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Modulation of Tumor Microenvironment through *Aurea helianthus* Extract and Induced Exosomes

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Abstract

Endometrial cancer (EC) cells metastasize to various regions, including ovaries, fallopian tubes, cervix, blood, liver, bone, and brain. Various carcinogens cause EC. Exosomes are released from several types of cells and contain different components depending on cellular types. Although tocopherol-α and rutin were high components in *Aurea helianthus*, the *Aurea helianthus* extract was enormously useful in modulating tumor microenvironment contrasted to the two substances. Notably, we established that the extract induced bioactive exosomes in EC cells, and profiling of miRNAs in the extract inducing exosomes (EIE) present potency to develop a biological drug. The extract and EIE contributed to the following five biological categories for EC cells: (1) suppressed migration and invasion; (2) activated cellular senescence by attenuating mitochondrial membrane potential and enhancing autophagy; (3) attenuated reproductive cancer activity; (4) activated drug susceptibility; and (5) EIE contained miRNA associated with decreasing inflammation.

Introduction

Endometrial cancer (EC) is classified into two types; type Ⅰ, the most common type, and Ⅱ [1]. The cancer cells metastasize to various regions, including ovaries, fallopian tubes, cervix, blood, liver, bone, and brain

[2, 3]. EC is caused by multiple carcinogens, including chemicals, radiation, biological reasons as an imbalance of hormones, and human papillomavirus (HPV) [4]. Recently, EC in female cancers has been increased steadily, and notably, the incidence rate of type Ⅰ EC has risen significantly globally [1, 5]. Significant EC incidence causes are obesity, excessive estrogen, high blood pressure, and diabetes mellitus [6].

The typical characteristics of activated cancer cells are evasion of cell death, healthy metabolism, limitless replication tissue invasion, and metastasis [7]. From many pieces of research, EC cells besides cervical cancer cells expressed specific markers including squamous cell carcinoma antigen (SCC), urinary gonadotropin fragment (UGF), Muc16 (CA-125; cancer antigen 125), cytokeratin 8 (CK8) [8, 9, 10, 11]. Metastasis is a significant characteristic of malignant tumors, and the character's prevention is an essential therapeutic point [12]. In metastasis' activation, cervical cancer cells are expressed several genes, including Homeobox (HOX) gene, PI3K/AKT/mTOR, epidermal growth factor receptor (EGFR), platelet-derived growth factor receptors (PDGFR), vascular endothelial growth factors (VEGF) besides vimentin [13, 14, 15, 16, 17].

Exosomes, one of the extracellular vesicles, are released from several types of cells[18]. In particular, cancer cells release more exosomes containing different molecular compositions compared to normal cells. Tumor-derived exosomes contribute to proliferation, inflammation, drug resistance, metastasis, tumorigenesis, and immune response [18, 19]. Tumor-derived exosomes possess bioactive compounds including proteins, single-strand and genomic DNA, retrotransposon elements, messenger RNA, long noncoding RNA, and microRNAs (miRNAs) [18, 19]. Exosomal miRNAs of tumor cells modulate the expression of oncogenes, activation of suppressors[18]. Likewise, alteration of exosomal miRNAs roles as a critical modulator for carcinogenesis, under a functional phytoextract, miRNAs in tumor cells is expected to be improved to alter tumor microenvironment.

Aurea helianthus belonging to the family *Malvaceae* serves various functions, including suppressing inflammation, fever, tumor, oxidation and melanogenesis, detoxification, modulation of lipid metabolism, and immune regulation[20]. Notably, the leaves have high tocopherol and rutin besides iron, vitamin A, and

vitamin C[20]. Tocopherol, the soluble phenolic, is a significant vitamin E structure and potent antioxidant and prevents cancer activity [21]. Additionally, rutin, known as the inhibitor against EGFR kinase, protects DNA structure and induced apoptosis of cancer cells by endoplasmic reticulum stress [22]. According to the reports[20], *A. helianthus* extract protects human vaginal cells under oxidative and fungal stress and strongly suppresses an expression of CK8, Muc-16, and vimentin. Furthermore, the extract modulates the differentiation of monocytes and activation of phagocytosis against HPV peptides[20].

This study has documented the effects of *A. helianthus* extract, α-tocopherol, and rutin for suppression of cancer activity, including down-regulation of EC markers, suppression of invasion, and migration in EC cells. The functions of EIE by the extract were established by miRNAs' patterns and exposure to EC cells. This research will play a role as an essential key to developing a biological drug to protect or heal gynecological cancers. Especially since the results of the study suggest that the EIE robustly protect and attenuate against endometrial cancer, and miRNAs in EIE utilize to develop a synthesis of liposomes for a biological drug.

Results

This study's concept was to document the roles of the *A. helianthus* extract and EIE for modulation of tumor microenvironments in the five categories (Table 1). Furthermore, based on the miRNAs profiling in EIE, significant miRNAs for the categories were identified in EC cells (Fig 1.).

The extracts effectively down-regulated squamous cell carcinoma antigen (SCC) and urinary gonadotropin fragment (UGF) genes in the EC cells (Fig 2.). Contrasted to rutin, 25 ug/mL (R25), tocopherol-α, 5 ug/mL (TP5) and the extracts (100, 500, 1000 ug/mL) effectively down-regulated SCC (Fig 2.). At 1000 ug/mL, the SCC level was lower about two times than those of TP5 (Fig 2.). In UGF levels, the

extracts (100, 500, 1000 ug/mL) down-regulated the levels, and notably, under the 1000 ug/mL, the gene was down-regulated strongly in EC cells (Fig 2.).

Compared with the expression of the markers, all substances down-regulated the markers in EC cells (Fig 3.). In the CK8 levels, R25 down-regulated two times higher than those of TP5, and under all extracts, the levels were four times higher than those of TP5 (Fig 3.a, d). In Muc-16, although TP5 and R25 down-regulated slightly, at AH 1000 ug/mL, the level down-regulated strongly the level in EC cells (Fig 3. b, d). Unlike R25 down-regulated slightly, the vimentin levels, at TP5 and AH 1000 ug/mL, were 1.28 and 5 times lower than those of R25, respectively (Fig 3.c, d).

Significantly, the extracts (100, 500, 1000 ug/mL) and TP5 inhibited the migration of EC (Fig 4a.). After the 36h, unlike the migrant activity of EC was enhanced firmly in control, the migration under TP5 and the extracts (100, 500, 1000 ug/mL) were inhibited intensely in the EC cells (Fig 4a.). Compared with the inhibitory areas among all conditions, at AH 1000 ug/mL, the value was seven times higher than the control, and at AH 100, 500 ug/mL, the values were about two times higher than those of TP5. Likewise, the vimentin result and the value in R25 were not different from those of the control (Fig 3d and Fig 4a.). Interestingly, at only one dose, AH 1000 ug/mL, EC cells' invasive activity was suppressed strongly, and the activity is 1.56 times higher than those of the control (Fig 4b.).

In considering metabolic modulation, the substances affected mitochondrial membrane potential (MMP) and cellular senescence. Without TP5, although R25 attenuated MMP at 1.5 times lower than in control, the inactive intensities of AH 500 and 1000 were more strongly lower than R25. (Fig 5a.). The senescence of the cancer cells was activated strongly at all substances. Notably, the activity at AH 1000 ug/mL was about 3.45 and 2.1 times higher than those of TP5 and R25, respectively (Fig 5b.).

Under the three bioactive substances, the induced exosomes were isolated from EC cells (Fig 6a.). The EC cells were exposed to the exosomes down-regulated the markers, including SCC, UGF, and IL2-receptor

(Fig 6b). Additionally, EIE down-regulated drug-resistant associated genes, including *NF-κB (P50, P52), mTORC2, and ABCB1* (Fig 6c.). Furthermore, the exosomes by the extract and TP down-regulated Muc-16+vimentin+ cells count at 0.37 and 0.45 times, respectively (Fig 6d). The clustering heatmap displayed patterns of significantly altered miRNAs based on analytic results for miRNAs in the induced exosomes by three substances (Fig 7a). Unlike the heatmap pattern by rutin, the heatmap was dramatically altered at AH1000 (Fig 7a). Moreover, the miRNA distribution between AH1000 and control on the scatter plot was the most different, wherein 112 and 102 miRNAs in EIE were up and down-regulated in EC cells (Fig 7b, c). Although some miRNAs by rutin were altered in various categories, the miRNAs in the categories were down-regulated at AH1000 and TP5 (Fig 7d). Several miRNAs associated with five biochemical categories were dramatically altered in EIE (Fig 8 and Table 2). Based on the results (Fig 8 and Table 2), EIE modulated specific bioactivities, including autophagy, drug transporting and carbolic process, cell migration, endocrinal metabolism, cellular respiration, and immune response. Notably, the level of hsa-miR-423-5p and hsa-miR-1908-5p were 100 times lower than those of control (Figure 8, Table2). Moreover, the dramatic alteration of miRNAs was involved in several categories (Fig 8 and Table 2).

Discussion

EC is the most popular malignancy in gynecological cancers, and the malignant tumor is associated with obesity [23]. The excess of estrogen causes EC, insulin resistance, and inflammation-driven by obesity, and the incidence rate of EC has been increasing in parallel with obesity[24]. This study documented the suppression of cancer activity by three substances to compare the relative efficacy of the extract and functional alteration of the induced exosomes in EC cells. Compared to tocopherol-α and rutin, the extract contained two significant characteristics. One is to suppress various cancer activities, including cancerous metabolism, migration, and invasiveness. Another is to induce a substantial alteration of miRNAs in EIE (Fig 1.). Unlike tocopherol-α and rutin, the extracts significantly down-regulated SCC and UGF,

particularly at AH1000 (Fig 2.). SCC bind to carbonyl reductase, which inhibits malignant behavior and TGF-β signaling in uterine cancer cells[25].

Additionally, SCC inhibits cellular apoptosis cells by irradiation, natural killer cells, an anticancer drug, irradiation, and promoting migration and invasion by decreasing E-cadherin [26, 27, 28]. UGF is known as the β-subunit of human chorionic gonadotropin (hCGβ), which also suppresses cellular apoptosis[29] and promotes migration, invasion, malignant transformation, and drug resistance[30, 31, 32]. These results suggest that the AH1000 effectively suppressed EC cells' malignant behaviors, and the extract plays good material for preventing malignant behavior and cancerous transformation of normal endometrial cells. These predicted functions were shown by the results in biological categories, including migration, invasion, and expression of malignant markers, cellular senescence, and mitochondrial membrane potential (Fig2-5.).

The extract and EIE modulate tumor microenvironments around EC cells through their effects on the biological five categories.

First, in migration and invasion, tocopherol-α and the extracts significantly inhibited the EC cells' malignant behaviors (Fig3.) In particular, the induced exosomes by AH1000 contained the inhibitory migration miRNAs such as hsa-miR-1908-5p and hsa-miR-20b-5p. Interestingly their levels were 100 times and 8.2 times higher than those of the control (Table 2 and Fig 7, 8.). Target genes of hsa-miR-1908-5p and hsa-miR-20b-5p are prostaglandin endoperoxide synthase 2 (*PTGS2*) and vascular endothelial growth factor A (*VEGFA*), respectively. The PTGS2 protein level is modulated by beta-2 adrenergic receptor (ADRB2) receptor signaling, and silencing PTGS2 suppresses migration and invasion of ovarian cancer cells[33]. VEGF protein is associated with critical roles, including migration, invasion, angiogenesis, endothelial cell proliferation, invasion, and migration of cancer cells[34]. Under EIE (Fig. 6), malignant markers, including SCC, UGF, interleukin-2 receptor (IL-2), Muc-16, and vimentin, were more strongly down-regulated in EC cells. These results showed that the two miRNAs in the induced exosome strongly suppress migration and invasion of the exosomes' peripheral EC cells.

Second, in cellular senescence, the three substances activated the senescence in EC cells, and the senescence was intensely triggered under AH1000 (Fig. 5). Based on miRNAs profiling in the induced exosomes, specific miRNAs such as autophagy suppression and DNA repair were up-regulated under AH1000 (Table 1). Interestingly, anti-Unc-51-like kinase 1 (ULK1)[35], hsa-miR-423-5p was down-regulated 100 times, and several anti-DNA repair genes hsa-miR-93-3p (Table 1) were up-regulated 8.1 times. The

exosomal results corresponded with the senescence results (Fig. 5), and the extract activated cellular senescence through activation of cytotoxic autophagy and suppression of DNA repair. Further, the induced exosomes contained potency for cellular senescence acceleration through suppression of DNA repair in EC cells. As described in this study (Table 1), hsa-miR-615-3p and hsa-let-7e-5p were up-regulated in the induced exosomes. The miRNAs targeted several proteins associated with DNA repair.

Regarding activity for mitochondrial membrane potential, at AH 500 and 1000, the activity significantly decreased, and the value was 2.92 times lower than in control at AH 1000. With the exosomal results, anti-ATP5A1 and ATP5I hsa-miR-877-3p were up-regulated at 6.7 times (Table 1). Documented researches[36, 37], *ATP5A1*, *ATP5I* genes activate ATP synthesis on the electron transporting system in mitochondria. Although cancerous activity is based on ATP synthesis activation, the extract suppresses ATP synthesis by inhibiting mitochondrial membrane activity.

Third, in reproductive cancer activity, miRNAs in EIE affect endocrinal modulation, such as estrogen and prostaglandin synthesis and signaling. Anti-*HSD17B12* hsa-miR-874-3p was significantly down-regulated but anti-*STRN3* hsa-miR-125b-5p, anti-*PHB2* hsa-miR-10a-5p and anti-*ISL1* hsa-let-7d-5p were significantly down-regulated in EIE (Table 1 and Fig. 8). Unlike HSD17B12 protein, estradiol synthesis enzyme[38], STRN3, PHB2, and ISL1 are associated with negative regulation of estrogenic intracellular signaling[39, 40, 41]. Likewise, excess of estrogen drives several diseases, including diabetes mellitus and gynecological cancers; these endocrinal modulations mean that EIE prevents carcinogenesis and cancer deterioration by the hormones. Furthermore, *PTGS2* targeting hsa-miR-1908-5p was strongly increased in EIE. Prostaglandin-endoperoxide synthase 2 (PTGS2) protein known as cyclooxygenase-2 (COX-2) synthesizes prostaglandin from arachidonic acid; the synthesized prostaglandin contributes to tumorigenesis including proliferation, metastasis, angiogenesis[42]. The dramatic increase of hsa-miR-1908-5p in EIE means a strong suppression of tumorigenesis by attenuated COX-2 in peripheral EC cells.

Fourth, the extract and EIE were involved in attenuating drug resistance and autophagy in EC cells. Under the extract, ha-miR-451a targeting *ABCB1* was increased in the induced exosomes. When exposed to an anti-cancer drug, cancer cells alter their phenotypes to resist the drug by increasing ATP binding cassette subfamily B member 1 (ABCB1) protein[43]. Furthermore, the expression of ABCB1 is modulated by various transcription factors, including P53[44], NF-κB[45], and YB-1[46]. Contrasted to the alteration of hsa-miR-451, hsa-miR-423-5p, hsa-miR-19b-3p, hsa-miR-320a in EIE were dramatically decreased, and notably, hsa-

miR-423-5p was 100 times lower than those of the control. Interestingly, corresponded to the results for miRNA profiling (Table 2), EIE down-regulated drug-resistant genes, including *NF-κB (P50, P52), mTORC2, and ABCB1* (Fig 6c.). Activated *NF-κB (P50, P52)* and *mTORC2* induce multidrug resistance[47] [48]and suppression of autophagy[49, 50]. These results documented that EIE intensely causes enhancing of autophagy and attenuation of drug resistance in around EC cells.

Fifth, the down-regulated hsa-let-7e-5p contributed to suppressing innate immune response by up-regulation of POLR3D protein, a subunit of RNA polymerase[51]. POLR3D activates interferon-β (IFN- β) production associated with the down-regulation of inflammatory responses [52]. In correspondence with the described research[20], the extract attenuates inflammatory response through EIE from EC cells.

Consequently, *Aurea Helianthus* extract intensely contributed to the modulation of tumorigenic microenvironments in EC cells. Although α-tocopherol and rutin were high components in *Aurea helianthus*[20], the extract effectively modulated the environment than the two substances. The extract's modulations were summarized in five biological categories: suppression of migration and invasion, increasing drug susceptibility, decreasing inflammation, attenuation of reproductive cancer activity, and activation of cellular senescence. The induced exosome effects suggest a biological cancer drug's potency to protect and heal gynecological cancers.

Materials and methods

- Cell culture

Human endometrial cancer cells (ATCC HTB115, VA, USA) were purchased and cultured in Dulbecco's Modified Eagle Medium (Gibco, Massachusetts, USA) containing high glucose. After ripening at 10 °C for 48 h, the flower of *A. helianthus* was exposed to infrared ray for 2 h and ground (400 mesh). The powder was extracted and concentrated under 0.08 MPa, 70 °C for 2 h, and filtered with a 0.2μm syringe filter (Nalgene, Massachusetts, USA). After treating of dosages; the extracts (100, 500, 1000 μg/mL) supported by Life Together (Gangwon-Do, Korea), tocopherol (Sigma, MO, USA) 5 μg and rutin (Sigma) 25ug, the endometrial cancer cells were cultured for 72 h at 37 °C, under 5 % CO2.

- Quantitative PCR

Total RNA in cells exposed to the three substances were isolated using the RiboEx reagent (GeneAll, Seoul, Korea). After synthesizing cDNA using Maxime RT PreMix (iNtRON, Seongnam, Korea), quantitative PCR was performed with primers (Table 1) at the cycling parameters: 1 min at 95℃, followed by 35 cycles of 35 s at 59℃ and 1 min at 72℃ for 35 cycles.

- Flow-cytometry analysis

To analyze the markers' expression levels, including CK8, Muc-16, and vimentin, all types of cells fixed with 2 % paraformaldehyde for 24h were treated with 0.02 % Tween 20 for 5 min. The fluorescence leveled three immunoglobulins, FITC anti-cytokeratin 8 (Abcam, Cambridge, UK), PE-anti-CA125 (Santacruz, CA, USA), and APC-anti-vimentin (Santacruz) were treated to the cells for 48h in room temperature, 85 rpm agitating. The washed samples were analyzed using a flow cytometer (BD FACScalibur) and FlowJo 10.6.1 (BD science).

- Migration test

In estimating the three substances' inhibitory efficacy for migration, EC cells were cultured in the Radius™ 24-well cell migration assay kit (CELL BIOLABS, INC. San Diego, CA, USA) under the substances. The migration was estimated with time intervals (0, 12, 24, 36, 48, 60 hrs) using Nikon ECLIPSE Ts2 (Tokyo, Japan) and imaging software Nikon NIS Elements V5.11.

- Invasiveness test

In estimating the three substances' inhibitory efficacy for invasiveness, EC cells were culture in CytoSelect™ 24-well cell invasion assay kit (CELL BIOLABS, INC. San Diego, CA, USA) under the

substances. The invasiveness was estimated using a flow cytometer (BD FACScalibur) and FlowJo 10.6.1 (BD science).

- Mitochondrial membrane potential test

After exposed to the three substances for three days, EC cells were stained with JC-1 Mitochondrial Membrane Potential Assay Kit (Invitrogen, MA, USA), and mitochondrial activity was estimated using a flow cytometer (BD FACScalibur) and FlowJo 10.6.1 (BD science).

- Senescence test

After exposure to the three substances during three days, EC cells were stained with CellEvent™ Senescence Green Flow Cytometry Assay Kit (Invitrogen, MA, USA). The stained cells were measured using a flow cytometer (BD FACScalibur) and FlowJo 10.6.1 (BD science).

- Exosome purification and microRNA profiling

The semi-confluent EC cells were cultured for three days under the substances without FBS. Their supernatants were collected, and the induced exosomes in the supernatants were isolated using the exoEasy Maxi kit (QIAGEN, Venlo, Netherlands). The induced exosomes from the sample size (n=3) were summated for their miRNAs profiling under each substance. Small microRNAs were sequenced by ebiogen Inc (Seoul, Korea) to analyze exosomal functions. Agilent 2100 bio-analyzer and the RNA 6000 PicoChip (Agilent Technologies, Amstelveen, The Netherlands) were used to assess RNA quality. RNA quantification was assessed using a NanoDrop 2000 Spectrophotometer system (ThermoFisher Scientific, Waltham, MA, USA). The Agilent 2100 Bio-analyzer instrument for the High-sensitivity DNA Assay (Agilent Technologies, Inc., USA) to prepare and sequence small RNA libraries, and NextSeq500 system

single-end 75 sequencings (Illumina, San Diego, CA., USA) were used for them. In obtaining a bam file (alignment file), the sequences were mapped by bowtie2 software, and read counts were extracted from the alignment file using bedtools (v2.25.0) and R language (version 3.2.2) to determine the expression level of miRNAs. miRWalk 2.0 was used for miRNA target study, and ExDEGA v.2.0 was used to deduce various results, including van diagram, heat map, scattering plots, and bar graphs.

- Statistical analysis

All experiments were performed thrice (n = 3), and the data were analyzed by paired T-test and analysis of variance (ANOVA), using the SPSS software v26 (IBM, NY, USA).

- Data availability

The datasets generated and/or analyzed during the current study might be availed from the corresponding authors, on reasonable request.

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Author contributions

Boyong Kim and Yoonjin Park: conceived this study and designed the experiments. Boyong Kim and Seung Gwan Lee: conducted the experiments. Kyunghwa Lee, Suhng Wook Kim, and Min woo Lee: analyzed the results.

Competing interests

The authors declare no competing interests.

Table 1. List of primers for quantitative PCR

Gene	Primer Sequence	
SCC (squamous cell carcinoma antigen)	F	ACACTGGTTCTTGTGAACGC
	R	GCCTGTACATCCTTGTTTGGC
UGF (urinary gonadotropin fragment)	F	ATGTGCGCTTCGAGTCCATC
	R	AGAAAGACCCGCCAGAGTGC
Il-2 receptor (interleukin-2 receptor)	F	CAGTTCGCCGCATCCTTCT
	R	TAAGTATTGGGCTGGCGTGT
mTORC2	F	AGTACGAGGGCGGAATGACA
	R	GCCACCACCTCTGGATTCTG
NF-κB(P50)	F	CGGAGCCCTCTTTCACAGTT
	R	TTCAGCTTAGGAGCGAAGGC
NF-κB(P52)	F	AGGTGCTGTAGCGGGATTTC
	R	AGCGGCACTGTATAGGGCAGA
ABCB1	F	TCTTGTCCAAACTGCCTGTGA
	R	TAATTGTGCCTCACCCCACC
GAPDH	F	AGTCTGTTATAACCCAGACGAG
	R	GCATCACAACCAATAGGTGTGA

Table 2. List of significant alteration of miRNAs in the induced exosomes by the extract and their targeted genes

Target gene Category		miRNA Symbol	Fold Change	Target Gene
Ⅰ	Positive regulation of cell migration involved in sprouting angiogenesis	hsa-miR-1908-5p	100	PTGS2
		hsa-miR-20b-5p	8.2	VEGFA
Ⅱ	Positive regulation of autophagy	hsa-miR-423-5p	100	ULK1
		hsa-miR-19b-3p	25	PRKAA1
		hsa-miR-320a	20	ULK1
	Positive regulation of DNA repair	hsa-miR-93-3p	8.1	NPM1, OTUB1, PARP1, SMC1A, TRIM28
		hsa-miR-877-3p	6.7	SUPT16H, TRIM28 HMGB1
	Mitochondrial ATP synthesis coupled proton transport	hsa-miR-877-3p	6.7	ATP5A1, ATP5I
Ⅲ	Estrogen biosynthetic process	hsa-miR-874-3p	7.2	HSD17B12
	Negative regulation of intracellular estrogen receptor signaling pathway	hsa-miR-125b-5p	10	STRN3
		hsa-miR-10a-5p	6.9	PHB2
		hsa-let-7d-5p	6.8	ISL1
	Positive regulation of prostaglandin biosynthetic process	hsa-miR-1908-5p	100	PTGS2
Ⅳ	Drug transmembrane transport	hsa-miR-451a	4	ABCB1
Ⅴ	Regulation of innate immune response	hsa-let-7e-5p	12.5	POLR3D

Up-Regulation **Down-Regulation**

Figure Legends

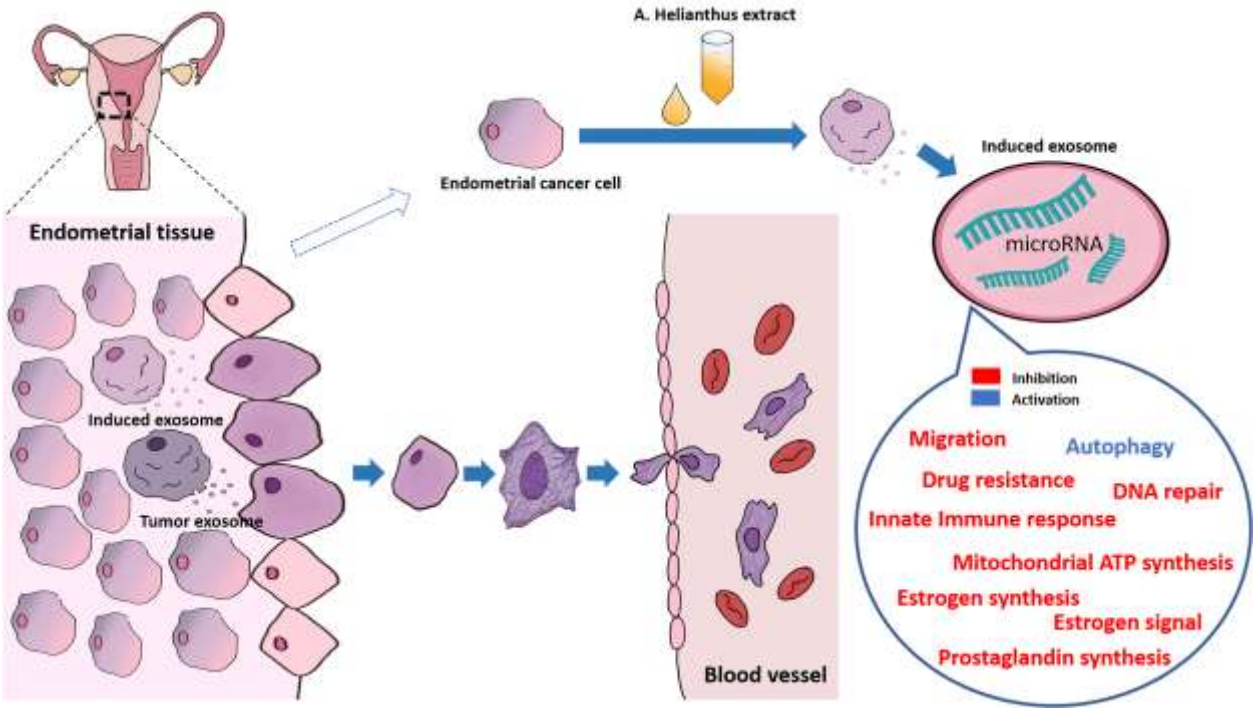


Figure 1. Schematic working flow for the research

Exposed to the *A. helianthus* extract, the endometrial cancer cells produced specific exosomes containing microRNAs involved in significant bioactivities.

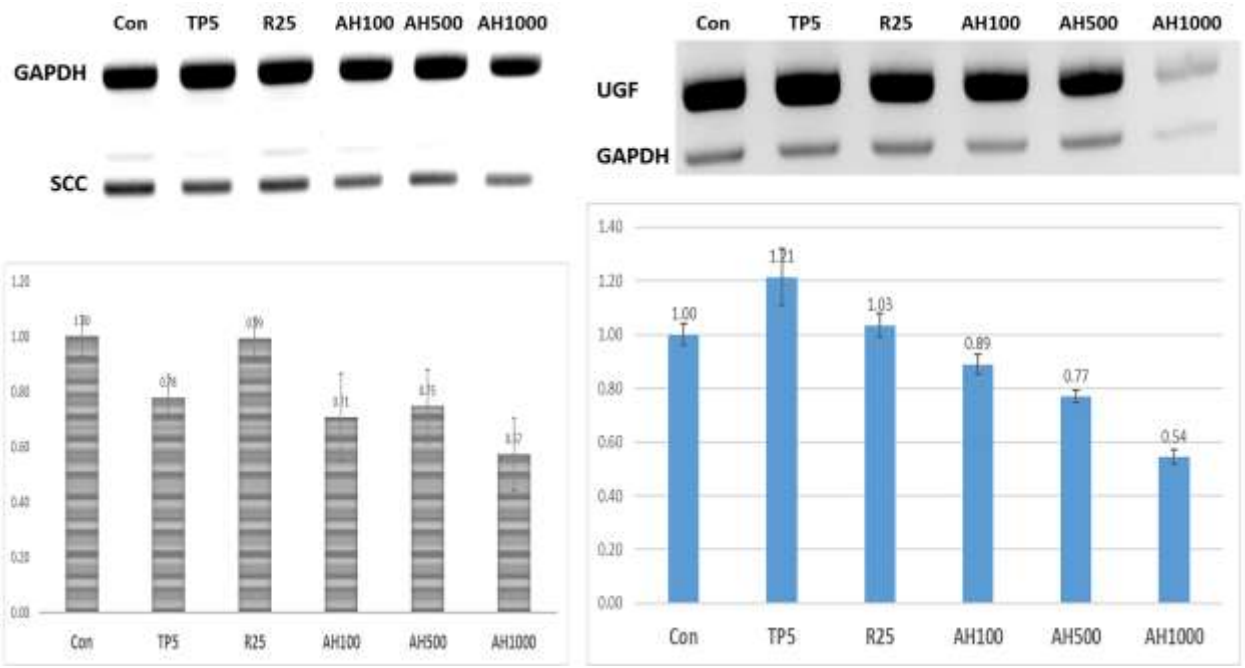


Figure 2. Expression levels of endometrial cancer cell markers under bioactive substances.

Exposed to the bioactive substances including tocopherol-α, 5 μg/mL (TP5), rutin 25 μ g/mL (R25) and *A. helianthus* extract (AH) 100, 500, 1000 μ g/mL, the markers of the endometrial cancer cells were down-regulated. squamous cell carcinoma (SCC), urinary gonadotropin fragment (UGF). ($P < 0.05$)

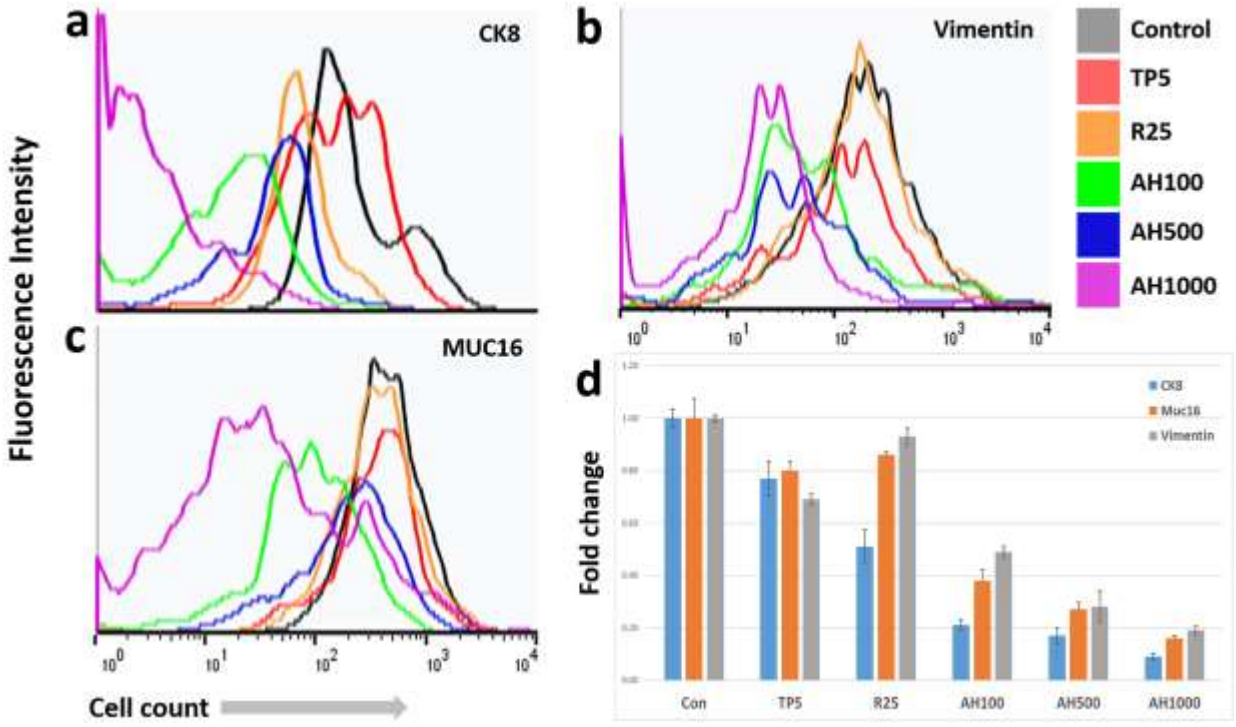


Figure 3. Expression of cancerous markers in endometrial cancer cells under bioactive substances

The CK8^{+}, Muc16^{+} and Vimentin^{+} cells were counted by a flowcytometer. Tocopherol-α, 5 μg/mL (TP5), Rutin 25 μ g/mL (R25) and *A. helianthus* extract (AH) 100, 500, 1000 μ g/mL. ($P < 0.05$)

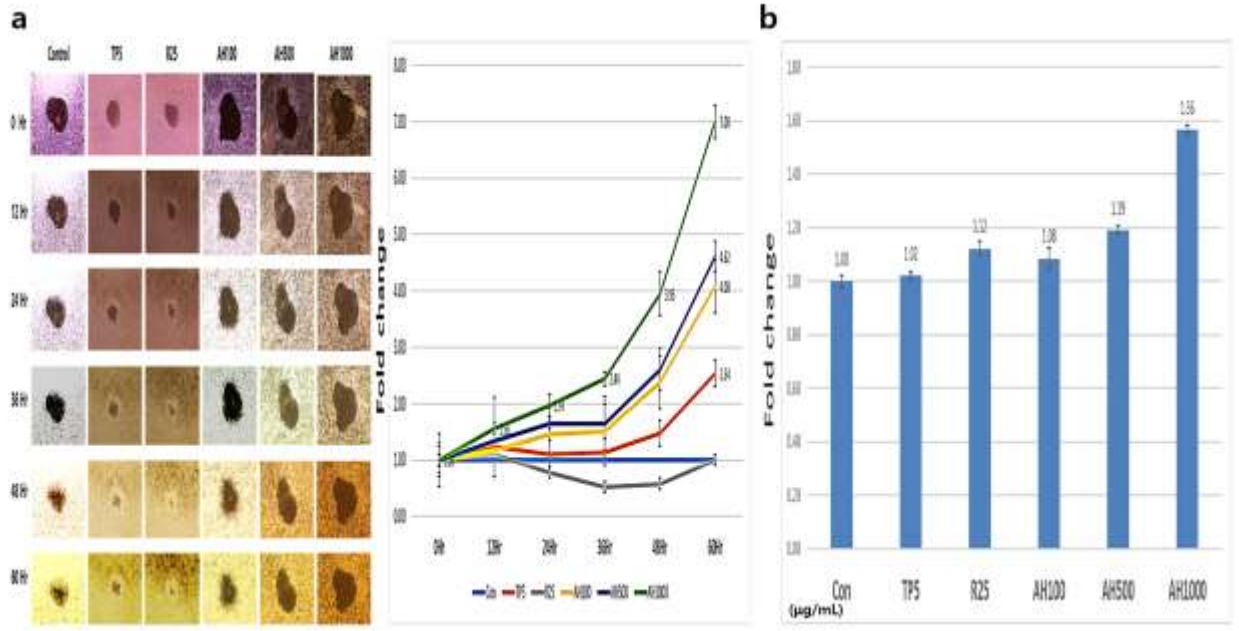


Figure 4. Metastatic and invasive inhibition of endometrial cancer cells by bioactive substances

a. The areas without migrated cells were measured with Nikon software and the measured values indicated the relative fold changes. b. The bar graphs displayed non-invasive cells under the substances, tocopherol-α, 5 μg/mL (TP5), rutin 25 μ g/mL (R25) and *A. helianthus* extract (AH) 100, 500, 1000 μ g/mL. ($P < 0.05$)

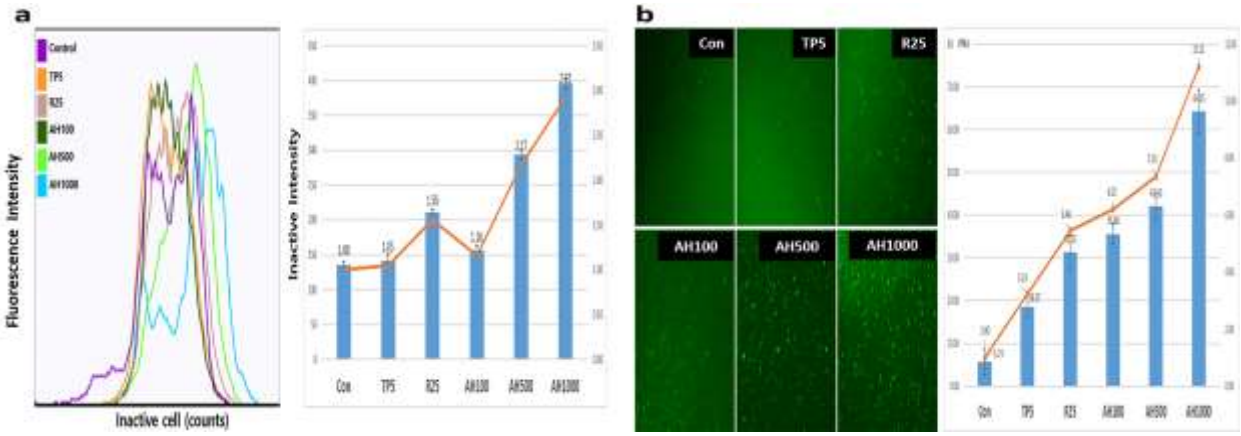
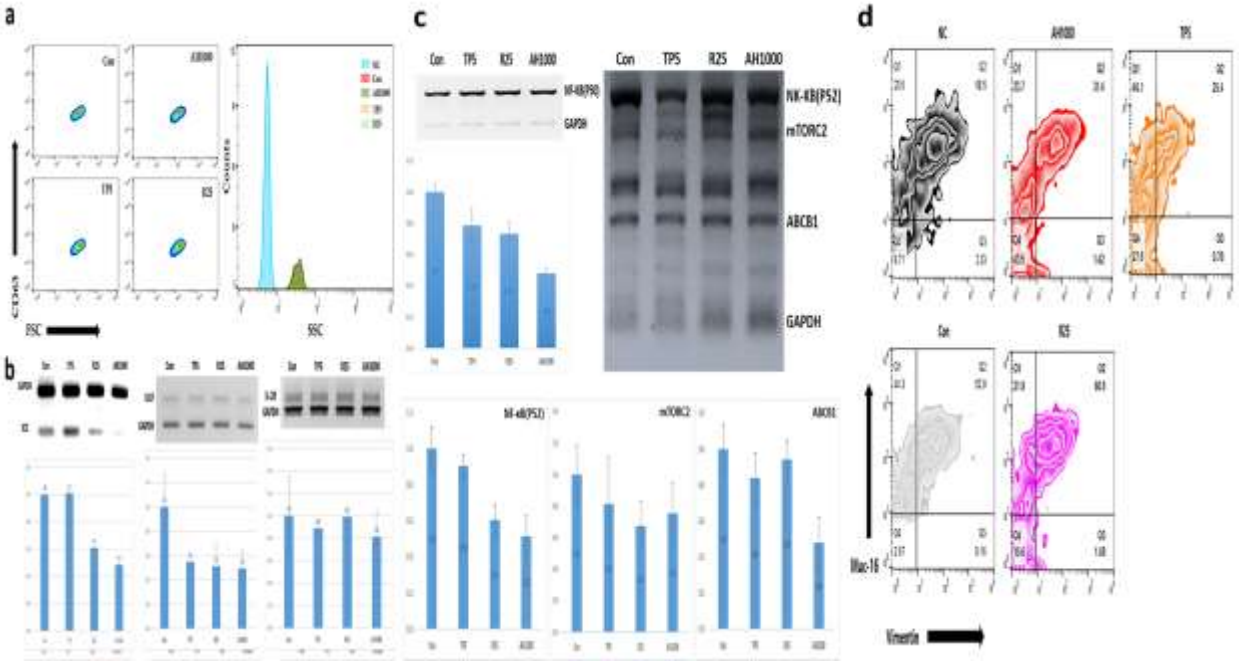


Figure 5. Effects of bioactive substances for mitochondrial membrane potential and senescence on endometrial cancer cells

a. The specific fluorescence was stained in the inactive cells. Tocopherol-α, 5 μg/mL (TP5), Rutin 25 μ g/mL (R25) and *A. helianthus* extract (AH) 100, 500, 1000 μ g/mL. b. The labeled cells indicated senescence cells. Tocopherol-α, 5 μg/mL (TP5), Rutin 25 μ g/mL (R25) and *A. helianthus* extract (AH) 100, 500, 1000 μ g/mL. ($P < 0.05$)



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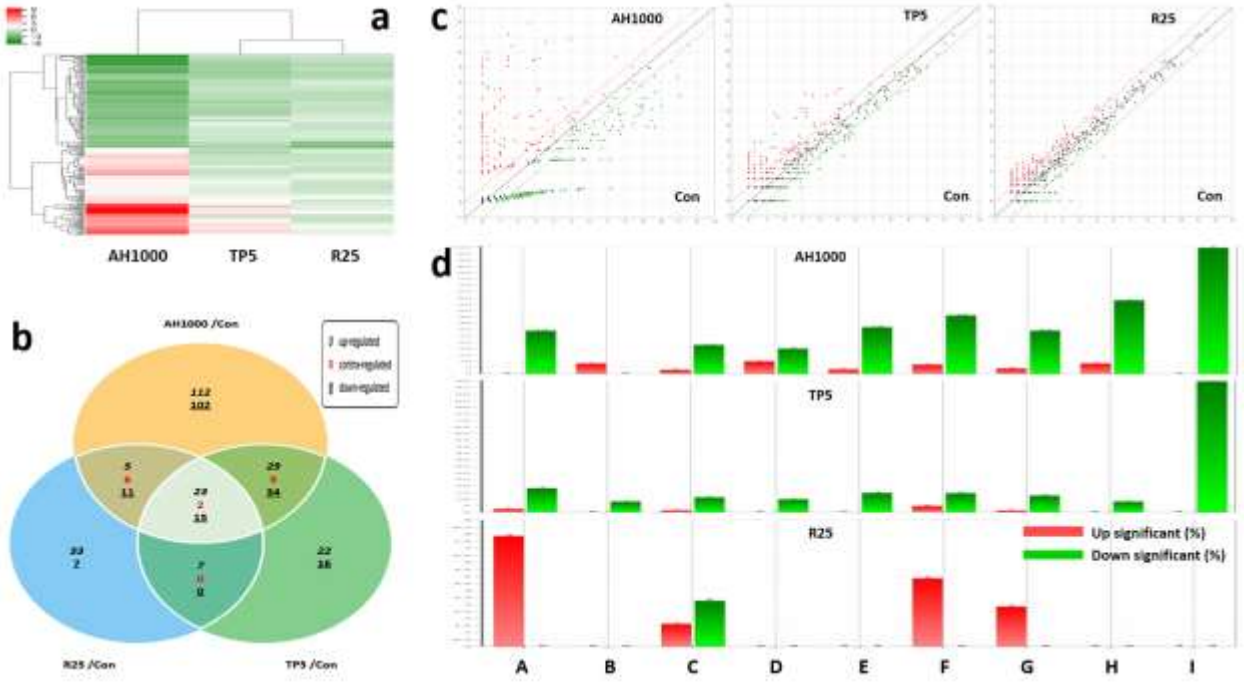

Figure 7. Profiling of miRNAs in the induced exosomes from endometrial cancer cells

a. The heat map for microRNA in exosomes induced by three substances. b. The van diagram for microRNAs in endometrial cancer cells under the three substances. c. The scattering plots between the control and bioactive substances. d. The microRNAs were involved in several categories (A: positive regulation of autophagy,B: drug transmembrane transport,C: positive regulation of cell migra tion involved in sprouting angiogenesis,D: estrogen biosynthetic process,E: positive regulation of prostag landin biosynthetic process,F: mitochondrial ATP synthesis coupled proton transport,G: positive regulati on of DNA repair regulation of innate immune response,H: negative regulation of intracellular estrogen

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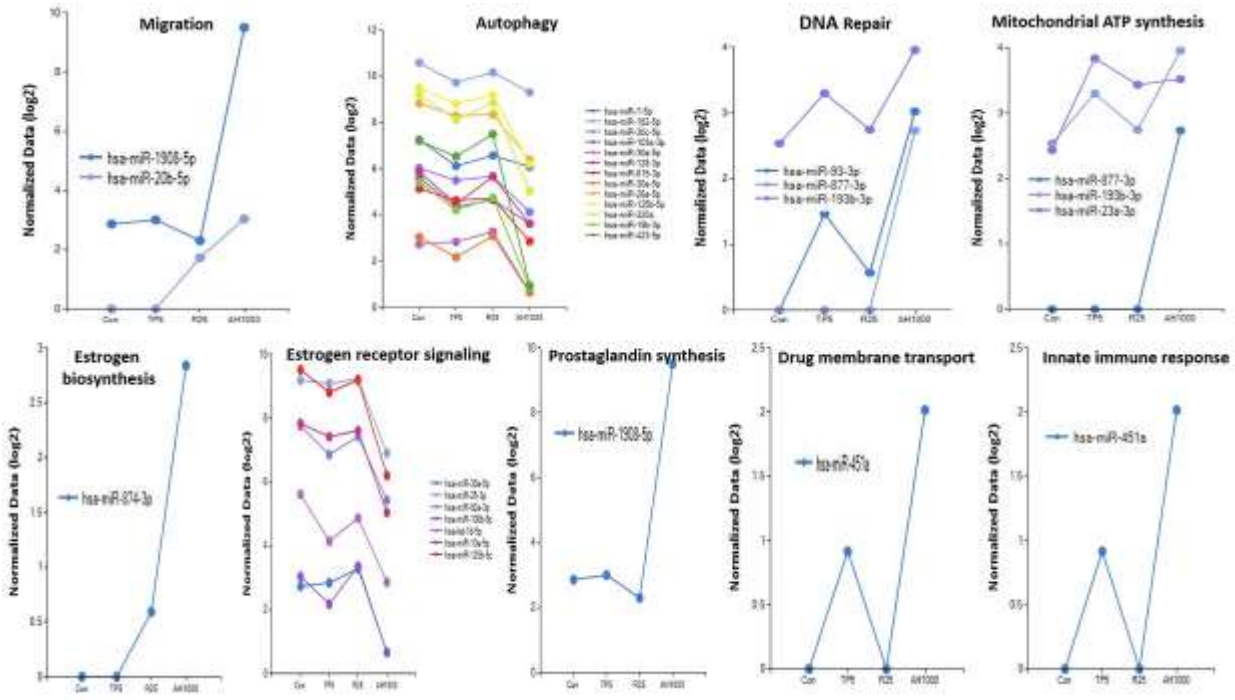

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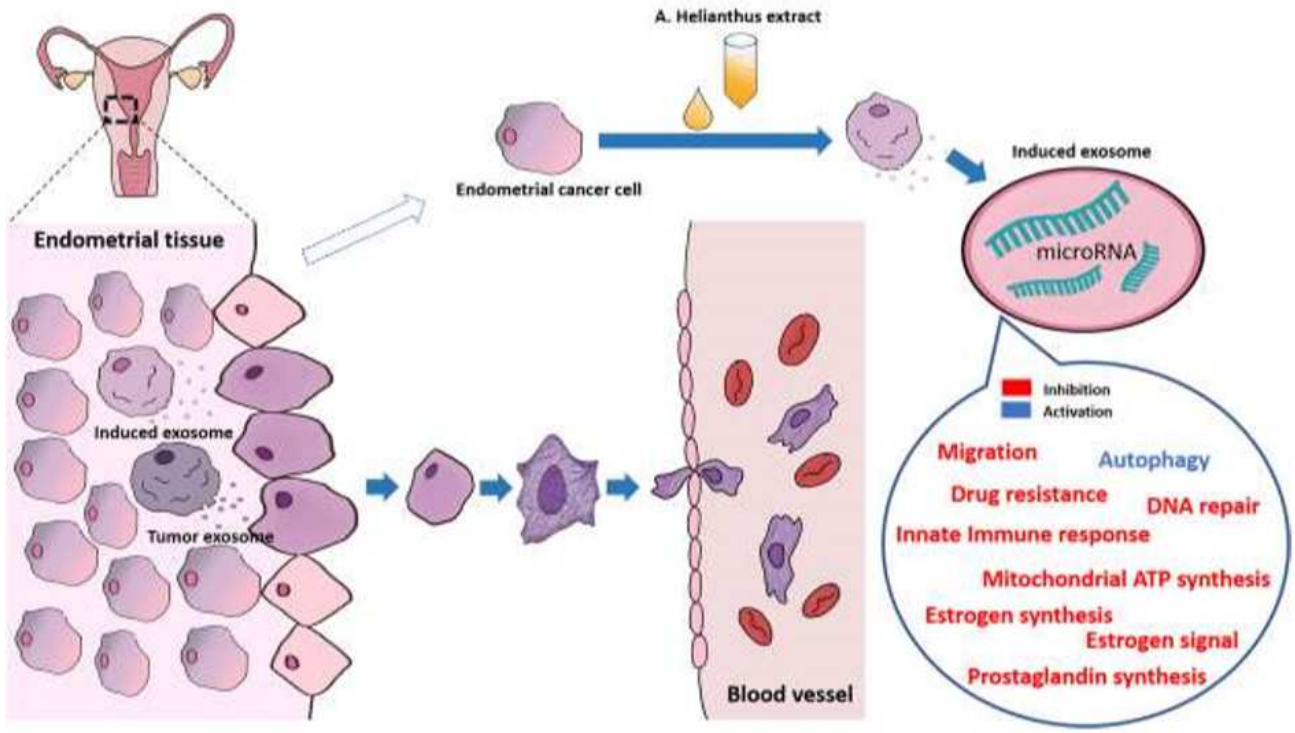
Figures



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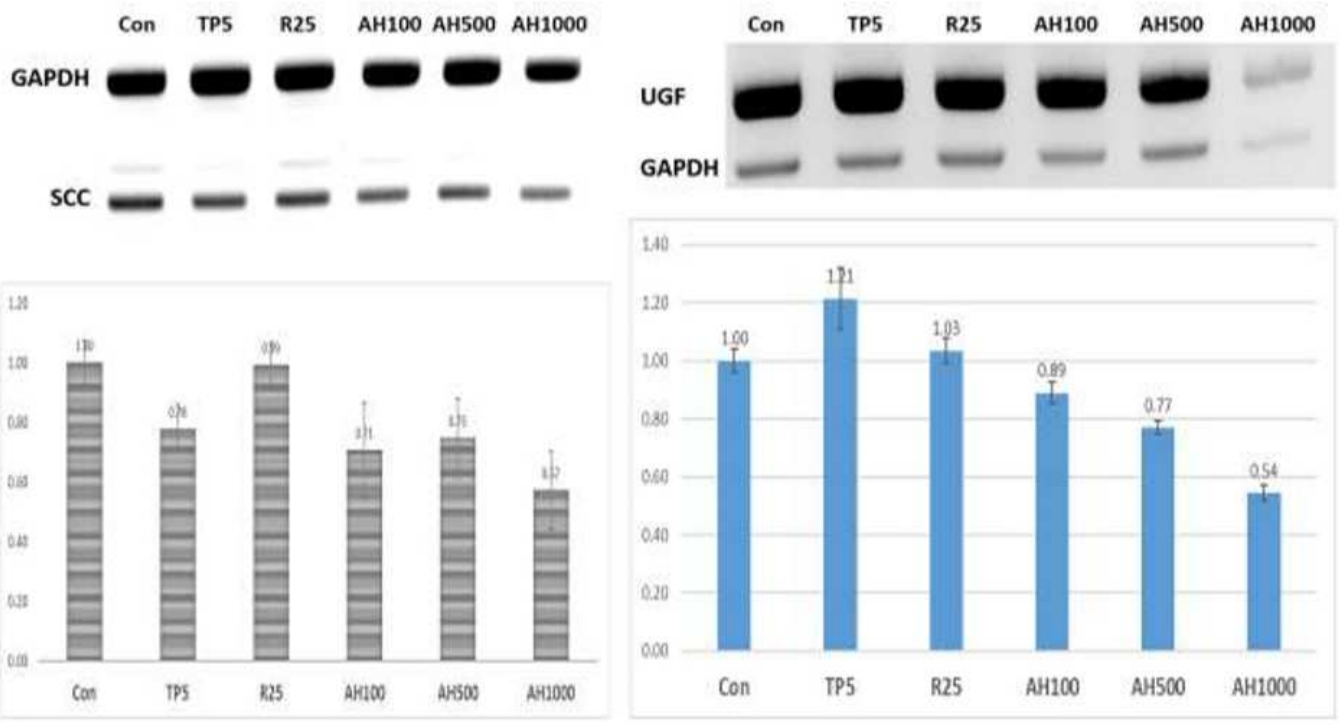

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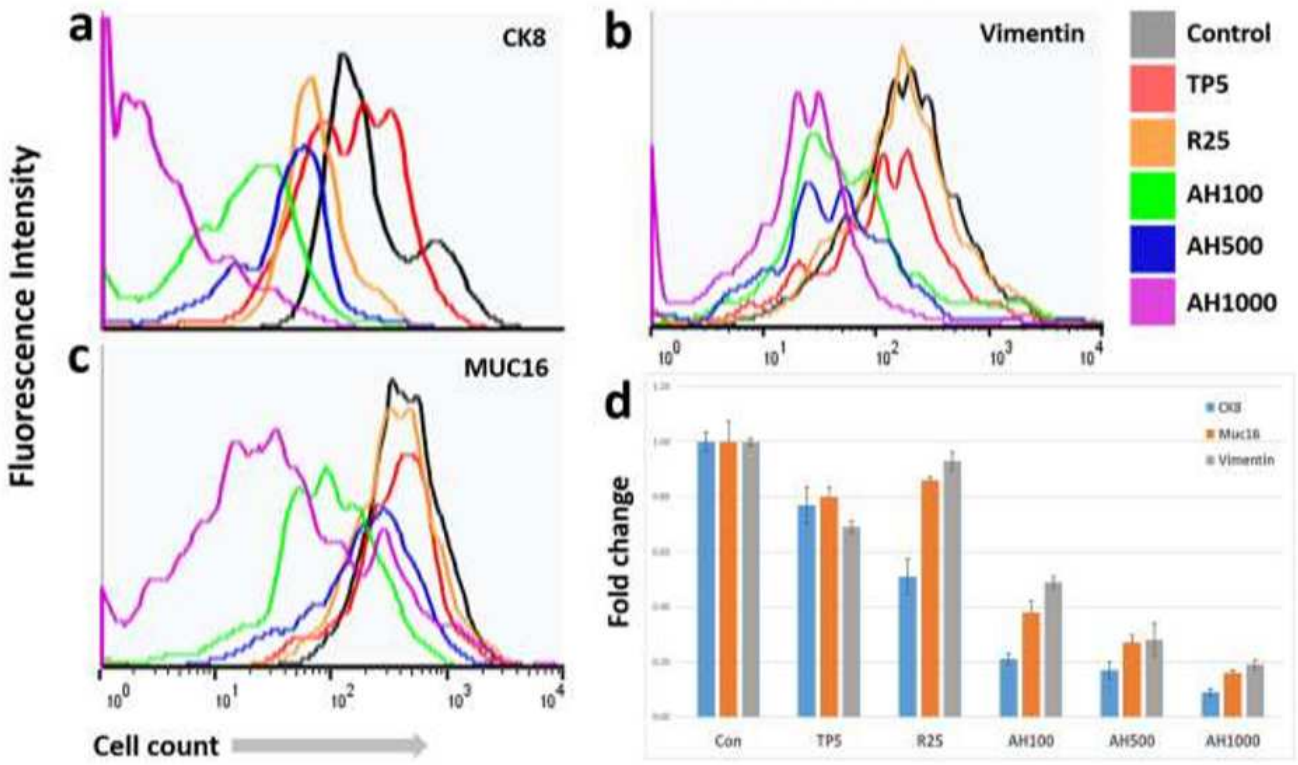

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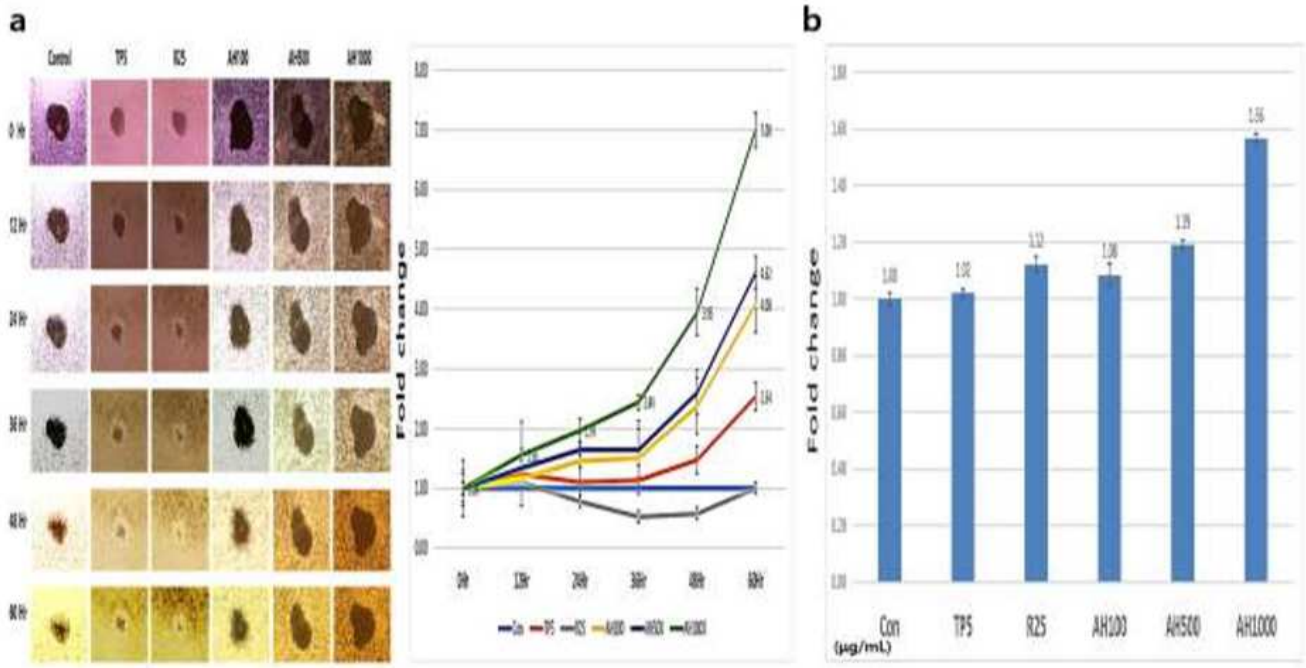

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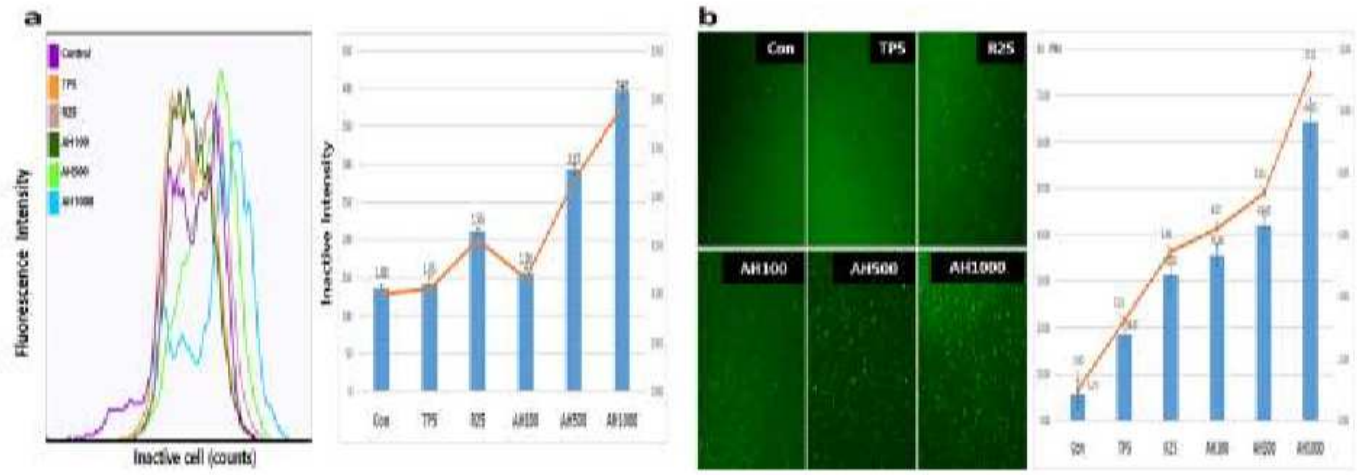

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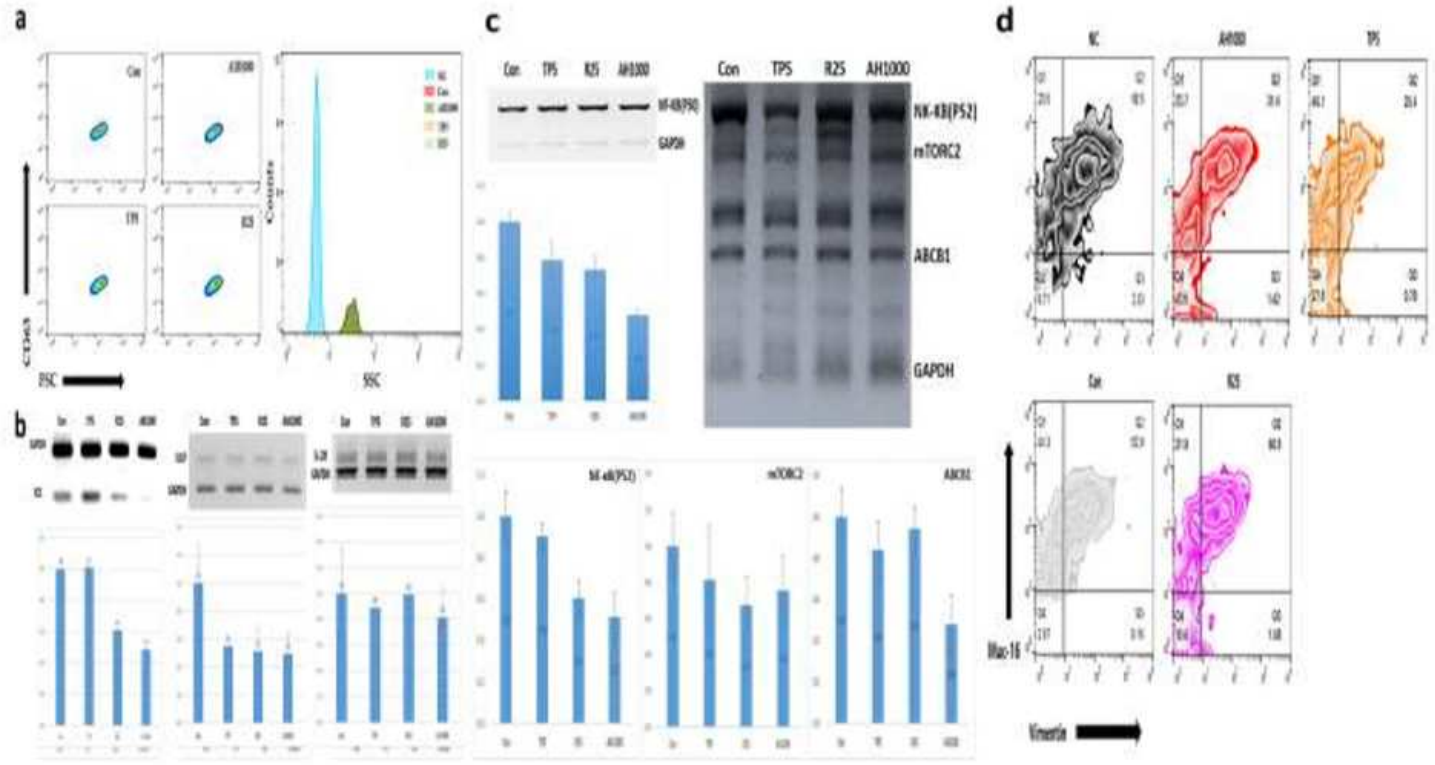

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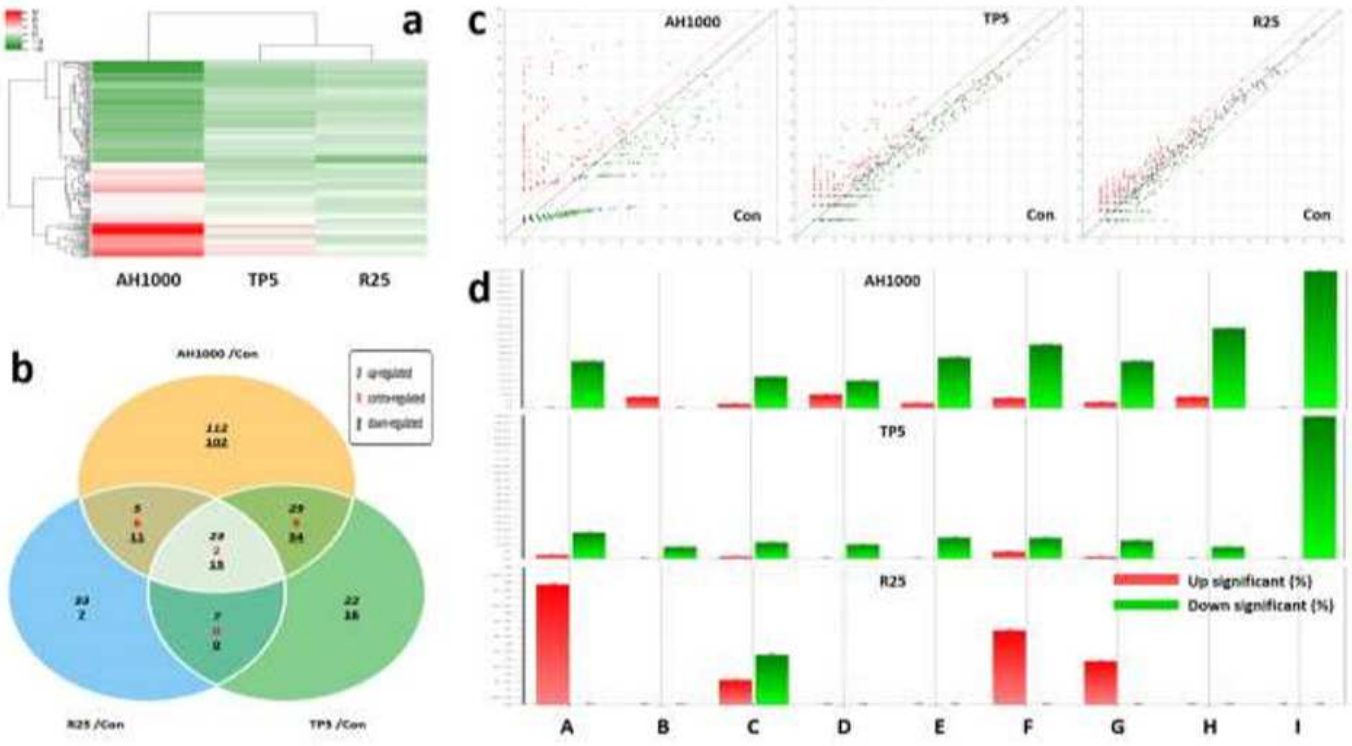

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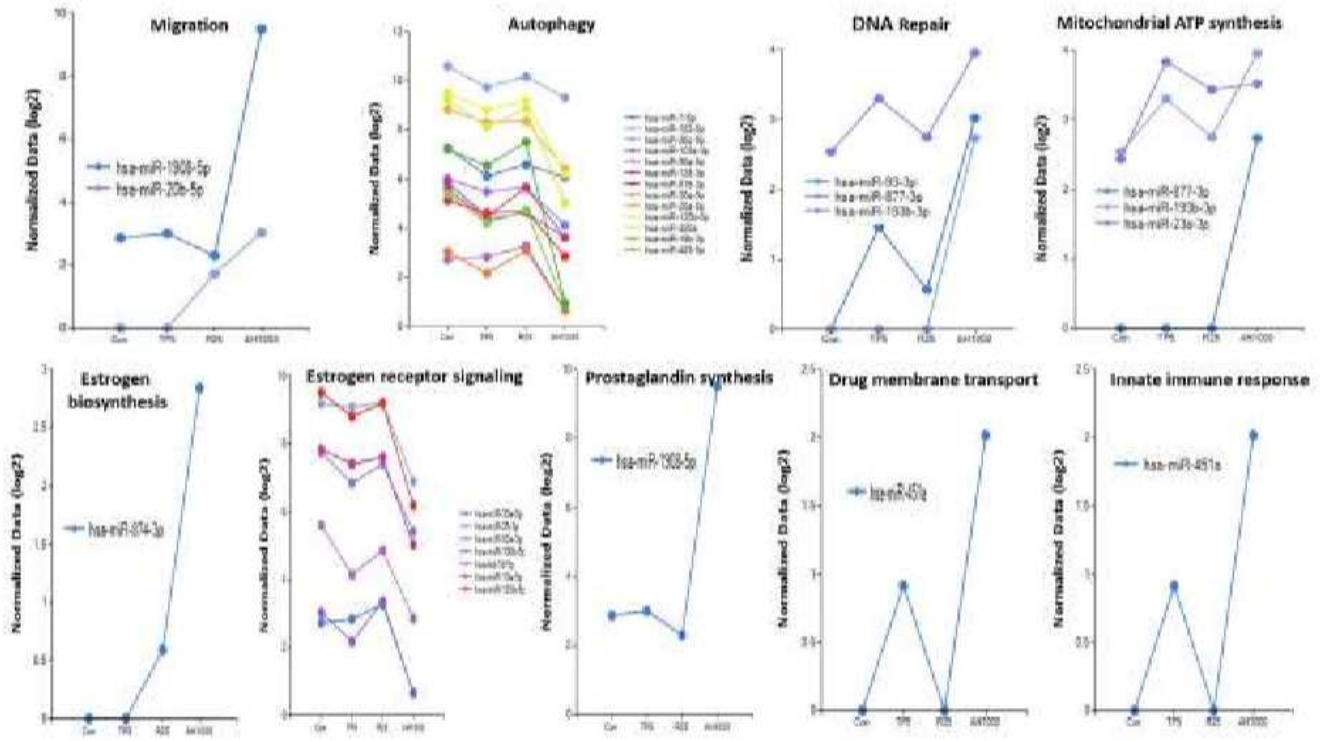

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