**Carthamus tinctorius** L. (Safflower) extracts inhibit expression of metastatic genes of MDA-MB-231 breast cancer cells

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**ABSTRACT**

Breast cancer is the most common type of cancer in women and the second cause of cancer-related death after lung cancer. Although the common methods used in the treatment of breast cancer are chemotherapy, radiotherapy and surgery, the search for alternative treatments continues. The leading alternative treatments are medicinal plants which inspire the production of many cancer drugs. In this study, the proliferative and metastatic effects of Carthamus tinctorius L., known for its many therapeutic properties, on metastatic breast cancer were investigated. Here, intending to evaluate the content and actions of different extracts of safflower leaf extracts were prepared by extracting in water, alcohol and oil and analysed by FTIR. Their antioxidant effect was tested and then the extracts were applied to metastatic breast cancer cells. FTIR spectrums of all three extracts have revealed the presence of organic compounds. It is found that all extracts but mostly the oil extract has antioxidant property. MTT assay, wound healing assay and gene expression analysis were performed to assess the antiproliferative and anti-metastatic effects of the extracts on breast cancer cells. It is found that there is no significant antiproliferative effect of extracts on MDA-MB-231 cells except the alcohol extract. However, all safflower extracts, especially the oil extract, significantly reduced the metastatic potential of breast cancer cells. It is concluded that safflower contents are potent chemicals that inhibit the cellular mechanisms underlying the spreading of cancer cells and further analysis may lead to new initiatives in drug design research.

**Introduction**

Breast cancer is the second cause of cancer-related casualties in women and is responsible for 15% of all cancer-related deaths in women (1). Despite the development of early diagnosis and comprehensive treatment strategies, the survival rate of metastatic breast cancer is only 25% (2). Treatment strategies for breast cancer such as surgery, radiotherapy, targeted therapies, chemotherapy, immunotherapy, hormonal therapy, and other biological therapies are applied alone or in combination (3). Since many treatment methods exert some type of side effects, the search for alternative treatment methods that can support classical methods or can be applied alone is ongoing (4). Medicinal plants, which are the source of drugs, are the most important alternative treatment methods (5). Natural products such as Gambogic acid, Curcumin, Silibinin, Emodin and β-elemene obtained from medicinal plants are shown as potential candidates for therapeutic drugs against cancer. β-element isolated from Curcuma wenyujin is currently used as a cancer medicine in China, and research on natural products that affect cancer is continuing (6).

Carthamus tinctorius L., also known as false saffron, is an oil seed plant belonging to the Asteraceae family; It is reported that there are about 25 species in the world (7). C. tinctorius L. has significant properties such as antioxidant, anti-inflammatory, analgesic, antidiabetic, hepatoprotective, antihyperlipidemic and immunomodulatory effects (8,9). More than 200 compounds have been isolated from the plant Carthamus tinctorius L., and the commonly known ones are flavonoids, phenylethanoid glycosides, coumarins, fatty acids, steroids, and polysaccharides (10). According to different extraction methods, lipophilic and hydrophilic compounds emerge. Therefore, the biological effects depend on the preparation technique of the extracts as well as the part of plant which is extracted (11). Extracts of C. tinctorius L. plant in dichloromethane, hexane and methanol solvents showed variable antiproliferative effects in SW620 colon cancer cells (12). C. tinctorius L. polysaccharides suppressed proliferation, invasion, and metastasis in the low metastatic potential benign MCF-7 breast cancer cells. In previous studies, it has been shown that polysaccharides obtained only from water extract in non-metastatic breast cancer cells inhibit metastatic potential by affecting TIMP-1 and MMP-9 proteins (13). There is a need to investigate the effects of active substances obtained from extracts such as water and oil on expressions of certain molecules involved in metastasis. The aim of this study is to investigate the antiproliferative and antimetastatic effects of C. tinctorius L. flower extracts prepared in water, alcohol and oil on MDA-MB-231, the human breast cancer cells with high metastatic potential.
Materials and Methods

C. tinctorius L. Extracts

C. tinctorius L., dried orange-coloured flowers were used to make the extracts. The flowers of the C. tinctorius plant were extracted in three different solvents: distilled water, pure ethanol (Sigma-Aldrich, Milan, Italy) and sunflower oil. The flowers of C. tinctorius L. (2g /50 ml) were added to each solvent. It was kept at 4°C for 24 hours. The solute was filtered and concentrated to 1/4 volume on a vacuum evaporator. The resulting solution was prepared for applications under sterile conditions by passing through an injector filter with a pore size of 0.22 µm (Nest Scientific USA Inc.). Sterile extract solutions were labelled and stored at 4°C for characterization and further analysis.

Fourier Transform Infrared (FT-IR) Spectrometer Analysis

FT-IR spectrums (Thermo Scientific Nicolet 6700 Smart iTR, USA) were obtained for each fraction via the attenuated total reflectance (ATR) technique in mode of transmittance to detect the characteristic peaks and their functional groups. A spectrum scan from 4000 to 500 cm⁻¹ was utilized adn the determinations were made in triplicate.

DPPH Scavenging Antioxidant Activity

The antioxidant activity of C. tinctorius L. Extract fractions was analyzed by using their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Milan, Italy). 100 µl of each fraction was added into 1 ml (0.1 M) DPPH solution prepared in 95% ethanol, incubated at 37°C for 30 min and kept away from light. Thereafter the mixtures were analyzed via UV-vis spectroscopy at 517 nm. DPPH radical scavenging activity was calculated by using Eq 1.

\[
\text{Antioxidant activity} \% = \frac{A_{\text{DPPH, sample}} - A_{\text{DPPH}}}{A_{\text{DPPH}}} \times 100
\]  

(Eq 1)

Cells

The triple negative, highly metastatic breast cancer cell line, MDA-MB-231, was purchased from the American Type Culture Collection (ATCC, Wesel, Germany). Cells were cultured in DMEM (Capricorn, Germany) containing 10% fetal bovine serum (BIOIND, Israel), 2 Mm L glutamine (Sigma-Aldrich, Milan, Italy) and 1% antibiotic-antimycotic solution (BIOIND, Israel). Culture conditions consist of 37 °C, 5% CO2 and 100% humidity. Analyses were carried out for seven experimental groups: water, alcohol and oil extract application groups and water, alcohol and oil alone and the untreated control.

Cell Proliferation Analysis

The extracts were applied to MDA-MB-231 cells cultured in 96 well plates yielding 40 µg in each well. The effects of different extracts on the proliferation of breast cancer cells were measured by spectrophotometer using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) test. Briefly, 2000 cells per well for each experimental group were seeded in 96-well plates (Nest Scientific USA Inc.) and incubated at 37°C with 5% CO2, 10 µl of MTT final concentration 0.5 mg/ml solution (Biotium,California, USA) was added to each well at 0, 24 and 48 hours. Plates were incubated for 4 hours in the dark at 37°C. Then, 100 µl of DMSO was added to each well to dissolve the formazan crystals. It was mixed by pipetting and left for 15 minutes to ensure complete dissolution. Absorbance was recorded at 570 nm by a microplate reader (BioTek Synergy H1, BioTek Instruments, Winooski, VT, USA).

Lateral Motility Assay

The lateral motility of MDA-MB-231 cells was examined by performing the wound healing assay. The cells (2 × 10⁵ cells per well) were seeded in six-well plates (Nest Scientific USA Inc.). After they reach confluency, three wounds were produced in each well using a pipette tip. The wells were rinsed once with fresh medium, and wound widths were recorded under an inverted microscope (Leica, Wetzlar, Germany). These cells were treated with 2µL of alcohol, water and oil extracts having a concentration of 0.08 mg/ml as well as alcohol and oil solvents. Pictures of wound areas at 0, 24 and 48 hours were measured with an inverted microscope. Mobility was expressed as the percent reduction of closed area at the wound sites compared to control cells.

Gene Expression Analysis

Total RNA was isolated using Bluezol (SERVA, Germany) according to the isolation protocol. To evaluate gene expression, 150 ng of RNA was used for timed PCR in accordance with kit protocol, which was performed with the Lightcycler® 96 system (Roche Diagnostic Systems, Indianapolis, IN), and A.B.T.™ 2X qPCR SYBR-Green Master Mix (ATLAS Biotechnology, Turkey). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TIMP metallopeptidase Inhibitor 1 (TIMP1) and matrix metallopeptidase 9 (MMP-9), osteopontin/secreted phosphoprotein-1(OPN), insulin like growth factor 2 receptor (IGF2R), kinase insert domain receptor (VEGFR2) and plasminogen activator (PLAU) primers were used at a final concentration of 0.25 µM. Data were normalized using the GAPDH gene threshold cycle (Ct) value. Samples were run in triplicate and the mean Ct value was used for calculations. The ratio of the Ct values of experimental groups is determined accordingly.

Statistical Analysis

All quantitative data are presented as mean ± standard deviation (SD) from three independent experiments. All graphics were designed using GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed using SPSS (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp.). Mann-Whitney U test and Kruskal-Wallis statistical tests were carried out. The value of p< 0.05 was considered as significant.

Results

Fourier Transform Infrared (FT-IR) Spectrometer Analysis

FT-IR analysis was performed to determine the functional groups of the active components in each extract fraction of C. tinctorius L (Fig.1) by using corresponding backgrounds for each fraction individually. In Figure 1,
the spectrums of all fractions showed same characteristic bands of *C. tinctorius* L., a band at 3300-3200 cm⁻¹ corresponding to the broad stretching of carboxylic acid and phenolic -OH stretching, bands between 3000-2870 cm⁻¹ of the C-H stretching bonds of alkene, alkane and aldehydes, the band at 1045 cm⁻¹ of the C-O bond, and bands between 880-770 are of the C-H/=C-H/N-H bonds. Moreover, in the oil and alcohol fraction, the band at 2972 cm⁻¹ corresponds to the oil-hydrocarbon skeletal stretching band as seen in Figure 1A, In is seen here that different from Figure 1B and C spectrums, there is a strong C=O stretching cyclopentanone peak at 1743 cm⁻¹. In addition, in the water fraction, a peak at 1721 cm⁻¹ corresponding to the C=O stretching of cyclohexanones/cyclopentanones constituents.

**DPPH Scavenging Activity**

Antioxidant capacities of *C. tinctorius* L. extracts were evaluated by determining their DPPH radical scavenging performance. As demonstrated in Figure 2, all water, alcohol and oil fractions have significant amount of antioxidant activity, 72 ± 2.9%, 65 ± 2.17%, and 90 ± 1.72%, respectively.

**Antiproliferative Effect of *C. tinctorius* L. Extracts on Breast Cancer Cells**

The antiproliferative effects of *C. tinctorius* L. extracts prepared in water, alcohol and oil on breast cancer cells were evaluated by MTT analysis for 48 hours. Results were expressed as average OD values and standard deviation (Fig. 3). There was no significant difference between solvent (alcohol and oil) and extract treated groups at 24 hours (p>0.05). However, after 48 hours, the antiproliferative effect was detected in alcohol extract treated cells only causing a decrease in cell growth by 48% and the growth of only alcohol treated cells decreased by 15%. Thus, there was no significant antiproliferative effect of extracts except the alcohol extract treated group at 48 hours.

**Effect of *C. tinctorius* L. Extracts on the Metastatic Potential of Cells**

Wound healing test was carried out to evaluate the effect of *C. tinctorius* L. extracts on the lateral motility of cells, which shows their metastatic potential. Micrographs of all the experimental groups are given at 0, 24 and 48 hours (Fig 4). The area at 0 hours of all groups was accepted as 100%. At all times the wound healing was greatly impaired in extract applied groups (Fig 4a). Percentage of cell-free areas at the end of 48 hours were 13%, 28% and 22% in control, only alcohol and oil treated groups. While the cell free areas in *C. tinctorius* L. Extract treated cells were 37%, 46% and 47% for water, alcohol and oil respectively (Fig 4b). There was a significant difference between the control cells and oil extract and water extract applied cells (p<0.05). When the extract applied groups were compared with their solvent groups, a significant difference was found for the water and oil extract applied groups (p<0.05), but no significant difference was found for alcohol extract and the only alcohol applied cells. Therefore, especially oil and water extracts of *C. tinctorius* L. flowers decreased the lateral motility of MDA MB breast cancer cells, which is an indicator of metastatic behavior.

**C. tinctorius** L. extracts reduce the ratio of MMP9/TIMP1 expression and the expressions of other genes involved in metastasis

The effects of *C. tinctorius* L. extracts on MMP9 and TIMP1 gene expression of breast cancer cells were evaluated by qRT-PCR after 48 hours. The sequence of the primers used for the MMP9 and TIMP1 genes is given in Table 1. The mean CT values of the genes were calculated and compared to each other. Results were expressed as the mean value of the ratios and standard deviation (Figure 5). After 48 hours, when the MMP9/TIMP1 expression rate was calculated by accepting the control group as 100%. The ratios of MMP9/TIMP1 mRNA were 56.9%, 62.5%, 41.2% in water, alcohol and oil extract treated cells. Their
solvents were also applied and the ratio of MMP9/TIMP1 expression ratio of cells were 87.5% and 91.6% for alcohol and oil (Fig. 5). There was a significant difference between control cells and extract applied cell groups (p<0.05). In paired comparisons, a highly significant decrease was found only between the control and the oil extract applied cells (p<0.05). Significant decrease in the expressions of OPN, VEGFR2, PLAU and IGF2R genes in all extracted cells compared to control groups (p<0.05) (Fig. 6). Furthermore, their expressions were decreased in alcohol control cells while in the oil control group, they were found to be increased. It is concluded that, extracts are able to decrease the metastatic potential of MDA MB 231 cells at gene expression level and the highest antimetastatic action was exerted by the oil extracts of \textit{C. tinctorius} L. Flowers.

**Discussion**

In a previous study using \textit{C. tinctorius} L seed oil, C–H, C=C and C–O bonds were detected. Similar functional groups were detected in all the extracts of flower used in this work. However, the cyclopentanone C=O bond having a peak at 1741 cm$^{-1}$ observed in that study was only detected in the oil extract of flowers (14). This indicates that oil extract is distinctive among the other extracts which may reflect some additional effects on cancer cells. The characteristic bands found by FTIR analysis predict the presence of structures such as proteins, alkaloids, lignans, organic acids, polysaccharides/sugars and phenolic compounds, especially flavonoids, in the extracts (15).

It was also reported that the methanol extract of \textit{C. tinctorius} L. flowers exerted detected antioxidant effect which was comparable to synthetic antioxidants, as well as high phenolic and flavonoid content(16). Yolci et al. has found that the total antioxidant activity of \textit{C. tinctorius} L. Flowers was 61.25 mg TE/g in the water extracted samples. Similarly, in our study, oil extract showed a high antioxidant activity (17). It is known that polysaccharides

![Figure 4](image_url)  
**Figure 4.** Effects of \textit{C. tinctorius} L. extracts on lateral mobility of MDA-MB-231 cells. Cells were treated with \textit{C. tinctorius} L. extracts in water, alcohol and oil (a) The micrographs of wound areas (b). The graphic representing the percent of cell free areas. The values represent mean ± SD, n=3 (p < 0.05).

![Figure 5](image_url)  
**Figure 5.** Effects of \textit{C. tinctorius} L. extracts on MMP9/TIMP1 gene expression ratio of MDA-MB-231 cells. Cells were treated with \textit{C. tinctorius} L. Water, alcohol and oil extracts of \textit{C. tinctorius} L flowers and with only oil and alcohol. The untreated and solvent treated cells served as control. Values represent mean ± SD, n=3 (p < 0.05).

![Figure 6](image_url)  
**Figure 6.** Effects of \textit{C. tinctorius} L. extracts on OPN, VEGFR2, OPN and IGF2R gene expression of MDA-MB-231 cells. Cells were treated with \textit{C. tinctorius} L. Water, alcohol and oil extracts of \textit{C. tinctorius} L flowers and with only oil and alcohol. The untreated and solvent treated cells served as control. Values represent mean ± SD, n=3 (p < 0.05).

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**Table 1.** Primers used in qRT-PCR.

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<td>MMP9</td>
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increase the expression of antioxidant genes through NF-kB signaling. It is thought that the polysaccharides of C. tinctorius L. protect cells from oxidative stress by reducing reactive oxygen species through enzyme activations (18). Here, although the antioxidant activity was detected in all the extracts, the maximum activity was found in oil extracts not only in the polysaccharide containing water extracts.

Metastasis of breast cancer is the leading cause of death, therefore it is important to determine an effective therapeutic approach for treatment (19). The majority of anticancer drugs with high efficacy in clinical use are derived from plant derived compounds. The anticancer effect of these natural products occurs through different mechanisms such as apoptosis induction, immune system modulation and angiogenesis inhibition (20). The antitumor effects of C. tinctorius L. are known to be via enhancing the immune response, inducing apoptosis of tumor cells, and preventing the migration of tumor cells (9, 20).

C. tinctorius L. seeds inhibited proliferation in colorectal cancer cells (21). The safflower polysaccharide content in water extract inhibited the proliferation of MDA-MB-231 and this effect was increased with the combination of cyclopaamine (22). Safflower polysaccharide at concentrations ranging from 0.02 to 1.28 mg/ml inhibited proliferation in HeLa cervical cancer cells depending on time and dosage (23). C. tinctorius L. extracts did not affect proliferation in MDA-MD-231 breast cancer cells except for cells treated with alcohol extract. It is noted that, all doses of C. tinctorius L. Polysaccharides (SPS) (0.04, 0.08, 0.17, 0.34, 0.68 or 1.36 mg/ml) inhibited proliferation in MCF-7 breast cancer cells at 24 and 48 hours (13). Furthermore, application of hydroxyl safflower yellow B, contained in C. tinctorius L, together with doxorubicin greatly reduced the proliferation of MCF-7 cells and induced apoptosis (24, 25). Extracts of C. tinctorius L. plant in dichloromethane, hexane and methanol solvents at a concentration of 0.1 mg/ml did not affect the proliferation of γδT lymphocyte cells (12). C. tinctorius showed an indirect antitumor effect by activating dendritic cells when applied at 5, 10 and 20 μg/mL (26). However, in our study no antiproliferative effect of flower extracts at 0.08 mg/ml concentration was detected for MDA-MB-231 cells, which are more aggressive than MCF 7 cells, except for the alcohol extract.

In this study, all extracts of C. Tinctorius decreased the migration of MDA-MB-231 breast cancer cells. This is in accordance with the findings of Fu et al. who reported the antimetastatic effect of safflower Yellow, the active ingredient of the plant, at doses of 0.19, 0.38, and 0.75 mg/mL (27). The similar effect seen in our study may be related to this active substance. It was reported that, in MCF7 breast cancer cells, MMP9 decreases and TIMP 1 related to this active substance. It was reported that, in this study the highest antimetastatic effect of Wnt/β-catenin signaling in gastric cancer (28). It is remarkable that in this study the highest antimetastatic effect was found in the oil extract. We have detected low ratio markable that in this study the highest antimetastatic effect of safflower yellow B, contained in C. Tinctorius L. has been already shown to be a potential candidate for the development of natural cancer therapeutics. The results of this study emphasize its potential to act as a antimetastatic gene regulating, cost effective and easy to reach type of herbal medicine. Here, it is shown that the extracts of C. Tinctorius L. is a potent regulator of a series of metastasis associated genes in metastatic breast cancer cells.

Conclusion

C. tinctorius L. has been already shown to be a potential candidate for the development of natural cancer therapeutics. The results of this study emphasize its potential to act as an antimetastatic gene regulating, cost effective and easy to reach type of herbal medicine. Here, it is shown that the extracts of C. Tinctorius L. is a potent regulator of a series of metastasis associated genes in metastatic breast cancer cells.

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Interest conflict
None declared.

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The author read and proved the final manuscript for publication.

Availability of data and material
All data generated during this study are included in this published article

Authors’ Contribution
Concept: AKÖ; Design: DK, AKÖ; Supervision: DK, AKÖ; Fundings: AKÖ, DK, DGK; Materials: AKÖ, DGK; Data Collection or Processing: DK, AKÖ, DGK; Analysis or Interpretation: DK, AKÖ; Literature Search: DK, AKÖ; Writing: DK, AKÖ, DGK; Critical Review: AKÖ.

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