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Inhibition activity of *Artemisia vulgaris* L. on Bhas 42 cell transformation

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Several factors can contribute to cancer development. Various methods are in use for cancer treatment, although their complications can be a cause of concern. *Artemisia vulgaris* L. is a medicinal herb proposed for treating several diseases, including cancer. This study investigated the inhibition activity of *A. vulgaris* on Bhas 42, a cell used to evaluate the carcinogenic potential of chemicals due to its ability to recapitulate to some extent of the multi-stage cell transformation process. Ethanolic extract of *A. vulgaris* was used to examine cytotoxicity and transformation inhibition activity on Bhas 42. Bhas 42 cells were induced by a cancer stimulator, 3-methylcholanthrene (MCA) or cancer promotor, 12-O-tetradecanoylphorbol-13-acetate (TPA), with and without *A.vulgaris* at various concentrations and date of addition. *A. vulgaris* can inhibit Bhas 42 cell transformation due to its smaller proportion of the transformed cells than those treated with only MCA or TPA. The most effective transformed foci inhibition condition was selected for proteomic assay. The proteomic study results also showed that the proteins related to the regulation of cancer cells, such as cancer development, proliferation, migration, transformation, and invasion of *A. vulgaris* treated groups, were dysregulated. Studies on the effects of *A. vulgaris* extract showed its ability to inhibit the transformation to cancer cells. However, further studies are required to understand these mechanisms better.

Keywords Cancer, *Artemisia vulgaris* L., Cell transformation assay, Bhas 42, Herbal extract

Cancer is caused by the transformation of normal cells into malignant cells, which then divide uncontrolled and spread into surrounding tissues[1]. Several factors contribute to cancer development, including genetic factors and external factors such as ultraviolet radiation, tobacco smoke, and infections from certain microorganisms[1–4]. In 2020, there was a report that cancer caused nearly 10 million deaths, or nearly one in six deaths globally[5]. In Thailand, the National Cancer Institute reported that the percentages of new cancer patients in 2018 and 2019 were 19.9% and 22.3%, respectively, based on a hospital-based registry[6].

Surgery, radiotherapy, and chemotherapy are the main methods used for cancer treatments[7]. However, some disadvantages of these methods, particularly the adverse effects on patient quality of life, are recognized. Developing cancer prevention methods, including immunotherapy such as cancer vaccines, remains challenging for researchers[8]. Medicinal herbs have been shown to play a role in cancer prevention and therapy due to their richness of bioactive compounds, and they are being applied integrated with conventional treatments[9].

Artemisia vulgaris L. is a plant utilized to relieve stomach pain, treat gastric ulcers, liver inflammation, seizure, and neonatal jaundice in Chinese traditional culture, combined with acupuncture treatment[10]. The crucial

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role of *A. vulgaris* in cancer treatment has been studied. Its potential to impact cancer cell growth, survival, and metastasis via specific molecular pathways offers benefits for cancer treatment. However, the study of the chemoprevention mechanisms of *A. vulgaris* has been limited. Therefore, we are interested in exploring whether *A. vulgaris* could inhibit the transformation of normal cells as a preventive measure.

This study investigated the inhibition ability of *A. vulgaris* on Bhas 42 cell transformation, which has been applied to evaluate the carcinogenicity of the chemicals based on its ability to recapitulate to a proportion extent of the multi-stage cell transformation process that takes place as normal cells transform into cancer cells. The transformation of Bhas 42 cell was induced by 3-methylcholanthrene (MCA) and 12-O-tetradecanoylphorbol-13-acetate (TPA), whereas *A. vulgrais* extract was added at various concentrations and time points to identify the most effective inhibition. In addition, proteomic analysis was conducted to identify the possible mechanisms of transformed foci inhibition of *A. vulgaris.*

Materials and methods

A. vulgaris collection and extraction

The *A. vulgaris* leaves were obtained and identified from Sireerukachat Botanical Natural Learning Park, Mahidol University. The voucher specimen was deposited in the Herbarium of Pharmaceutical Botany Mahidol University (PBM No. 005476). Adapted from the study of Abubakar and Haque, air-dried leaves of *A. vulgaris* (100 g) were extracted using 80% ethanol, filtered, evaporated, dissolved with DMSO and stored at -20 °C until use[11].

Identification of major phytochemical components

An Ultrahigh-performance liquid chromatography with diode-array detector quadrupole orbitrap (UHPLC-DAD-Orbitrap) was performed on Vanquish UHPLC system (Thermo Fisher Scientific Inc.) equipped with Thermo Scientific Vanquish- Binary Pump F, Thermo Scientific Vanquish- Split Sampler FT, Thermo Scientific Vanquish- Column Compartment H, and Thermo Scientific Vanquish- Diode Array Detector FG, coupled with Thermo Scientific Orbitrap ExplorisTM 120 mass spectrometer. The separation was done on a BDS Hypersil C18 column (50 × 2.1 mm i.d., 2.4 µm). The mobile phases were (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. A mobile phase time program was set up with gradient elution with 100% A for 2 min, linear increasing from 0 to 100% B in A for 8 min, then 100% B for 2 min. Before each injection, the column was equilibrated with 100% A for 2 min. The column temperature was controlled at 25 °C with a constant 1.0 mL/min flow rate. DAD detection was set at the wavelength of 230 nm. The injection volume setting was 2 µL for all samples.

For the mass spectrometer, mass analysis was done in both positive and negative modes using internal mass calibration EASY-IC™. The ion source type was Heated-ESI. The spray voltage setting was static with positive ion 3500 V and negative ion 2500 V. The nitrogen gas mode was static with the flow setting: sheath gas 60 Arb, Aux gas 15 Arb, and sweep gas 2 Arb. The ion transfer tube temperature was 350 °C. The vaporizer temperature was 350 °C. The full scan mode range was 200–1000 m/z with a resolution of 60,000 and RF Len 70%. $ddMS^2$ mode was triggered with an intensity threshold of 5.0×10^5. The MS^2 parameters were isolation window: 1.5 m/z, collision energy type: normalized, orbitrap resolution: 15,000, and scan range mode: automatic.

The instrument was controlled and analyzed using Chromeleon™ Chromatography Data System software. The recorded chromatogram was visualized and analyzed using FreeStyle software. Compounds were identified or tentatively identified by their mass spectral data.

Cell culture condition

Bhas 42 cells were purchased from JCRB Cell Bank, National Institute of Biomedical Innovation, Japan. All cell culture media and supplements were supplied by Gibco® (Life Technologies, Grand Island, NY, USA). Routinely, Bhas 42 cells were cultured in MEM with 10% FBS supplement and 1% PS (M10F) in a humidified incubator with 5% CO_2 at a temperature of 37 °C. Before reaching the confluence, the cells were subcultured using 0.25% trypsin. For transformation assays, Bhas 42 cells were cultured in media, DMEM/F12 with 5% FBS supplement and 1% PS (DF5F), and allowed to reach about 70% confluence before seeding them for further testing.

Cytotoxicity assay

Cell Counting Kit-8 (CCK-8) (Dojindo Laboratory, Japan) was performed for cell cytotoxicity testing according to the manufacturer's instructions. Briefly, Bhas 42 cells (5,000 cells/well) were grown in 96-well plates for 48 h at 37 °C and 5% CO_2 and treated with media DF5F containing *A. vulgaris* at 0.0041 to 2.0591 mg/mL, 16 samples per concentration. DF5F with 0.1% DMSO was used for negative control, while 1 µg/mL of 3-methylcholanthrene (MCA) (Sigma-Aldrich, St. Louis, MO, USA) and 50 ng/mL of 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma-Aldrich, St. Louis, MO, USA) were used for positive control of initiation and promotion assay. After 48 h, CCK-8 solution was added to each well, which was incubated for 4 h, with the absorbance determined with a microplate reader (Tecan, Switzerland) at a wavelength of 450 nm. The cell survival rate (%) was calculated for dose setting in further studies[12].

Inhibition activity of *A. vulgaris* on bhas 42 cell transformation

The transformation inhibition activity of *A. vulgaris* in Bhas 42 cells was investigated using the transformation assay protocol previously described[13], which added *A. vulgaris* extract at different concentrations and dates of addition (Figs. 1 and 2). Bhas 42 cell suspension in DF5F was seeded into 96-well plates at 200 cells/well and 400 cells/well for testing the initiation and promotion stages, respectively. They were then incubated at 37 °C and 5% CO_2 in a CO_2 incubator for 21 days with DF5F changed according to treated conditions, as shown in Figs. 1 and 2. On day 21, the cells were fixed with methanol and stained with a 5% Giemsa solution. The transformed

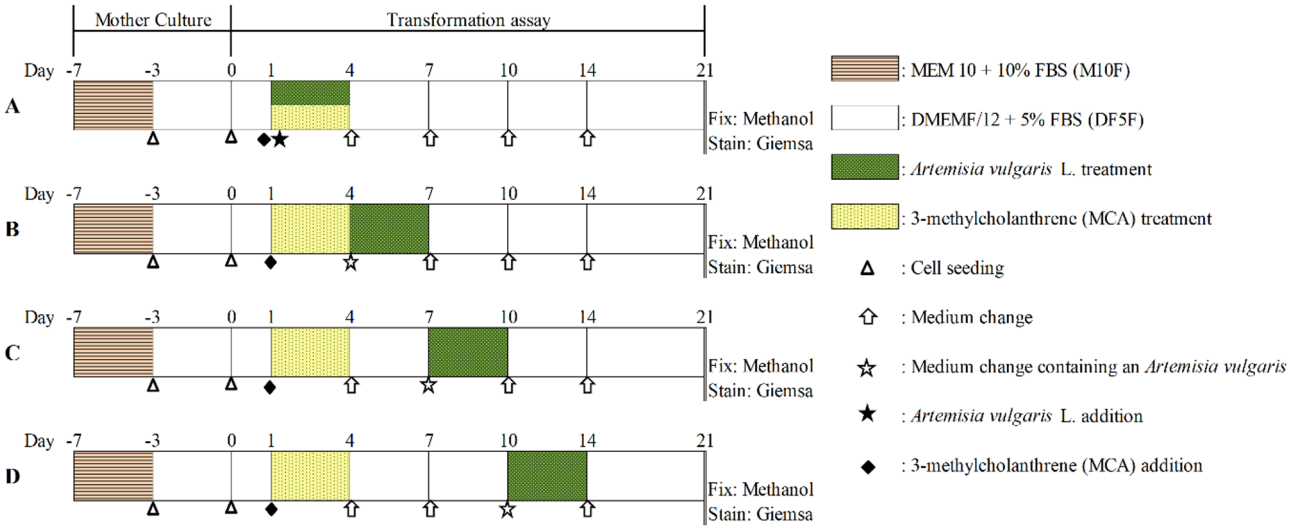

Fig. 1. Bhas 42 cell treated conditions for the initiation stage. Bhas 42 cells were exposed to MCA from Day 1 to Day 4. The *A. vulgaris* extract was added on Day 1 (Treatment A), Day 4 (Treatment B), Day 7 (Treatment C) or Day 10 (Treatment D).

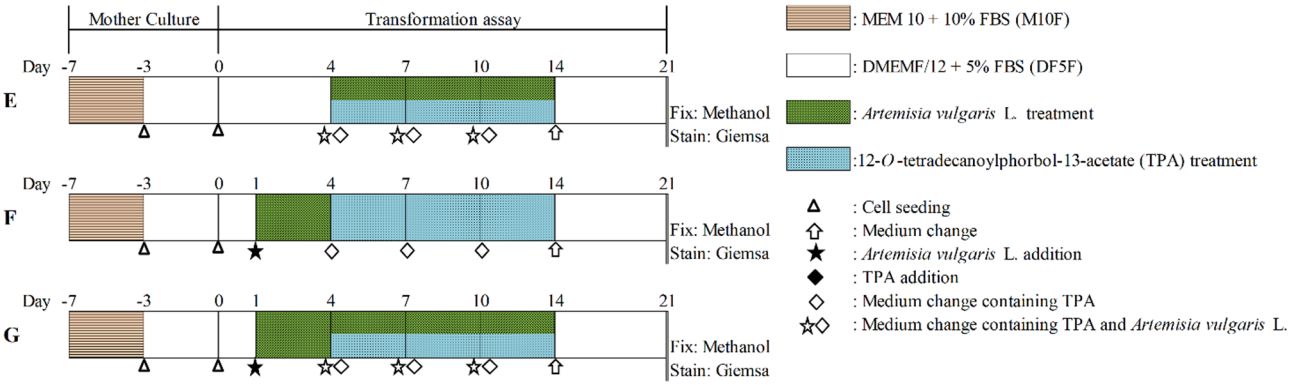

Fig. 2. Bhas 42 cell treated conditions for the promotion stage. Bhas 42 cells were exposed to TPA from Day 4 to Day 14. The *A.vulgaris* extract was added at the same time as TPA (Treatment E), 3 days before adding TPA (Treatment F) or 3 days before adding TPA and continuously incubated with TPA for 14 days (Treatment G).

foci were examined and counted under an inverted microscope based on the photo catalog of foci in Bhas 42 cell transformation assay (CTA)[14]. All conditions were performed in triplicate, including blank (DF5F), negative control (0.1% DMSO), and positive control (1 μg/mL MCA or 50 ng/mL TPA). In addition, the cell growth assays of initiation and promotion were also conducted in parallel to check the cell viability of Bhas 42 cells when exposed to MCA (1 μg/mL) or TPA (50 ng/mL), a cancer stimulator and promoter[11,13] together with/without *A. vulgaris* extract. The inhibition rate (%) was calculated compared with positive control as follows.

$$Inhibition\ rate\ (\%) = \left(\left(1 - \left(\frac{Ns}{Nn}\right)\right) \times 100\right)$$

Ns is a number of wells with transformed foci in the plate treated with various concentrations of *A. vulgaris* extraction, Nn is a number of wells that transformed foci in the plate treated with initiator or promoter (positive control).

The most effective transformed foci inhibition conditions of initiation and promotion stages were selected for further analysis by proteomic assay.

Statistical analysis

The statistical significance of Bhas 42 cell viability and transformation inhibition was analyzed by multiple comparisons using the two-sides Dunnett's test ($p < 0.05$).

Proteomic assay

Proteomic analysis was performed using in-gel digestion, coupled with mass spectrometric analysis (GeLC-MS/MS) to identify and characterize the proteins. Briefly, Bhas 42 cells cultured under the most effective transformed foci inhibition condition were collected for protein denaturing and precipitation. Then, SDS-PAGE fractionation, band excision, destaining, and in-gel enzymatic digestion were conducted before introducing the products to an UltiMate 3000 nano-LC system (Dionex, Surrey, UK) coupled with a micrOTOF-Q (Bruker Daltonics, Bremen, Germany). Separation was performed on a 58 min gradient with a flow rate of 200 nL/min with mobile phase A (2% acetonitrile and 0.1% formic acid in HPLC grade water) and mobile phase B (0.1% formic acid in HPLC grade acetonitrile). Data acquisition was controlled using Hystar software (Bruker Daltonics, Bremen, Germany). MS and MS/MS spectra covered the mass range of m/z 400–2000 and m/z 50–1500, respectively.

The results derived from the proteomic assay were analyzed using bioinformatics programs. The downregulated and upregulated proteins of cell transformation inhibition assays were submitted to the KEGG Automatic Annotation Server (Ver. 2.0; http://www.genome.jp/tools/kaas/)[15]. PANTHER was applied to identify the gene ontology in relation to cellular components, biological processes, and molecular function. String and David were used to predict protein-protein interactions and functional annotation, respectively.

Results

Identification of major phytochemical components of *A. vulgaris*

The phytochemical profile of *A. vulgaris* was analyzed using ultra-high-performance liquid chromatography coupled with a photodiode array detector and a high-resolution tandem mass spectrometer (UHPLC-DAD-MS/MS). The representative chromatogram is shown in Fig. 3. Identification of peaks was done using mass spectral data compared with the National Institute of Standards and Technology (NIST) and the mzCould database. However, none of the mass spectral could be identified. The results are demonstrated in Table 1, and their mass spectral were demonstrated in Supplementary material 1.

Cell viability and cytotoxicity of *A. vulgaris*

The cell viability and cytotoxicity of the Bhas 42 cell line were determined using the CCK-8 assay following 48 h treatment of *A. vulgaris* extract at 0.0041 to 2.0591 mg/mL. The results showed that the cytotoxicity of *A. vulgaris* extract on the Bhas 42 cell line was dose-dependent (Fig. 4). The Lethal Concentration 50% (LC50) value was 1.0021 mg/mL.

Bhas 42 cell transformation Inhibition ability

Bhas 42 cells were induced to transform in the initiation (Fig. 1A and D) or promotion (Fig. 2E and G) stage. The *A.vulgaris* extract was added in different periods and concentrations.

The initiation stage results revealed that Bhas 42 cells treated with *A. vulgaris* extract at 0.2265 mg/mL after 10 days of MCA addition (Treatment D) had the highest inhibition rate (87%) among all treatments. Adding the same concentration of the extract after 7 days of MCA addition (Treatment C), the inhibition rate was slightly decreased to 73%. However, at the concentration of 0.2265 mg/mL, the Bhas 42 cells in treatments A and B could not survive. Bhas 42 cells incubated with MCA simultaneously with 0.0453 mg/mL of the extract for 3 days (Treatment A) show a transformation inhibition rate of 73%. The variation of cell transformation inhibition of the extract concentration and each treatment was observed (Fig. 5).

For the promotion stage, the results of Bhas 42 cell transformation showed the highest inhibition rate of 92% in Treatment G, in which Bhas 42 cells were incubated with 0.0453 mg/mL *A. vulgaris* extract for 3 days before being induced with TPA and were continuously incubated with *A. vulgaris* for 14 days. The lowest inhibition rate in the promotion stage was observed in the treatment E (8–67%). In treatment E, the *A.vulgaris* extract was added at the same time as TPA, while in the other treatments, the extracts were added before (Fig. 6).

Proteomics analysis using GeLC-MS/MS

Bhas 42 cells from Treatment A in the initiation stage and Treatment G in the promotion stage at the concentration of 0.0453 mg/mL *A. vulgaris* extract were used for protein and pathway analysis with the application of GeLC-MS/MS.

For the initiation stage, the results showed 1,055 detected spots, among which 63 protein spots were remarkably upregulated and 55 were remarkably downregulated compared to the control ($p < 0.05$). When comparing the fold change of upregulated proteins with the control, Annexin A3 expressed the highest, whereas Ras-related protein Rap-1 A showed the maximum fold change of down-regulated proteins (Table 2).

The results revealed 961 detected spots for the promotion stage with 63 upregulated proteins and 23 down-regulated proteins compared to the control ($p < 0.05$). ADP-ribosylation factor 5 (Arf-5) expressed the maximum fold change of the upregulated proteins, while Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed the most remarkable fold change in down-regulated proteins (Table 3).

Proteomic assay: functional analysis of protein network

Gene ontology, according to the Panther classification system.

The gene ontology of the molecular function category of initiation and promotion stages demonstrated that the largest group of proteins are involved in binding, followed by catalytic activity and structural molecular activity.

In the biological process category, the largest share of proteins plays a vital role in cellular processes, followed by metabolic processes and biological regulation.

The protein class in the cellular component category of the initiation and promotion stage were cellular anatomical entities, intracellular entities, and protein-containing complexes (Fig. 7).

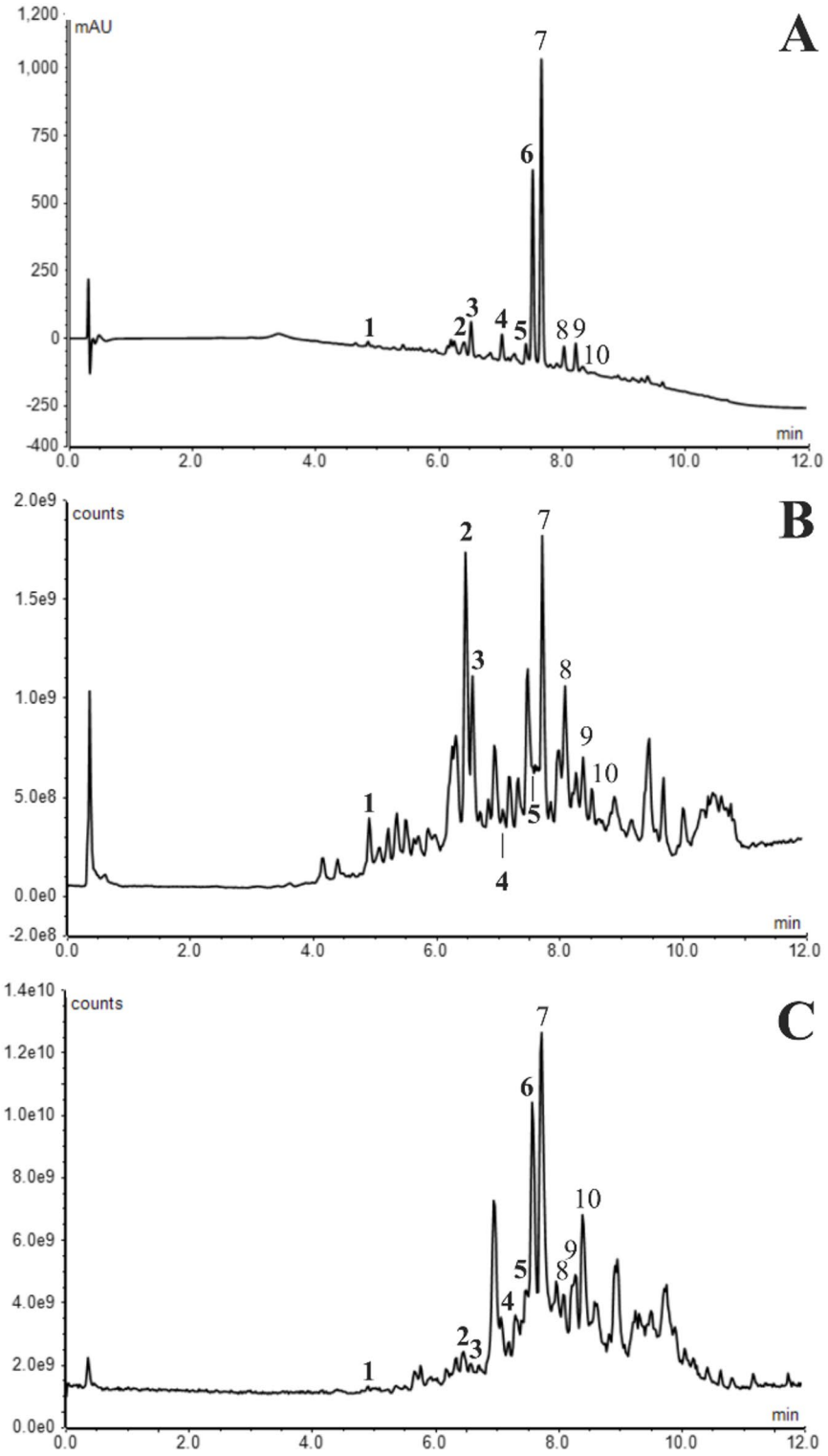

Fig. 3. LC-DAD-MS chromatograms of *A. vulgaris* extract. (A) detected at 220 nm, (B) full scan with negative mode, and (C) full scan with positive mode.

STRING protein-protein interaction networks functional enrichment analysis

The results showed that the protein-protein interactions of the upregulated proteins expressed in the initiation stage were related to translation and ribosomal protein. Protein-protein interactions of down-regulated proteins expressed in initiation were related to translation. (Fig. 8).

For the promotion stage, protein-protein interactions of upregulated proteins were related to endocytosis, while down-regulated proteins were related to the regulation of the actin cytoskeleton and translation (Fig. 9).

Peak no.	Retention time (min)	Mode	Base peak (m/z)	Results
1	4.850	Negative	$[M-H]^-$ = 353.0884	$C_{23}H_{15}O_4$ MW = 354 Calc for $[M-H]^-$ = 353.08193 Calc for $[M+H]^+$ = 355.09649 Calc for $[M+Na]^+$ = 377.07843
		Positive	$[M+H]^+$ = 355.1035 $[M+Na]^+$ = 377.0856	
2	6.407	Negative	205.0511, 161.0612	Unidentified
		Positive	245.1179	
3	6.520	Negative	723.5051, 713.4764, 515.1214, 313.1667, 287.0571	Unidentified
		Positive	701.4953, 340.2606, 301.1420, 198.1859	
4	7.020	Negative	836.5892, 723.5051	Unidentified
5	7.410	Negative	$[M-H]^-$ = 345.0623	$C_{17}H_{15}O_8$ MW = 346 Calc for $[M-H]^-$ = 345.06159 Calc for $[M+H]^+$ = 347.07614
		Positive	$[M+H]^+$= 347.0774	
6	7.520	Negative	503.1517	$C_{39}H_{42}O$ MW = 526 Calc for $[M+H]^+$ = 527.33084
		Positive	$[M+H]^+$ = 527.3361	
7	7.660	Negative	329.0676, 252.1613, 299.0570	$C_{39}H_{43}O_3$ MW = 558 Calc for $[M+H]^+$ = 559.32067
		Positive	$[M+H]^+$ = 559.3260	
8	8.027	Negative	$[M-H]^-$ =373.0939 $[MS^2]$ 373.0939 -> 358.0703, 343.0467	$C_{26}H_{14}O_3$ MW = 374 Calc for $[M-H]^-$ = 373.08072 Calc for $[M+H]^+$ = 375.10157
		Positive	$[M+H]^+$ = 375.1086	
9	8.217	Negative	329.2346	Unidentified
		Positive	359.1134	
10	8.333	Negative	293.1770	$C_{39}H_{43}O_3$ MW = 558 Calc for $[M+H]^+$ = 559.32067
		Positive	$[M+H]^+$ = 559.3259	

Table 1. Characterization of major components of *A. vulgaris* with LC-MS/MS.

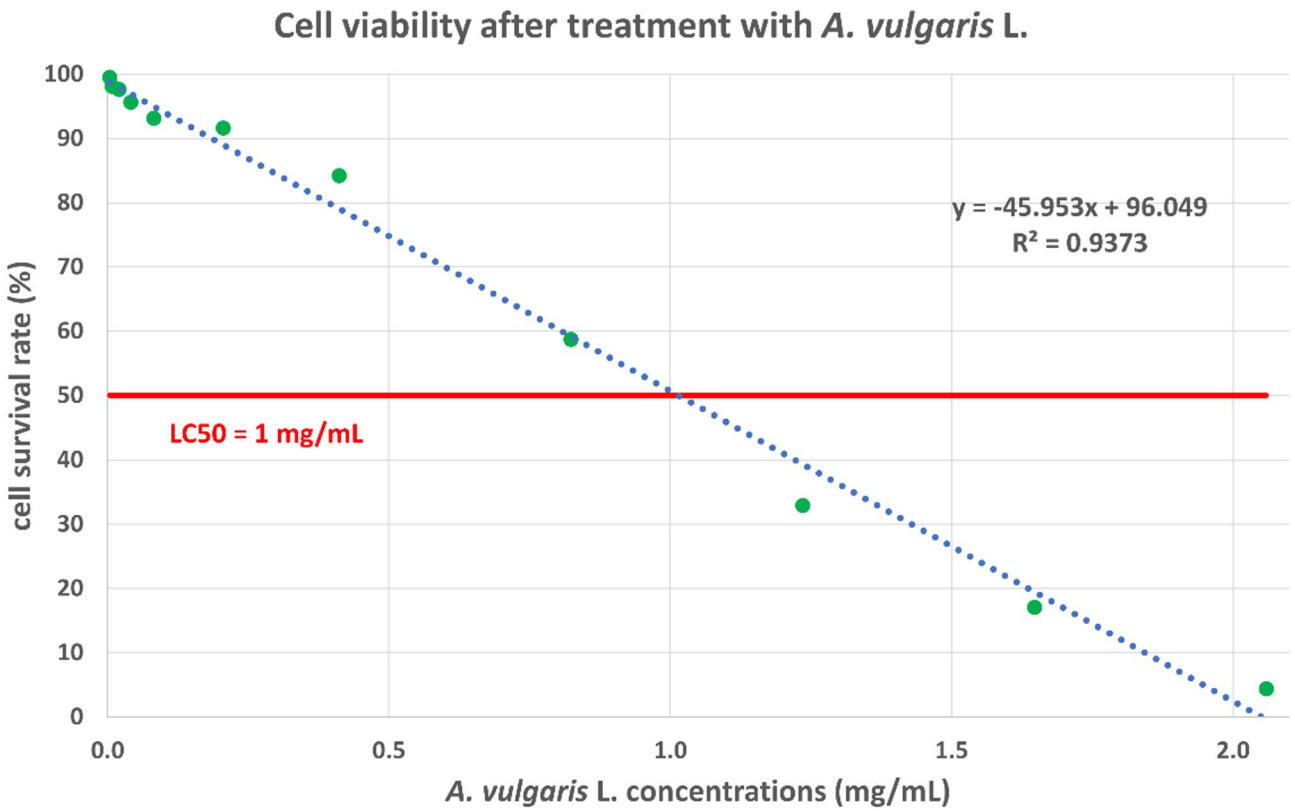

Fig. 4. Bhas 42 cell viability after treatment with *A. vulgaris* extract. The Lethal concentration 50% (LC50) of *A. vulgaris* extract was 1 mg/ml.

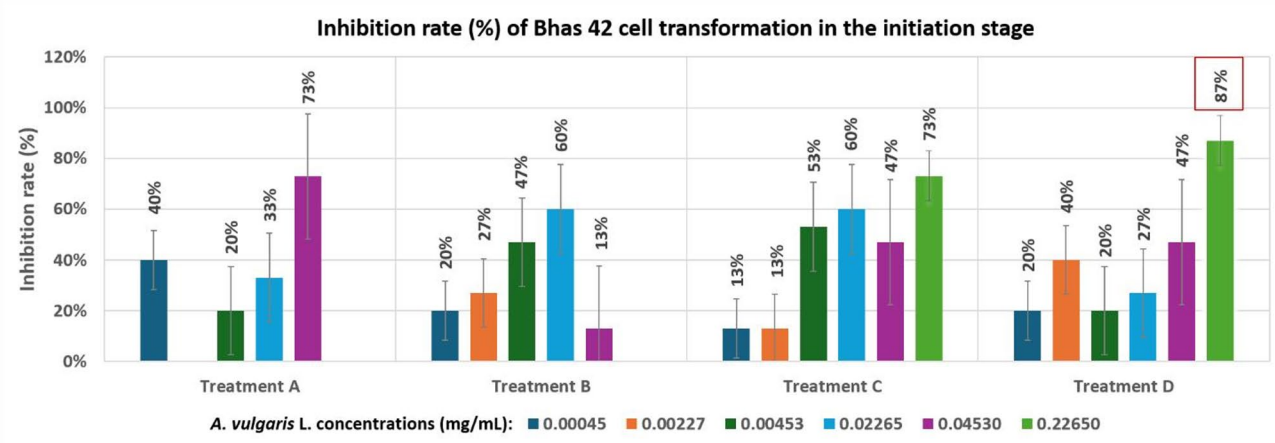

Fig. 5. Inhibition rate (%) of Bhas 42 cell transformation after treatment with *A. vulgaris* extract in the initiation stage compared with positive control. Bhas 42 cells were exposed to MCA from Day 1 to Day 4. The *A. vulgaris* extract was added on Day 1 (Treatment A), Day 4 (Treatment B), Day 7 (Treatment C) or Day 10 (Treatment D).

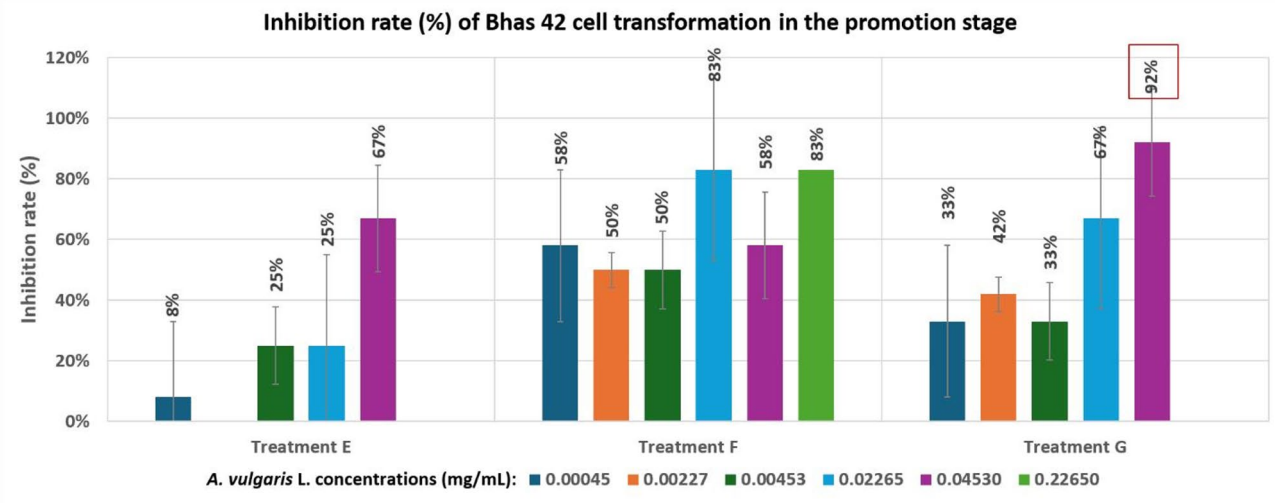

Fig. 6. Inhibition rate (%) of Bhas 42 cell transformation after treatment with *A. vulgaris* extract in the promotion stage compared to positive control. The *A.vulgaris* extract was added at the same time as TPA (Treatment E), 3 days before adding TPA (Treatment F) or 3 days before adding TPA and continuously incubated with TPA for 14 days (Treatment G).

	Upregulated proteins		Downregulated proteins	
No	Proteins description	Fold change	Proteins description	Fold change
1	Annexin A3	5.59	Ras-related protein Rap-1 A	5.06
2	Prohibitin-2	3.96	Spectrin beta chain, brain 1	3.67
3	40 S ribosomal protein S12	3.83	Histone H1.3	3.24
4	ATP-dependent RNA helicase A	3.67	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	3.12
5	60 S ribosomal protein L21	3.61	Keratin, type II cytoskeletal 75	3.00
6	Gremlin-1	3.56	Spectrin alpha chain, brain	3.00
7	Transgelin-2	3.56	V-type proton ATPase catalytic subunit A	2.73
8	60 S ribosomal protein L26	3.55	14-3-3 protein eta	2.71
9	Proteasome subunit alpha type-2	3.38	Mitochondrial inner membrane protein	2.63
10	60 S ribosomal protein L5	3.20	Actin, cytoplasmic 2	2.46

Table 2. Top 10 upregulated and downregulated proteins from the initiation stage.

No	Upregulated proteins		Downregulated proteins	
	Proteins description	Fold change	Proteins description	Fold change
1	ADP-ribosylation factor 5	5.19	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	4.43
2	Peptidyl-prolyl cis-trans isomerase B	5.07	Transketolase	2.49
3	Keratin, type II cytoskeletal 4	4.33	Annexin A6	2.39
4	L-lactate dehydrogenase B chain	4.06	Actin, cytoplasmic 2	2.31
5	Cytochrome c oxidase subunit 5 A, mitochondrial	3.67	ATP-dependent RNA helicase A	2.30
6	Cytochrome b5 type B	3.62	Cofilin-2	2.27
7	Histone H1.1	3.60	Obg-like ATPase 1	2.21
8	Lactoylglutathione lyase	3.56	Cell division control protein 42 homolog	2.20
9	6-phosphogluconolactonase	3.54	60 S ribosomal protein L28	2.18
10	F-actin-capping protein subunit alpha-2	3.30	Ras-related protein Rab-10	2.13

Table 3. Top 10 upregulated and downregulated proteins from the promotion stage.

Western blot analysis

Annexin A3 showed the most significant fold change, so Western blot analysis was performed to validate the expression of Annexin A3 using the Hoefer TE22 wet tank transfer system. Then, the protein bands were quantified with the GelQuantNET program. The results showed that cells treated with *A. vulgaris* had approximately 2.3-fold greater upregulated Annexin A3 expression than control (Fig. 10 and Supplementary material 2).

Discussion

Cancer is caused by the transformation of normal cells into tumor cells. Herbal extracts are a source of pharmacological potential bioactive compounds, including anticancer activity. This study determined the ability of *A. vulgaris* extract to inhibit the Bhas 42 cell transformation induced by the initiator (MCA) or promotor (TPA) for cancer prevention.

A. vulgaris is a medicinal plant traditionally used for various therapeutic purposes. The leaf extracts of *A. vulgaris* demonstrated its ability to inhibit cancer cell growth. Studies have reported several bioactive compounds of *A. vulgaris*, such as 1,8 cineole, α-terpinene, Quercetin, and Camphor[16]. In this study, the leaves of *A. vulgaris* were used to evaluate its cancer-preventive effects. The level of the bioactive compound was analyzed using UHPLC-DAD-MS/MS, but the content of the major reported composition, 1,8 cineole, could not be detected. This could be attributed to the low concentration of 1,8 cineole in *A. vulgaris* and the sensitivity limitation of the analytical equipment used. Previous studies have reported varying concentrations of 1,8 cineole in *A. vulgaris* depending on geographic and developmental factors. For instance, 1,8 cineole was detected at a concentration of 1.8% in sample grown in Pennsylvania using GC-MS analysis[17] and 9.9–24.3% in samples from Vietnam at various vegetation stages, analyzed with GC/RI and GC/MS methods[18]. *A. vulgaris* should be harvested before the flowering stage and all above-ground parts of the plant should be used in future studies to maximize the content of bioactive compounds.

Furthermore, the ethanol extraction employed in this study may have influenced the yield of bioactive compounds since the solvents and methods for medicinal plant extraction are key to the quality and quantity of bioactive compounds[11,17–21]. The impact of different geographical and developmental stages of the plants and different extraction methods may cause active compounds to be undetected. Method optimization should be a concern. Further evaluation by advanced techniques such as targeted metabolomics could enhance characterization.

The dose of exposure is also a critical factor influencing cell survival. High doses of *A. vulgaris* extract exhibit cytotoxic effects on Bhas 42 cells. At the lower concentration, ranging from 0.0041 to 0.205 mg/L, the cell survival rate remained above 90%. However, in some treatments where the extract was used at concentrations of 0.00227–0.22650 mg/L, the Bhas 42 cells could not survive. This could be attributed to the synergistic toxicity of *A. vulgaris* in combination with MCA or TPA, which likely contributed to cell death[19]. On the other hand, using the extract in combination with other anticancer agents may reduce the amount of extract used, thus reducing its toxicity and enhancing the efficacy of anticancer drugs[22]. Further testing for synergy effects with other anticancer agents is needed.

The ability of *A. vulgaris* to inhibit cancer cell proliferation has been reported[20]. It was found to suppress the proliferation of the Human Gastric Adenocarcinoma cell line (AGS), the Human Cervix Carcinoma cell line (Hela), the human colon adenocarcinoma cell line (HT-29 cancer cells), the human breast carcinoma cell line (MCF-7), and mouse fibroblast-like cell line (L929). However, the extract's effectiveness varied across these cancer cell lines. Unlike these findings, our study demonstrated that *A. vulgaris* extract could inhibit the transformation of Bhas 42 cells during the cancer initiation and promotion stage, indicating a potential protective role against the transformation of normal cells into cancerous cells. The ethanolic extract of *A. vulgaris* inhibits MCA- nd TPA- induced Bhas 42 cell transformation, suggesting its anticancer potential.

Bhas 42 cells from Treatment A of the initiation assay and Treatment G in the promotion assay, exposed to *A. vulgaris* extract at a concentration of 0.0453 mg/mL, were selected for proteomic analysis to investigate the mechanism underlying transformed foci inhibition and protein expression changes, focusing on the protein fold change in the treated group compared to the control. The analysis revealed protein expression alterations in

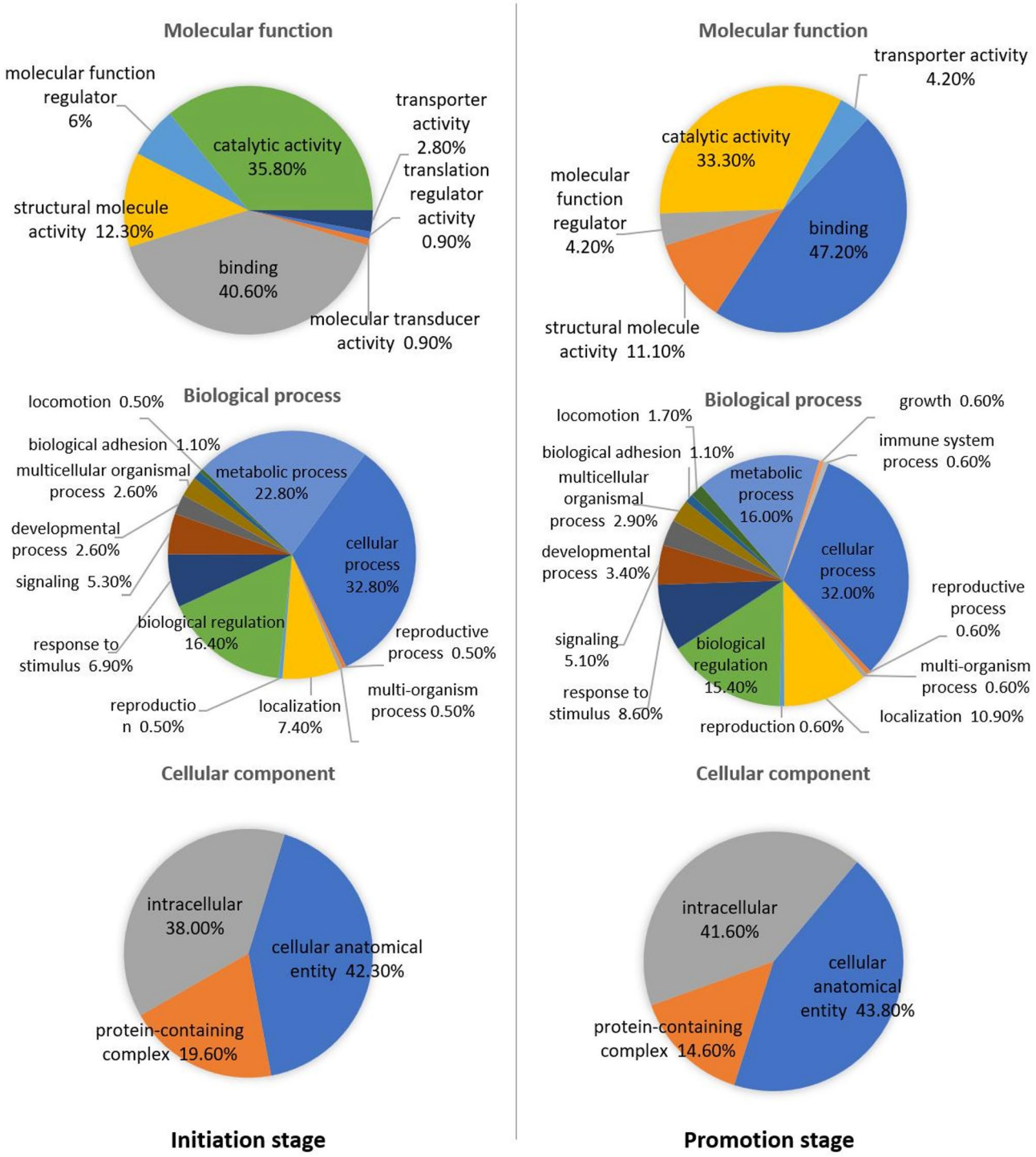

Fig. 7. Gene ontology according to the Panther classification of the Bhas 42 cells treated with *A. vulgaris* in the initiation and promotion stage.

the initiation and promotion assays, with some proteins being up or downregulated. Notably, the greatest fold change observed was a 5.59 times increase in protein expression in the treated group compared to the control group.

Among the proteins that exhibited remarkable changes in expression compared to the control, Prohibitin-2 and Arf-5 were notably upregulated in the treated group. *A. vulgaris* may exert its potential to inhibit cell transformation into cancer cells during the initiation and promotion stages through these proteins' functions, respectively. Since Prohibitin-2 is closely linked to key cancer-related processes such as cell proliferation, apoptosis, mitophagy, and metastasis[23], overexpression of Prohibitin-2 induced by *A. vulgaris* extract may facilitate the elimination of damaged or transformed cells through mitophagy, potentially preventing their progression into

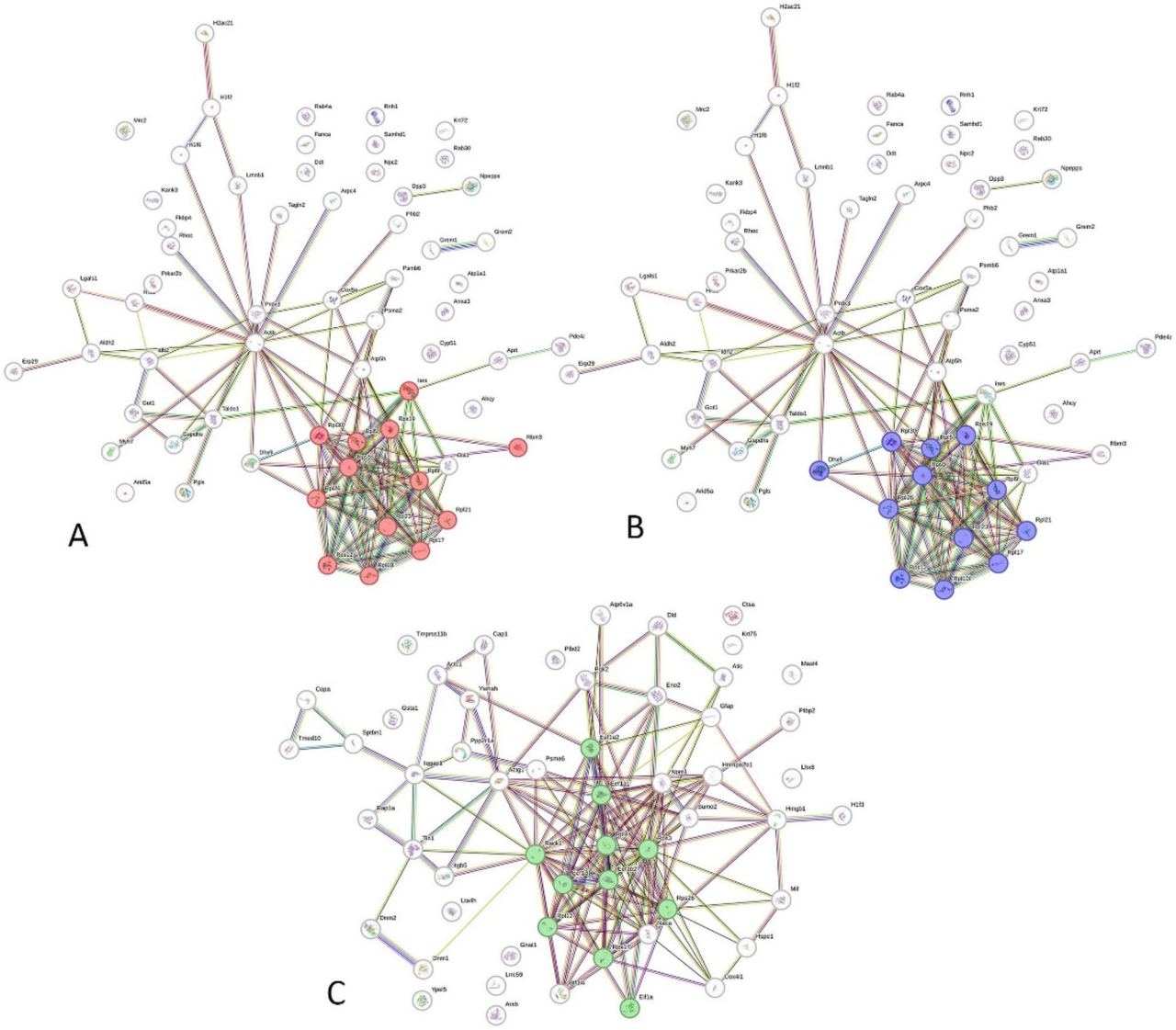

Fig. 8. Protein-protein interaction networks of initiation assay; upregulated proteins in translation (A) and ribosome (B) and down-regulated proteins in translation (C).

cancerous cells. Whereas Arf-5 is involved in regulating cell adhesion, migration, invasion and proliferation[24,25]. The upregulation of Arf-5 induced by the *A.vulgaris* extract may interfere with the transformation of Bhas 42 cell by modulating endocytosis, thereby disrupting processes critical for cell transformation. Upregulation of Prohibitin-2 and Arf-5 suggests roles in mitophagy and endocytosis modulation, inhibiting cancer cell transformation.

The other upregulated proteins related to cancer development are Annexin A3, Peptidyl prolyl cis/trans isomerases (PPIases), and Glyoxalase (GLO1). These proteins are typically linked with cancer initiation, growth, and proliferation and promote cancer progression by regulating biological activities[26–30]. However, their increased expression after being treated with *A. vulgaris* demonstrated that the inhibition of cell transformation by *A. vulgaris* extract might not involve these particular proteins.

The study results also exhibited the lower expression of Ras-related protein Rap-1 A, Cofilin-2, Cell division control protein 42 homolog, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transketolase, and Annexin A6. The down-regulation of these proteins following exposure to *A. vulgaris* extract is associated with the inhibition of cell transformation inhibition through multiple mechanisms, including the induction of cell apoptosis, reduction of glycolytic flux, suppression of nucleotide synthesis, and increased cellular stress leading to cell death[31–39].

Interestingly, the Spectrin beta chain, brain 1, a known tumor suppressor[40], was also down-regulated, suggesting that the cell transformation inhibition effect of *A.vulgaris* may not involve this specific pathway.

Functional analysis of protein networks in cancer pathways further revealed the upregulation of ribosomal proteins RPS12, RPL21, RPL26, and RPL5. These proteins play essential roles in ribosomal biogenesis and protein synthesis, influencing tumor progression and inhibition[41–43]. Following exposure to *A.vulgaris* extract,

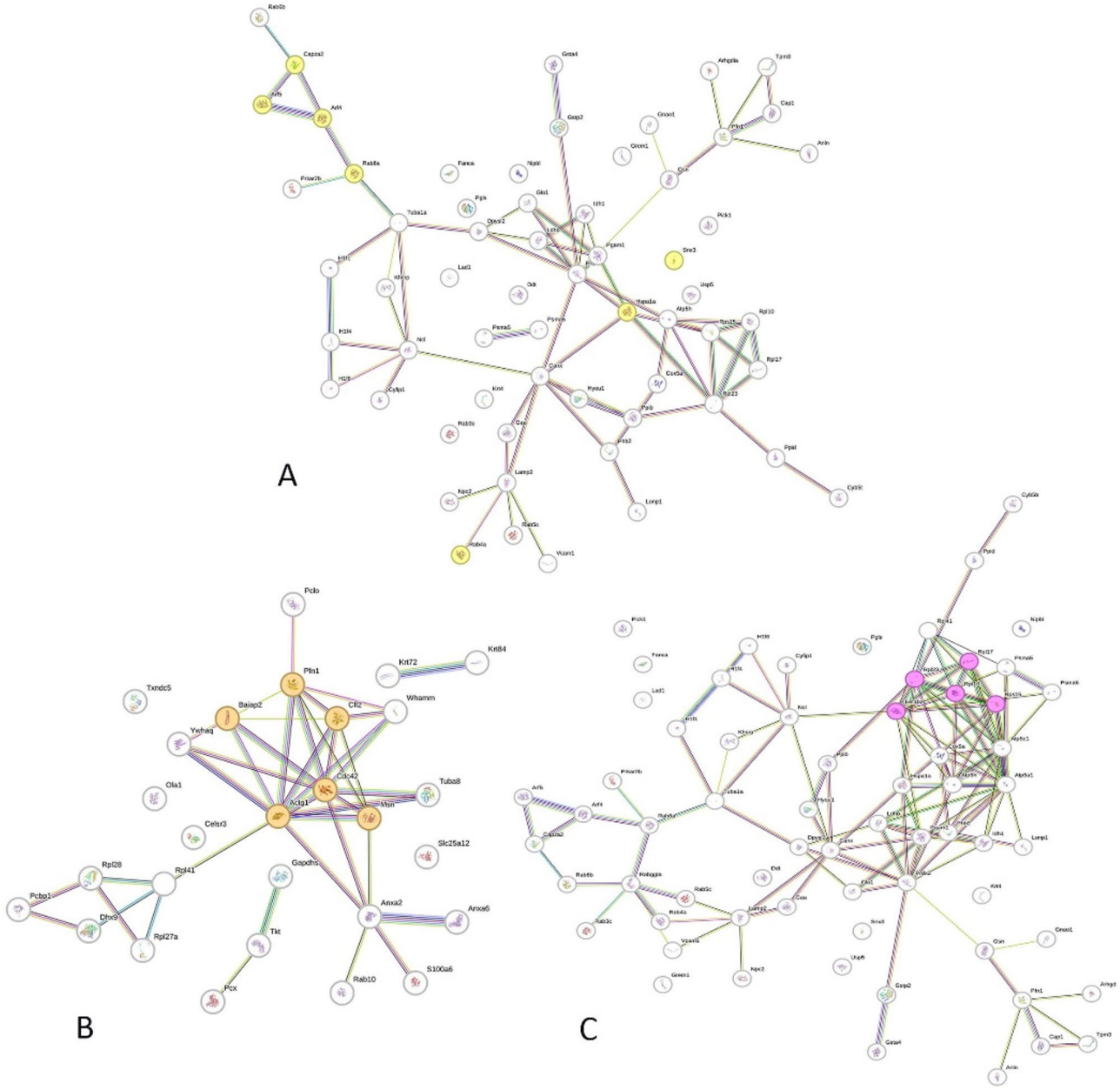

Fig. 9. Protein-protein interaction networks of promotion assay; upregulated proteins in Endocytosis (A) and down-regulated proteins in Regulation of actin cytoskeleton (B) and Translation (C).

the upregulation of these proteins suggests a protective role in inhibiting cell transformation by activating the tumor suppressive pathways (e.g., p53), inducing ribosomal stress, and ensuring translational fidelity. It may counteract the molecular mechanisms driving cell transformation, thereby preventing the initiation or progression of cancerous growth. Upregulation of tumor-suppressive ribosomal proteins and downregulation of glycolysis/apoptosis-related proteins suggest a multi-targeted inhibitory mechanism.

Cancer progression relies on a multifaceted process involving multiple components[25,44]. Similarly, the dysregulation of several proteins observed during Bhas 42 cell transformation inhibition following *A.vulgaris* treatment underscores a complex mechanism in which various factors mediate the inhibitory effect. In this study, Bhas42 cell transformation was induced by MCA and TPA and treated with *A. vulgaris* extracts. The results showed that *A. vulgaris* can inhibit cancer cell proliferation at the molecular level through several potential mechanisms. The ethanolic extract of *A. vulgaris* likely interferes with key signaling pathways involved in cell transformation and carcinogenesis. It may exert its effects by modulating the expression of proteins involved in cell growth, apoptosis, and cell cycle regulation, such as activating an oncogene or suppressing the activity of tumor suppressor genes. In the initiation and promotion stages, the proteomic study results showed cancer progression-related protein changes between the *A. vulgaris* treated and the control groups. The dysregulated proteins were involved in regulating cancer cell progression, transformation, proliferation, migration, and invasion, which are

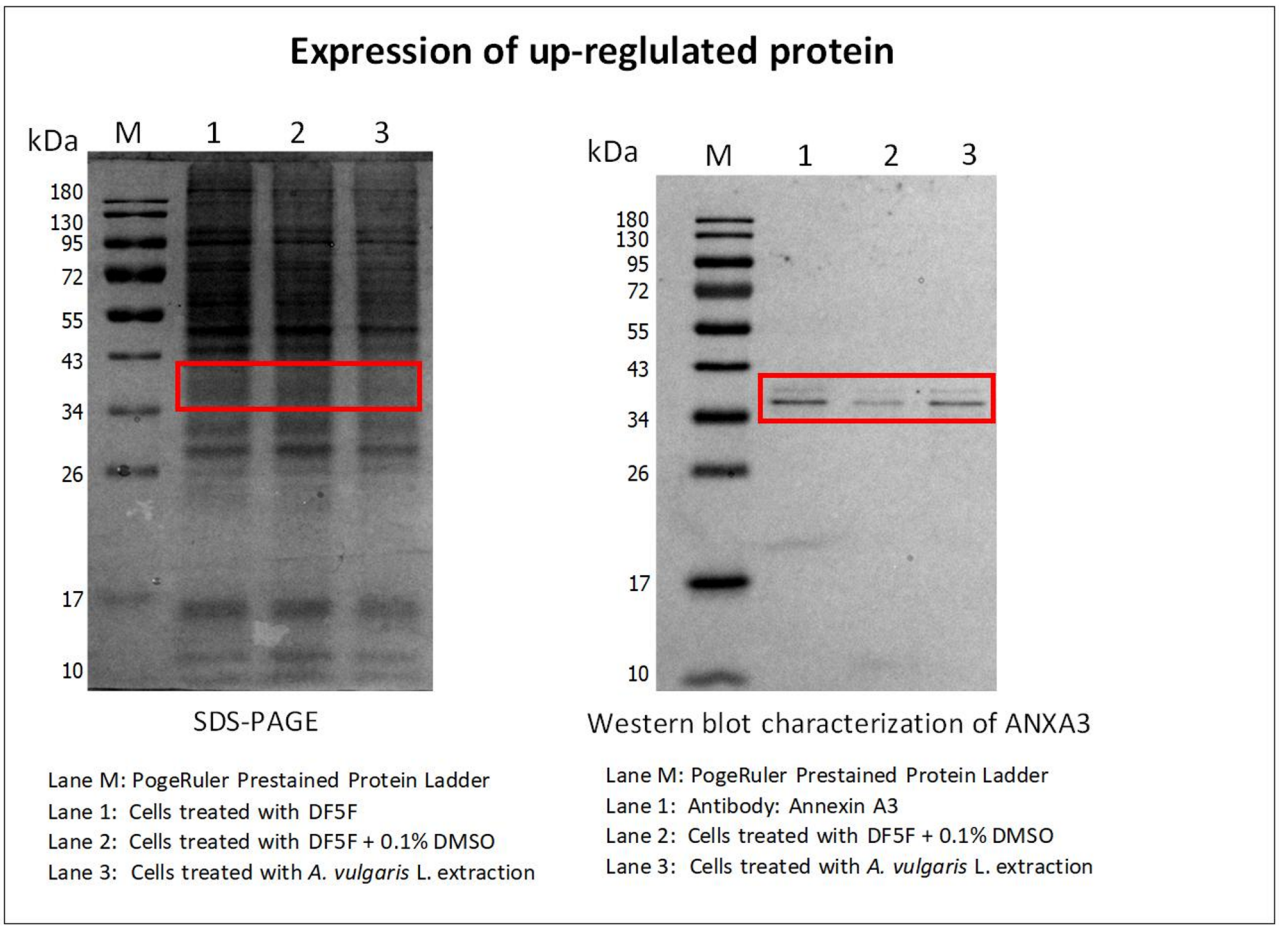

Fig. 10. Expression of upregulated protein (Annexin A3) in cells treated with *A. vulgaris*.

the hallmarks of cancer cells. However, further studies are required to develop an understanding of the complex mechanisms of *A. vulgaris* extract in inhibiting cell transformation. In addition, in vivo studies that administered *A. vulgaris* extract may be conducted to confirm its cancer-preventive effects, pharmacokinetics and toxicity[45,46].

Data availability

Available on request from the corresponding author.

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Author contributions

P.P., S.W. and Y.L. conceptualized the study. P.P., S.K., SW., O.R., N.K., R.M. and N.K. perform experiments. P.P., O.R., K.T. and S.K. analyzed the data. S.K., S.W., O.R., N.K., K.T. and Y.L. supervised the experiments and manuscript preparation. S.W. acquired funding. P.P. prepared the draft manuscript and figures. S.W., S.K. and Y.L. finalized the manuscript. All authors approved the final version of the manuscript.

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Competing interests
The authors declare no competing interests.

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