancers (da Rocha et al. 2001). In the past decade, various fruit extracts have been screened for potential use as alternative remedies for the treatment of infectious diseases and as cancer chemopreventive agents. Several studies have reported that the lower incidence of gastric cancer could be associated with citrus fruit intake (Bae et al. 2008) and have found that citrus fruits exhibit anticancer effects by inducing apoptosis in cancer cells (Patil et al. 2009; Ko et al. 2010; Lim et al. 2009). *Citrus unshiu,* locally known as "unshiu" or "onjumilgam", is one of the most popular citrus fruits in Korea and has been reported to possess diverse therapeutic activities including anticancer effects (Lee et al. 2011a, b; Kim et al. 2011). Its peel has been used for traditional medicines against cancer in East Asia (Kamei et al. 2000; Morley et al. 2007; Park et al. 2008).

Citrus leaves are reputedly a general tonic for gastrointestinal disorders and fevers (Lee et al. 2011a). The leaf extract is consumed as an herbal tea, and leaf hydrolate from numerous *Citrus* species is used in the preparation of some traditional dishes in Tunisian cuisine (Hosni et al. 2013). Although there are some reports on the biological effects and chemical compositions of certain species of citrus leaves (Arias and Ramon-Laca 2005; Moon et al. 2009; Lota et al. 2001; Yang et al. 2009; Kim et al. 2010), very little information about the anticancer properties of *C. unshiu* leaves has been reported. *C. unshiu* leaves are readily available agricultural byproducts because the appropriate tree canopy

for *C. unshiu* is maintained by skirting and pruning to create a suitable microclimate for better quality and quantity of fruit production. Therefore, *C. unshiu* leaves can be exploited for potential uses in food and pharmaceutical industries.

Apoptosis is programmed cell death and cancer cells elude apoptosis through a variety of pathways. The induction of apoptosis and the inhibition of cancer cell proliferation have been used to evaluate phytochemical anticancer activities and develop new anticancer drugs (Sun et al. 2004; Beevi et al. 2010). On the other hand, autophagy is an evolutionarily conserved and genetically programmed process that degrades long-lived cellular proteins and organelles (Park et al. 2011). In response to stress conditions, cytoplasmic organelles are engulfed by autophagosomes and subsequently fused with lysosomes, resulting in their degradation and recycling to promote cell survival (Yorimitsu and Klionsky 2005; Meijer and Codogno 2004; Mariño and López-Otín 2004). During the initial stages of autophagy, cellular proteins, organelles, and cytoplasm are sequestered and engulfed by intracellular double-membrane-bound structures called autophagosomes. Autophagosomes mature by fusing with lysosomes to form autolysosomes, in which the sequestered proteins and organelles are digested by lysosomal hydrolases and recycled to sustain cellular metabolism (Mizushima 2004). The role of autophagy in cancer is quite complicated and controversial; autophagy is assumed to be tumor suppressive during cancer development but to contribute to tumor cell survival during cancer progression (Yang and Klinosky 2010). Regardless of whether they promote cell survival or cell death, the two processes engage in complex and poorly understood molecular cross talk (Maiuri et al. 2007).

With the aim of exploring the anticancer activity of *C. unshiu* leaves, we analyzed the chemical composition of methanolic extracts of *C. unshiu* leaves (MECL) using GC–MS and sought to elucidate the ability of MECL to induce apoptosis and/or autophagy in human gastric adenocarcinoma AGS cells. Our results demonstrated for the first time that MECL contain bioactive compounds capable of suppressing the proliferation of AGS cells by inducing autophagy. Thus, autophagic cell death must be considered a therapeutic approach to reduce AGS proliferation and a promising strategy to develop an anticancer reagent from MECL.

Materials and methods

Plant samples

Citrus unshiu Marc. cv. Miyagawa leaves were collected from National Institute of Subtropical Agriculture, Jeju Province, Korea. Botanical samples were taxonomically identified and a voucher specimen (number SKC. 111022) was deposited in the herbarium of the Subtropical Research Institute of Jeju National University. The lyophilized leaves were pulverized using a milling machine and extracted with 80 % methanol by sonication for 45 min. The extract was filtered, concentrated with a vacuum rotary evaporator, and lyophilized. The extract was refrigerated (4 °C) until use. The extract was dissolved in DMSO to a concentration of 200 mg/mL during the experiments.

Cell lines and cell culture

AGS, HeLa, MCF-7, HepG2, and human dermal fibroblast cells were obtained from the Korean Cell Line Bank (Seoul, Korea). AGS and MCF-7 cells were cultured in RPMI-1640 and HeLa, HepG2, and fibroblast cells were cultured in DMEM medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 1 % antibiotics at 37 °C in a humidified atmosphere containing 5 % CO_2.

Reagents

RPMI 1640 medium, DMEM medium, trypsin/EDTA, and fetal bovine serum (FBS) were purchased from Invitrogen. Hoechst 33342 dye, acridine orange dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI), and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO). The Annexin V-FITC Apoptosis Detection Kit I was purchased from BD^{TM} Biosciences (Franklin Lakes, NJ).

Cell viability assay

The effect of MECL on cell viability was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, which involves the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Briefly, cells were plated in 96-well plates at an initial density of 5×10^4 cells/mL per well. After 24 h incubation, the cells were pre-treated with or without 1 mM 3-methyladenine for 2 hand then various concentrations (from 0 to 200 μg/ml) of MECL were added to the cells. At the indicated time points, 20 μL of MTT solution (5 mg/mL) was added to each well and the cells were kept in a humidified environment for 3–4 h. The supernatant was removed and dissolved in 150 μL DMSO. Absorbance was detected in a microplate reader at 570 nm (Tecan, Salzburg, Austria).

Cell morphology analysis

AGS cells were placed in 6-well plates at 5×10^4 cells/mL and treated with the *C. unshiu* leaf extract after 24 h of seeding. After 24 h, 10 μM of Hoechst or AO was added to the solution in each well, and the plates were incubated for

10 min at 37 °C. The stained cells were then observed under a fluorescence microscope (Olympus, UK).

Flow cytometry

For sub-diploid population detection, cells were detached from plates by the addition of trypsin–EDTA, washed in phosphate-buffered saline (PBS), fixed in 70 % ethanol, treated with RNase A (25 ng/mL), and stained with propidium iodide (PI) (40 μg/mL). The annexin V-FITC apoptosis detection kit I was used according to the manufacturer's protocol to detect phosphatidylserine translocation from the inner to the outer plasma membrane. For each assay, cells were washed with PBS, diluted in annexin V binding buffer containing annexin V and PI, and incubated for 15 min at room temperature (RT). For autophagy detection, we stained the cells with 10 μM acridine orange, harvested the cells, and kept them in 2 mM EDTA-PBS containing 10 % FBS. Data from 10,000 cells/sample were analyzed with Cell Quest software (Becton Dickson, USA). Each experiment was repeated at least 3 times.

LC3-GFP transfection

Vector pEGFP-LC3B was a kind gift from Professor Tamotsu Yoshimori (Hayama, Japan) and Junsoo Park (Yonsei University, Wonju, Republic of Korea). The transfection was carried out with Lipofectamine (Invitrogen) according to the manufacturer's protocol. After 24 h of incubation with the plasmid-Lipofectamine complex, the indicated doses of extract were applied, followed by further incubation for 24 h before visualization of the LC3-II in autophagosomes under a fluorescent microscope (Olympus, UK).

GC–MS analysis

Chromatographic analysis was carried out using a Shimadzu GC–MS (Model QP-2010, Shimadzu Co., Kyoto, Japan) in EI (Electron Impact) mode. The ionization voltage was 70 eV and the temperatures of the injector and detector were 250 and 290 °C, respectively. The capillary column used was an RTX-5MS (30 m long × 0.25-mm internal diameter and 0.25 μm, film thickness). The oven temperature programmed at 60 °C (isothermal for 2 min) was ramped to 250 °C at 5 °C/min and then to 310 °C at 5 °C/min (isothermal for 12 min). Helium was used as the carrier gas at a flow rate of 1 ml/min, with an injector volume of 1 μL using 1:10 split ratio. The dried methanolic extract powder was dissolved in methanol, filtered through a 0.20-μm syringe filter (Advantec, Tokyo, Japan), and aliquots were injected onto the GC–MS. The mass spectra of each compound obtained from GC–MS were tentatively identified using the

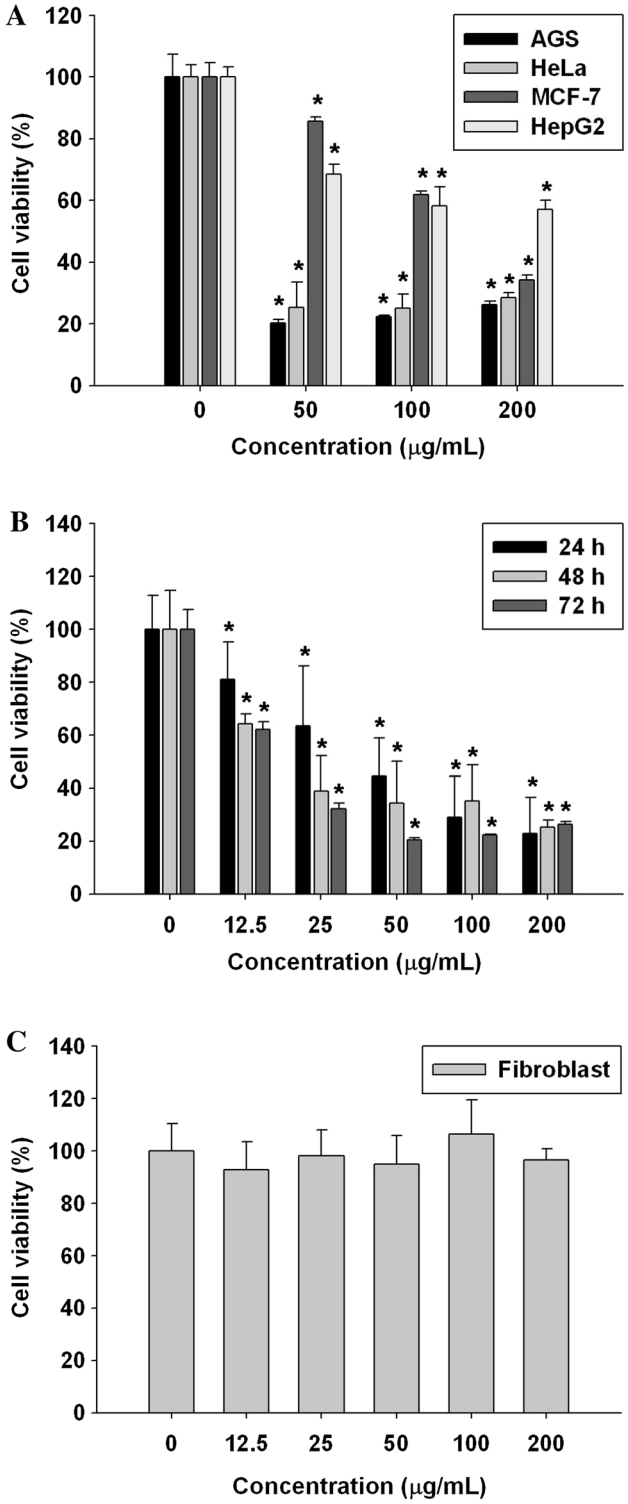


Fig. 1 Antiproliferative activities of MECL. Cell viability was assessed based on MTT reduction. **a** Several cancer cell *lines* were treated with the indicated concentrations of MECL for 72 h. The statistically significant differences are presented as $*p < 0.05$. **b** AGS cells were treated with the indicated concentrations of MECL for 24, 48, 72 h. $*p < 0.05$. **c** Normal human dermal fibroblast cells were treated with the indicated concentrations of MECL for 72 h

spectral data of compounds contained within the WILEY7 and NIST libraries. Furthermore, tentative identification was completed by comparison of their mass spectra with authentic published data (Adams 1995).

Statistical analysis

Data are expressed as mean ± standard deviation of three independent determinations. The significance of differences between groups was determined through one-way analysis of variance (ANOVA).

Results and discussion

C. unshiu leaves extract reduced viability of AGS cells

To investigate the cytotoxic effects of *unshiu* leaves on human cancer cell lines, MTT assay was conducted. The cells incubated with various concentrations of the methanolic extract of *C. unshiu* leaves (MECL) showed a dose-dependent reduction in cell viability. The AGS cells appeared to be the most sensitive among the cancer cell lines tested with treatment of MCEL for 72 h (Fig. 1a). MECL reduced cell viability both time- and dose-dependently in AGS cells (Fig. 1b), and no obvious cytotoxicity in normal human dermal fibroblasts was observed even after treatment for 72 h (Fig. 1c).

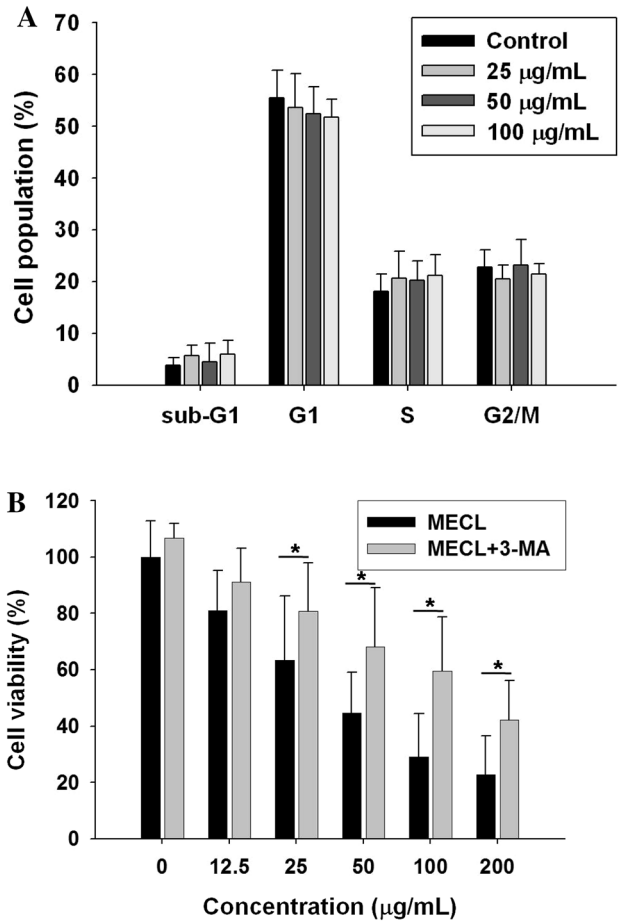
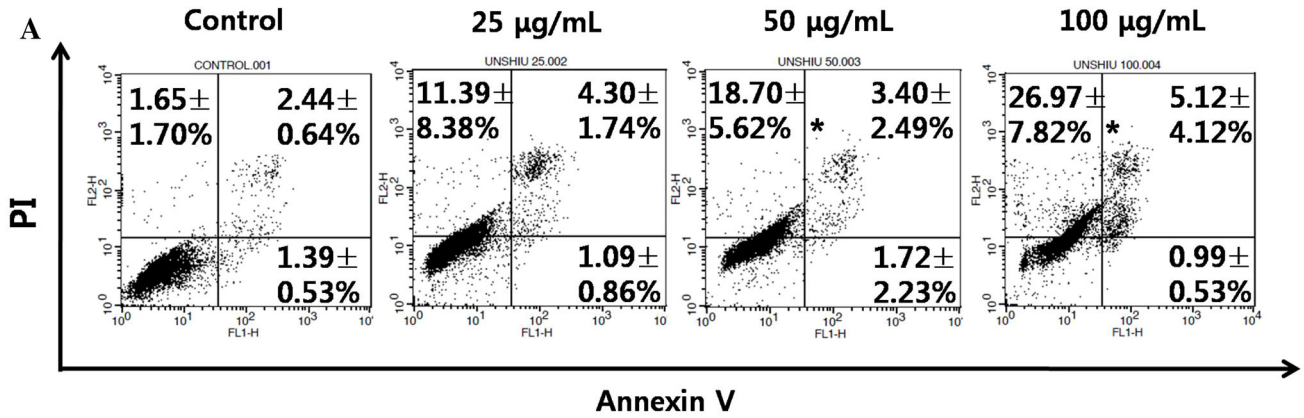


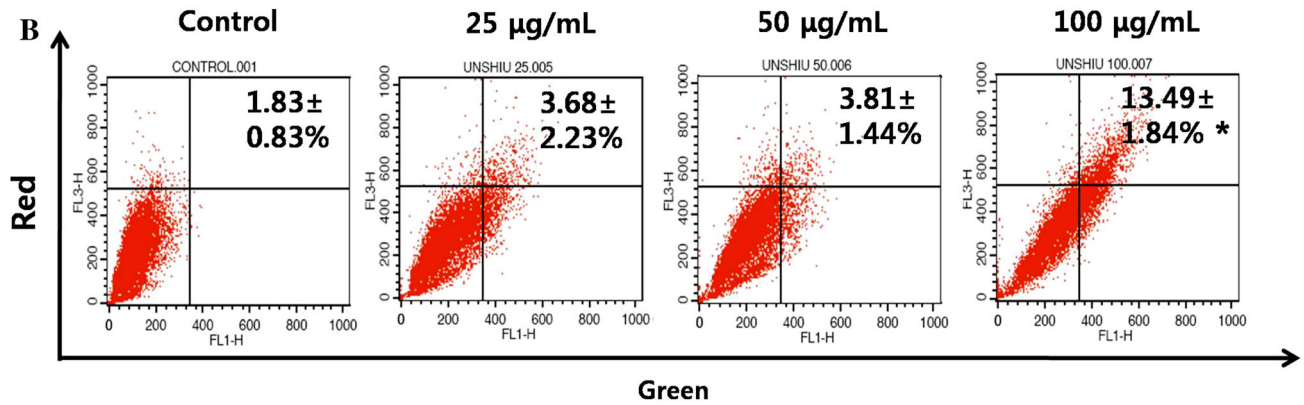
Fig. 2 Cell cycle analysis and the effect of 3-MA on MECL-induced cell death. **a** AGS cells were treated for 24 h and analyzed for DNA content using flow cytometry. **b** AGS cells were pre-treated with or without 1 mM 3-MA for 2 h and then incubated with the various concentrations (from 0 to 200 μg/ml) of MECL for 24 h. Cell viability was detected using MTT assay. $*p < 0.05$

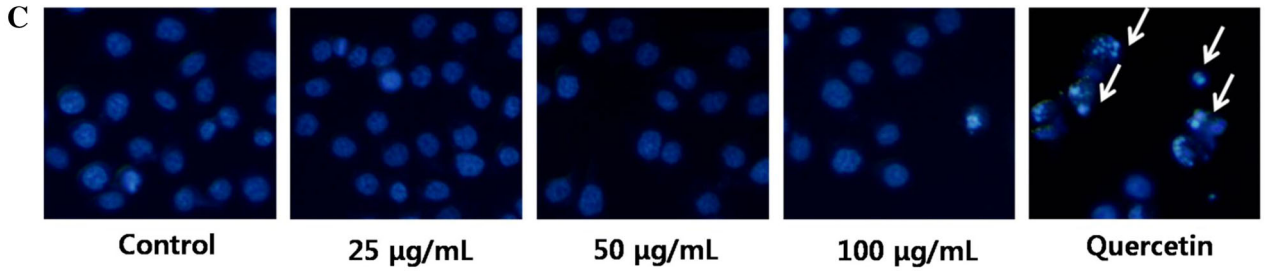
Fig. 3 MECL induces non-apoptotic but autophagic cell death. ▶ **a** Cells were treated with 0–100 μg/mL MECL for 24 h. After treatment, the cells were collected and stained with annexin V and PI and then analyzed by flow cytometry. $*p < 0.05$. **b** Quantification of AVO-positive cells by flow cytometry. Cells were seeded in 60 mm dishes and treated with the indicated concentration of MECL. After 24 h, the cells were stained with 10 μM of AO, trypsinized, and analyzed. $*p < 0.05$. **c** Nuclear Hoechst staining. Cells were treated with the indicated concentrations of MECL, DMSO (*control*), or Quercetin 50 μM (*positive control*) for 24 h, then stained with Hoechst 33342 and observed under a fluorescent microscope. Arrows indicate the formation of apoptotic bodies. **d** Representative images for increased AVO-positive cells. Cells were seeded in 60 mm plates for 24 h and treated with the indicated concentrations of MECL for an additional 24 h. *Arrows* indicate the formation of AVO. **e** AGS cells were transfected with an expression construct for LC3 fused to green fluorescent protein (GFP-LC3) for 24 h. Cells were treated with or without MECL for 24 h and visualized under a fluorescent microscope. Arrows indicate the puncta of LC3. **f** AGS cells were treated with 0–200 μM phytol for 24 h. After treatment, stained with acridine orange dye and observed under a fluorescent microscope. Arrows indicate the formation of AVOs

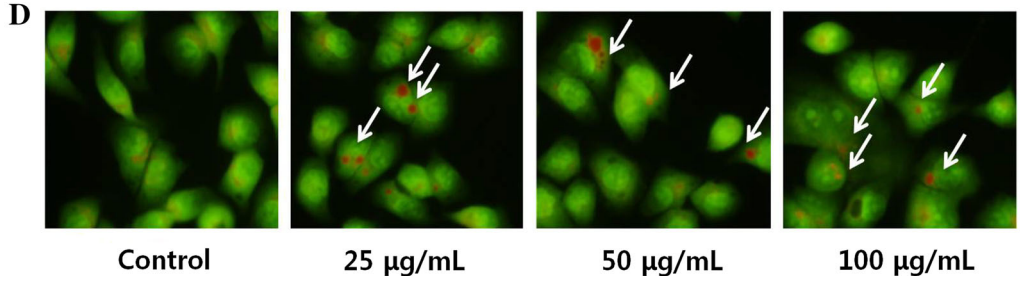
Analysis of cell cycle and the effects of 3-MA on MECL-induced cell death

To evaluate whether the MECL-induced decrease in viability was attributable to apoptosis, we first investigated cell cycle distribution. There were no significant changes in the cell cycles of MECL-treated cells (Fig. 2a). Thus, the inhibition of growth observed in response to MECL is not associated with sub-G1 arrest of the cell cycle. Next, we investigated whether MECL induces autophagy in AGS cells since autophagy has been reported to increase as a result of chemotherapy (Liu et al. 2011). Initiation of the autophagy process requires class III PI3K (PI3KC3) and the initiation process can be suppressed by 3-MA, a specific inhibitor of endogenous lysosomal protein degradation that targets PI3KC3 but not the other PI3Ks (Seglen and Gordon 1982; Araki et al. 2006). For this reason, we measured and compared the viability of cells pre-treated with an autophagy inhibitor, 3-methyladenine (3-MA) to the viability of cells without pretreatment of 3-MA. The cell viability of AGS cells pre-treated with 3-MA was reversed, indicating that the MECL induced autophagic cell death in AGS cells (Fig. 2b).

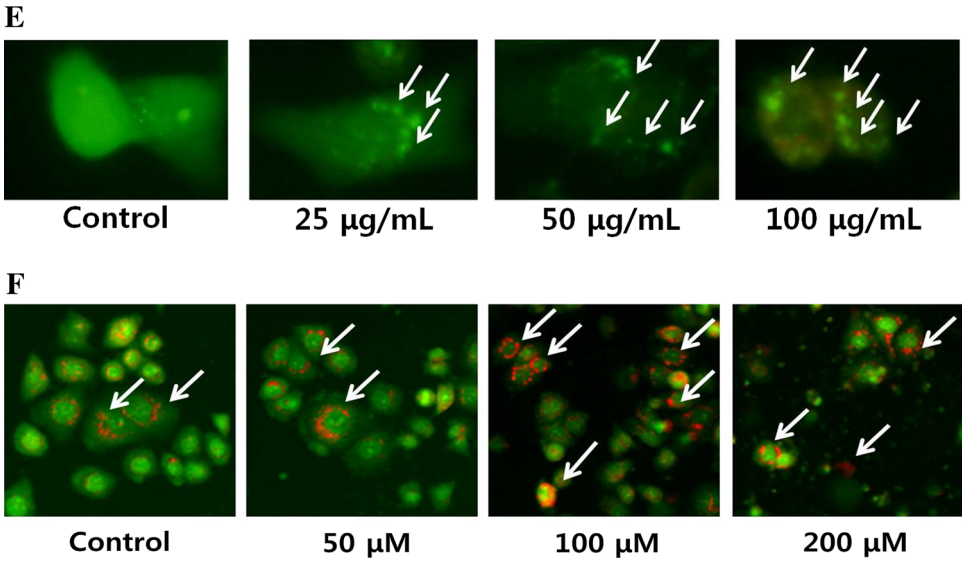

A
Control
25 μg/mL
50 μg/mL
100 μg/mL
CONTROL.001
UNSHIU 25.002
UNSHIU 50.003
UNSHIU 100.004
1.65± 1.70%
2.44± 0.64%
1.39± 0.53%
11.39± 8.38%
4.30± 1.74%
1.09± 0.86%
18.70± 5.62% *
3.40± 2.49%
1.72± 2.23%
26.97± 7.82% *
5.12± 4.12%
0.99± 0.53%
PI
Annexin V


B
Control
25 μg/mL
50 μg/mL
100 μg/mL
CONTROL.001
UNSHIU 25.005
UNSHIU 50.006
UNSHIU 100.007
1.83± 0.83%
3.68± 2.23%
3.81± 1.44%
13.49± 1.84% *
Red
Green


C
Control
25 μg/mL
50 μg/mL
100 μg/mL
Quercetin


D
Control
25 μg/mL
50 μg/mL
100 μg/mL

◀**Fig. 3** continued



MECL induced autophagy in AGS cells

To confirm that the MECL-induced decrease in viability was attributable to autophagy, we performed flow cytometric analysis and staining of the cells. Flow cytometric analysis using annexin V/PI double-staining revealed that the percentage of annexin V-positive/PI-negative AGS cells under apoptosis only slightly exceeded the control level (Fig. 3a), whereas acidic vesicular organelles (AVO) increased by about 13.49 % in the AGS cells treated with 100 μg/mL of MECL (Fig. 3b), which is characteristic of autophagy (Herman-Antosiewicz et al. 2006; Yo et al. 2009). Nuclear staining with Hoechst 33342 revealed fragmented and condensed nuclei in the quercetin-treated cells, whereas no morphological changes of cells or condensed nuclei were found when the cells were treated with the high concentration of MECL (Fig. 3c). As shown in Fig. 3d, higher numbers of AVOs were detected in the AGS cells treated with MECL. The LC3 has been proposed to serve as a marker for autophagic vesicles and the lipidated form of LC3 has been considered an autophagosomal marker because of its localization and aggregation on autophagosomes (Mizushima 2004; Li et al. 2010; Kabeya et al. 2000). Therefore, we transiently transfected the AGS cells with a pEGFP-LC3 plasmid and observed that the control cells revealed diffused and weak GFP-LC3 punctuate dots, whereas MECL-treated cells exhibited green LC3 punctuate dots in the cytoplasm (Fig. 3e). Collectively, these results indicate that MECL induced not apoptotic cell death but autophagic cell death in the AGS cells.

Compositional analysis by GC–MS

These data suggest that *C. unshiu* leaves-derived phytochemicals play a vital role in preventing human gastric cancer cell proliferation. Thus, we analyzed the composition of MECL by GC–MS and tentatively identified 30 compounds that account for nearly 90 % of the constituents (Fig. 4; Table 1). MECL contain different groups of phytochemicals including hydrocarbons, alcohols, lactones, pyrans, flavones, and sterols. Phytol was the major compound and the sequence of compounds in relative concentration was 4-ethenyl-2-methoxyphenol, hexadecanoic acid, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, and vitamin E. Stigmasterol, γ-Sitosterol, and δ5-ergostenol were identified as minor steroidal compounds. Although, the present investigation revealed that phytol might be the responsible ingredient for induction of the autophagic cell death in AGS cells (Fig. 3f), several ingredients are known for induction of cell death. For example, 4-ethenyl-2-methoxyphenol (2-methoxy-4-vinylphenol) can arrest growth of NIH3T3 cells (Jeong and Jeong 2010), hexadecanoic acid is inducer of apoptotic cell death in leukemic cell line (Harada et al. 2002), and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one has also been reported to have an antiproliferative and anti-apoptotic effect in human colon cancer (Ban et al. 2007). Vitamin E may protect against ovarian cancer growth and progression by reducing cancer cell growth and/or promoting apoptosis (Kline et al. 2007). Furthermore, vitamin E was reported as a novel enhancer of autophagy (Karim et al. 2010). In addition, nobiletin, the only flavone identified, has been reported to inhibit the proliferation of several kinds of human cancer cell lines (Kandaswami et al. 1991, 1992; Yanez et al. 2004). It has also been reported to decrease the incidence of chemically induced tumors in mice and rats (Murakami et al. 2000) and to control many human cancer cell lines (Manthey and Guthrie 2002). It is possible that therapeutic properties are expressed by the constituents of MECL, in which case the

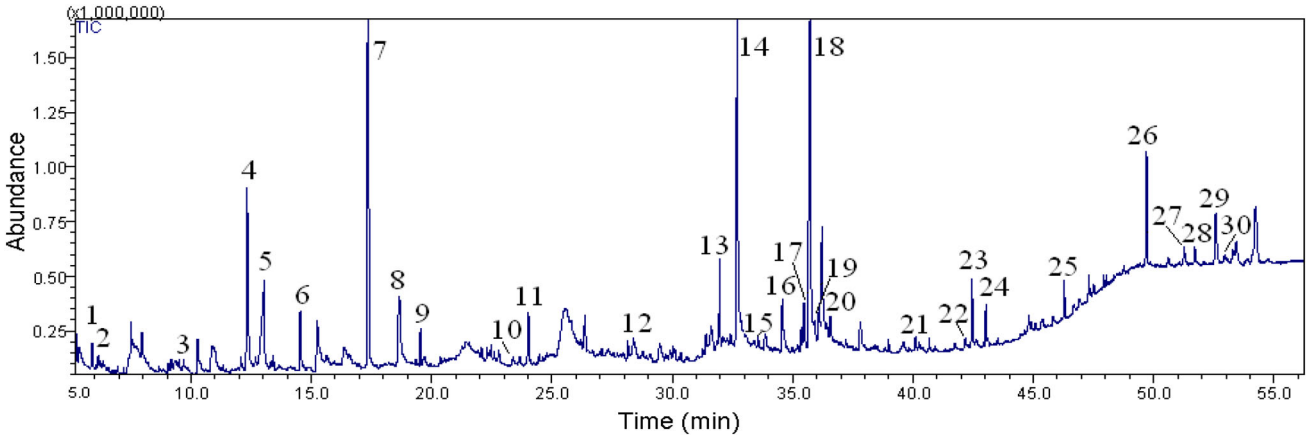


Fig. 4 GC–MS chromatogram of MECL

Table 1 Compounds identified from the methanolic extract of *C. unshiu* leaf by GC–MS

No.	RI[a]	Name[b]	Area %[c]
1	923	Butyrolactone	0.47
2	933	1,2-Cyclopentadione	0.22
3	1,060	Furaneol	0.38
4	1,147	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	3.28
5	1,170	Benzoic acid	1.53
6	1,219	4-Vinylphenol	1.06
7	1,316	4-Ethenyl-2-methoxyphenol	13.81
8	1,363	p-Hydroxybenzaldehyde	1.17
9	1,394	β-Elemene	0.69
10	1,554	Elemol	1.25
11	1,568	3,4-Dimethoxyacetophenone	0.90
12	1,743	Coniferyl alcohol	0.34
13	1,919	Hexadecanoic acid, methyl ester	1.57
14	1,955	Hexadecanoic acid	6.58
15	1,964	Bis(2-methoxyethyl)-phthalate	0.18
16	2,054	Heptadecanoic acid	0.31
17	2,090	(Z,Z)-9,12-octadecadienoic acid methyl ester	0.42
18	2,109	(E)-Phytol	47.35
19	2,128	(Z,Z)-9,12-octadecadienoic acid	0.54
20	2,155	(Z)-9-Octadecenoic acid	0.47
21	2,398	Tetracosane	0.31
22	2,499	Pentacosane	0.31
23	2,507	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	1.23
24	2,551	Bis(2-ethylhexyl) phthalate	0.72
25	2,826	Squalene	0.70
26	3,000<	Vitamin E	2.18
27	3,000<	delta.5-Ergostenol	0.28
28	3,000<	Stigmasterol	0.29
29	3,000<	gamma-Sitosterol	0.91
30	3,000<	Nobiletin	0.15
		Total	89.60

[a] Retention index

[b] Compounds tentatively identified based on parent molecular ions, retention times, retention indices and elution order, and fragmented spectra compared with the literature

[c] Peak area percentage (peak area relative to the total peak area %)

above compounds, either individually or synergistically, would be responsible for the anticancer activity. We are currently exploring the effects of the five major compounds and the potential of these compounds as therapeutic agents for the prevention of gastric cancer.

Conclusion

This is the first report on the antiproliferative activity of *C. unshiu* leaves extract against AGS human gastric adenocarcinoma cells. Leaves of *C. unshiu* are rich in phytol, 4-ethenyl-2-methoxyphenol, hexadecanoic acid, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, and vitamin E. MCEL induced autophagy was evidenced by the increase in acidic vacuole organelle formation and GFP-LC3 puncta as well as the inhibition of MECL-induced cell death by pre-treatment with the autophagy inhibitor 3-MA. Our results suggest that *C. unshiu* leaves extract could be effective as a preventive agent against cancer cells. Further studies must be carried out to isolate and purify the compounds that are responsible for the autophagy.

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