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OPEN PEER REVIEW

# Cytotoxic Activity and Phytochemical Analysis of *Artemisia biennis* Essential Oils at Different Growth Stages

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## About this report

This open peer review (OPR) report shows the public comments made on this article during peer review, by the reviewers, the associate editor and the editor-in-chief, for every revision round, together with the authors' replies. Comments appear only with the consent of the people who wrote them. Reviewers who chose to sign their report are named; the others are shown as "Referee". Comments are edited for language only, and confidential remarks to the editors are not included.

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## 1 Original submission

3 comments · 20 Apr 2024 – 1 May 2024

### Referee 1 Reviewer

MAJOR REVISION 20 Apr 2024

Dear Editor
Have a nice day.
The author investigated Cytotoxic Activity and Phytochemical Analysis of Artemisia biennis Essential Oils in Different Growth Stages.
I would suggest reconsidering the manuscript for publication only after the following major revisions.
2. It is necessary to specify the number of repetitions of experiments.
3. There are many problems in the cytotoxicity test steps.
4. Report the SD value for all quantitative data.
5. It is necessary to mention the source of the materials used in a separate section.

### Saeed Tavakoli Reviewer

MAJOR REVISION 30 Apr 2024

Dear author
I read your article. According to previous studies, your article is not very innovative. However, with corrections to the items mentioned and depending on the editor's opinion, it is possible to accept the article.
Why did you extract essential oil from the aerial part of the plant in all cases? (Naturally, at different times, depending on the presence of flowers or fruits, the composition of aerial organs is different.)
Explain the reason for choosing these cell lines.
Mention the herbarium number of the plant.
2 figures are mentioned in the article, but these figures are not available in the article.
A normal cell line must be added to the article so that a better conclusion can be drawn.
Add the following references:
Cytotoxic and apoptotic effects of different extracts of Artemisia biennis Willd. on K562 and HL-60 cell lines
Preparation and characterization of pullulan-soy protein concentrate blended film incorporated with Zataria multiflora and Artemisia biennis essential oils

### Associate Editor

MAJOR REVISION 1 May 2024

Dear Authors,

We have received the reports from our reviewers on your manuscript, "Cytotoxic Activity and Phytochemical Analysis of Artemisia biennis Essential Oils in Different Growth Stages," which you submitted to Jundishapur Journal of Natural Pharmaceutical Products.
Based on the advice received, we have decided that your manuscript could be reconsidered for publication should you be prepared to make major revisions. When preparing your revised manuscript, you are asked to carefully consider the reviewers' comments and submit a list of responses to the comments. Please highlight all changes in the revised manuscript in yellow so that they can be detected by the reviewers.

With kind regards,
Associate Editor

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# Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

**Jundishapur Journal of Natural Pharmaceutical Products**

Manuscript ID: **147988**

Dear Editors and Reviewers,

Thank you very much for your comments and suggestions on our manuscript entitled "**Cytotoxic Activity and Phytochemical Analysis of *Artemisia biennis* Essential Oils in Different Growth Stages**". Those comments are all valuable and very helpful for revising and improving our paper. We have studied comments carefully and have made corrections which we hope meet with approval. Revised portions are marked by text highlights in the revised version of the manuscript. We have tried our best to revise our manuscript according to the comments, and the detailed corrections are listed below:

## Reviewer #1

Dear Editor

Have a nice day.

The author investigated Cytotoxic Activity and Phytochemical Analysis of Artemisia biennis Essential Oils in Different Growth Stages. I would suggest reconsidering the manuscript for publication only after the following major revision.

1. There are many similarities with previous research works.

**Response**;

While Artemisia species have been studied for their medicinal properties, research specifically focusing on *Artemisia biennis* essential oils is relatively limited. Investigating the cytotoxic activity and phytochemical composition of essential oils derived from *Artemisia biennis* represents a novel approach within the field of

phytochemistry. On the other hand, Examining the cytotoxic activity and phytochemical composition of *Artemisia biennis* essential oils across various growth stages adds a dynamic dimension to the research. By analyzing how these factors vary throughout the plant's development, the study provides insights into potential fluctuations in bioactivity and chemical composition, which can impact the plant's therapeutic potential.

The identification of cytotoxic activity in *Artemisia biennis* essential oils suggests potential therapeutic applications, particularly in the context of cancer treatment. This aspect of the research contributes to the exploration of natural sources for novel anticancer agents, addressing an ongoing need for alternative treatments with reduced side effects. Different ecotypes of a plant species may exhibit variations in their phytochemical composition, leading to diverse biological activities. So, by studying the cytotoxicity of essential oils from different plant ecotypes on a cell line, researchers can identify bioactive compounds within the oils that exhibit cytotoxic effects. This information is valuable for understanding the pharmacological potential of *Artemisia biennis* essential oils, the importance of ecological conditions on their cytotoxicity, and their potential applications in medicine.

2. It is necessary to specify the number of repetitions of experiments.

**Response**;

The number of repetitions of experiments was added to the text as follows;

-refer to the Statistical Analysis section

Statistical analyses were performed by one-way analysis of variance test. All experiment was repeated three times and the average result was calculated. The numerical data are presented as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

-refer to the Cytotoxicity Test section;

### 3.4. Cytotoxicity Test

#### 3.4.1. Cell Cultures

The human colon adenocarcinoma (HT-29), breast adenocarcinoma (MCF-7), and normal fibroblast were obtained from Pasture Institute (Tehran, Iran) and maintained in DMEM-F12 medium with 10% v/v fetal bovine serum and 100 u/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % $CO_2$. The cells were sub-cultured two to three times per week.

#### 3.4.2. Cytotoxicity Evaluation Using Viability Assay

MTT assay was used to investigate the EO toxicity, a standard method previously reported (11, 12). Cell suspension with a concentration of $5 \times 10^3$/mL were plated in each well of 96-well plates, and 100 µL of DMEM-F12 culture medium with 10% FBS was added to each well. Plates incubated for 5 h at 37 °C, 90% humidity with 5% $CO_2$. Different concentrations of the Eos with series dilution (100, 50, 25, 12.5, 6.25 µg/ml) were added to wells and incubated for another 24 h. After an incubation period, the cells were replaced with 100 µL of MTT (10%) solution followed by another 24-hour incubation period. After aspiration, dimethyl sulfoxide was added to dissolve the formazan product. Color intensity was by the plate reader device at a wavelength of 570 nm. Color intensity was proportional to living cell number. Each assay was repeated three times and the average result and standard deviation were calculated. The $IC_{50}$ was calculated by Prism software.

3. There are many problems in the cytotoxicity test steps.

**Response**;

Thank you for your feedback. The cytotoxicity test section was revised as follows;

### 3.5. Cytotoxicity Test

#### 3.5.1. Cell Cultures

The human colon adenocarcinoma (HT-29), breast adenocarcinoma (MCF-7), and normal fibroblast were obtained from Pasture Institute (Tehran, Iran) and maintained in DMEM-F12 medium with 10% v/v fetal bovine serum and 100 u/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % $CO_2$. The cells were sub-cultured two to three times per week.

#### 3.5.2. Cytotoxicity Evaluation Using Viability Assay

MTT assay was used to investigate the EO toxicity, a standard method previously reported (11, 12). Cell suspension with a concentration of $5 \times 10^3$/mL were plated in each well of 96-well plates, and 100 µL of DMEM-F12 culture medium with 10% FBS was added to each well. Plates incubated for 5 h at 37 °C, 90% humidity with 5% $CO_2$. Different concentrations of the Eos with series dilution (100, 50, 25, 12.5, 6.25 µg/ml) were added to wells and incubated for another 24 h. 1% DMSO was used as a positive control. After an incubation period, the cells were replaced with 100 µL of MTT (10%) solution followed by another 24-hour incubation period. After aspiration, dimethyl sulfoxide was added to dissolve the formazan product. Color intensity was by the plate reader device at a wavelength of 570 nm. Color intensity was proportional to living cell number. Each assay was repeated three times and the average result and standard deviation were calculated. The IC50 was calculated by Prism software.

4. Report the SD value for all quantitative data.

**Response**;

quantitative data of cytotoxicity results within text was modified.

Refer to page 1

Results: Data from GC/MS revealed that (Z)-nerolidol (19.16-54.09%), (E)-β-farnesene (6.38-13.87%), and (Z)-tonghaosu (11.73-17.25%) were the most abundant chemical constituents accounting about 96 % of the oil composition. The EO of *Artemisia biennis* was characterized by a high content of oxygenated sesquiterpenes of which nerolidol was the main component identified. Based on the MTT assay, The EO of the plant species collected during June showed the most potent cytotoxicity against MCF-7 (IC50= 2.84 ± 0.15 µg/ml) and HT-29 cell lines (IC50= 2.82 ± 0.2 µg/ml).

Refer to page 12

The percentage of cell viability after 24 hours of exposure to different concentrations of the EO (0, 6.25, 12.5, 25, 50, and 100 µg/ml) was evaluated by the MTT method and the results were analyzed by Prism software (Figure 3). Cytotoxicity effects on both cell lines were found to be dose-dependent. According to the data presented in Table 2, the most significant reduction in cell viability across all concentrations was observed with the essential oil extracted in June, demonstrating an IC50 of 2.82 ± 0.2 and 2.84 ± 0.15 µg/ml against HT-29 and MCF-7 cells, respectively (* $p<0.05$).

But if the Dear Reviewer means the GC/MS data, which according to the articles listed below, such an SD value has not been reported.

- Screening of chemical composition, antimicrobial and antioxidant activities of Artemisia essential oils
- Essential oil profile of oregano (*Origanum vulgare* L.) populations grown under similar soil and climate conditions
- Chemical Composition and Antioxidant and Antibacterial Potencies of the *Artemisia ordosica* Aerial Parts Essential Oil during the Vegetative Period

- A comprehensive study on essential oil compositions, antioxidant, anticholinesterase, and antityrosinase activities of three Iranian Artemisia species

5. It is necessary to mention the source of the materials used in a separate section.

**Response**;

3.1. Materials

All solvents and other chemicals were purchased from Merck company (Darmstadt, Germany). All cell culture media and supplements namely Dulbecco's modified Eagle's medium (DMEM-F12), fetal bovine serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin-streptomycin, and trypsin/EDTA were obtained from Bonyakhteh (Iran).

### 1.1.1. Cell Cultures

The human colon adenocarcinoma (HT-29), breast adenocarcinoma (MCF-7), and normal fibroblast were obtained from Pasture Institute (Tehran, Iran) and maintained in DMEM-F12 medium with 10% v/v fetal bovine serum and 100 u/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % $CO_2$. The cells were sub-cultured two to three times per week.

6. The reviewed methods are not based on valid references.

**Response**;

Valid references were added to essential oil preparation, GC/MS analysis, and MTT assay as follows;

- Guenther E, Kulka K, Rogers JA. Essential oils. Analytical Chemistry. 1959;31(4):679-87.

- Daferera DJ, Ziogas BN, Polissiou MG. GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on Penicillium digitatum. Journal of agricultural and food chemistry. 2000;48(6):2576-81.

- Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry: Allured publishing corporation Carol Stream; 2007.

- Plumb JA. Cell sensitivity assays: the MTT assay. Cancer cell culture: methods and protocols. 2004:165-9.

- Plumb JA. Cell sensitivity assays: the MTT assay. Cytotoxic Drug Resistance Mechanisms. 1999:25-30.

## Reviewer #2:

Dear author

I read your article. According to previous studies, your article is not very innovative. But by correcting the mentioned items and with the opinion of the editor, it is possible to accept the article.

1. Why did you extract essential oil from the aerial part of the plant in all cases? (Naturally, at different times, according to the presence of flowers or fruits, the composition of aerial organs is different).

**Response**;

The aerial component of plants refers to the plant's structures above ground, such as stems, leaves, petioles, flowers, fruit, and seeds. The aerial parts of *A. biennis* contain a higher concentration of essential oils compared to other parts of the plant such as the roots. The essential oils extracted from the aerial parts of the *A. biennis* typically contain a unique blend of compounds that are responsible for the characteristic aroma and therapeutic properties of the plant. Additionally, the composition of the essential oils

can vary depending on the specific stage of growth, the presence of flowers or fruits, and environmental factors, so extracting from the aerial parts allows for the most consistent and optimal composition of the essential oil.

2. Explain the reason for choosing these cell lines.

**Response**;

We appreciated the reviewer's constructive comment. As it was known, Colorectal cancer is the 3rd most common cancer worldwide. It is the 3rd most common cancer in men and the 2nd most common cancer in women. Moreover, breast cancer is the most common cancer in women in 157 countries out of 185 in 2022. It must be noted that recent studies have shown that female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%) and colorectal (10.0 %) cancer. Therefore, according to the above, it was decided to study the cytotoxic effect of Artemisia biennis Essential Oils on breast and colon human cancer cell lines.

Refer to "Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries".

3. Mention the herbarium number of the plant.

**Response**;

The aerial parts of *A. biennis* were collected from the mountainous areas of Jaghargh (36°18′43″N 59°19′21″E), Mashhad, Iran, in June (early vegetative stage), July (pre-flowering stages), August (full-flowering stages), and October (late vegetative stage) 2021 with herbarium number 12570. The samples were identified by Dr Seyed Ahmad Emami, Mashhad University of Medical Sciences.

4. 2 figures are mentioned in the article, but these figures are not available in the article.

**Response**;

These figures are uploaded separately with captions according to the journal process, and it seems that the lack of viewing of the images is related to the journal system.

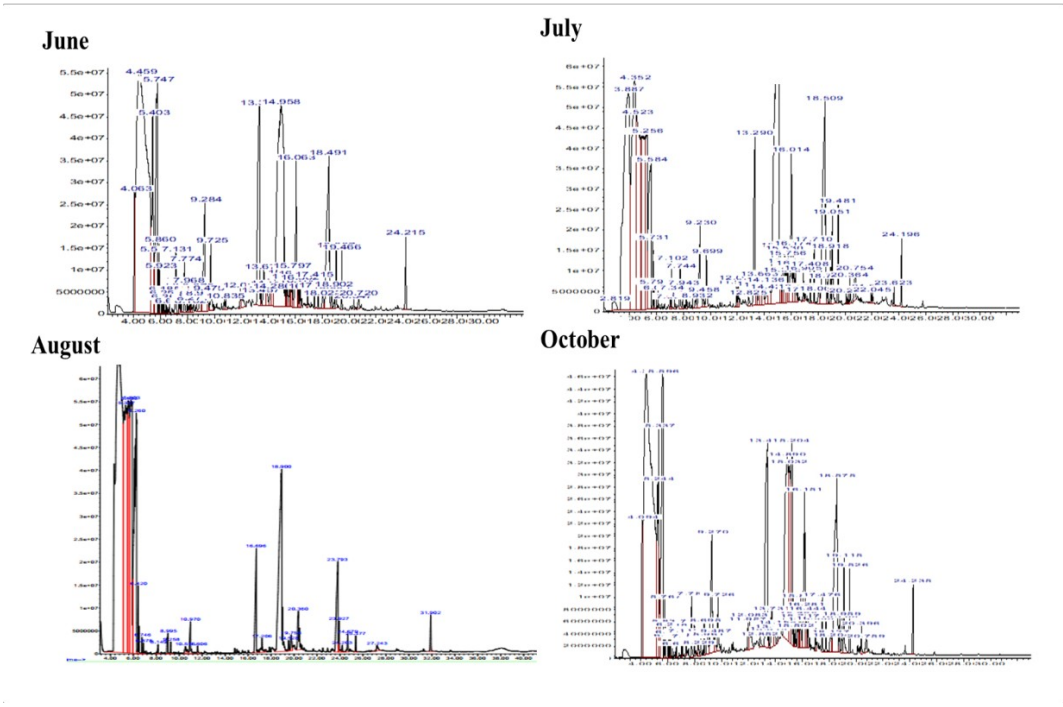



Fig. 1. The GC/MS chromatogram of the *A. biennis* EO under various growth stages during June, July, August, and October.

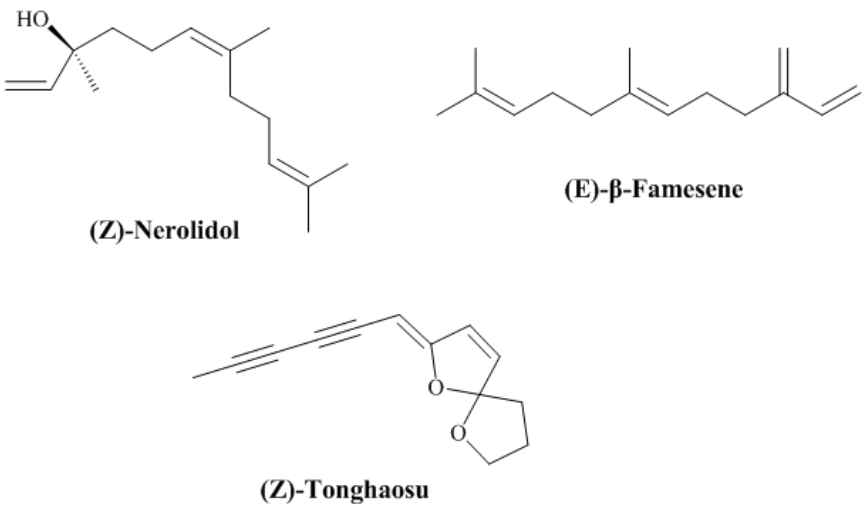


Fig. 2. Major chemical constituents identified in the EO of *A. biennis.*

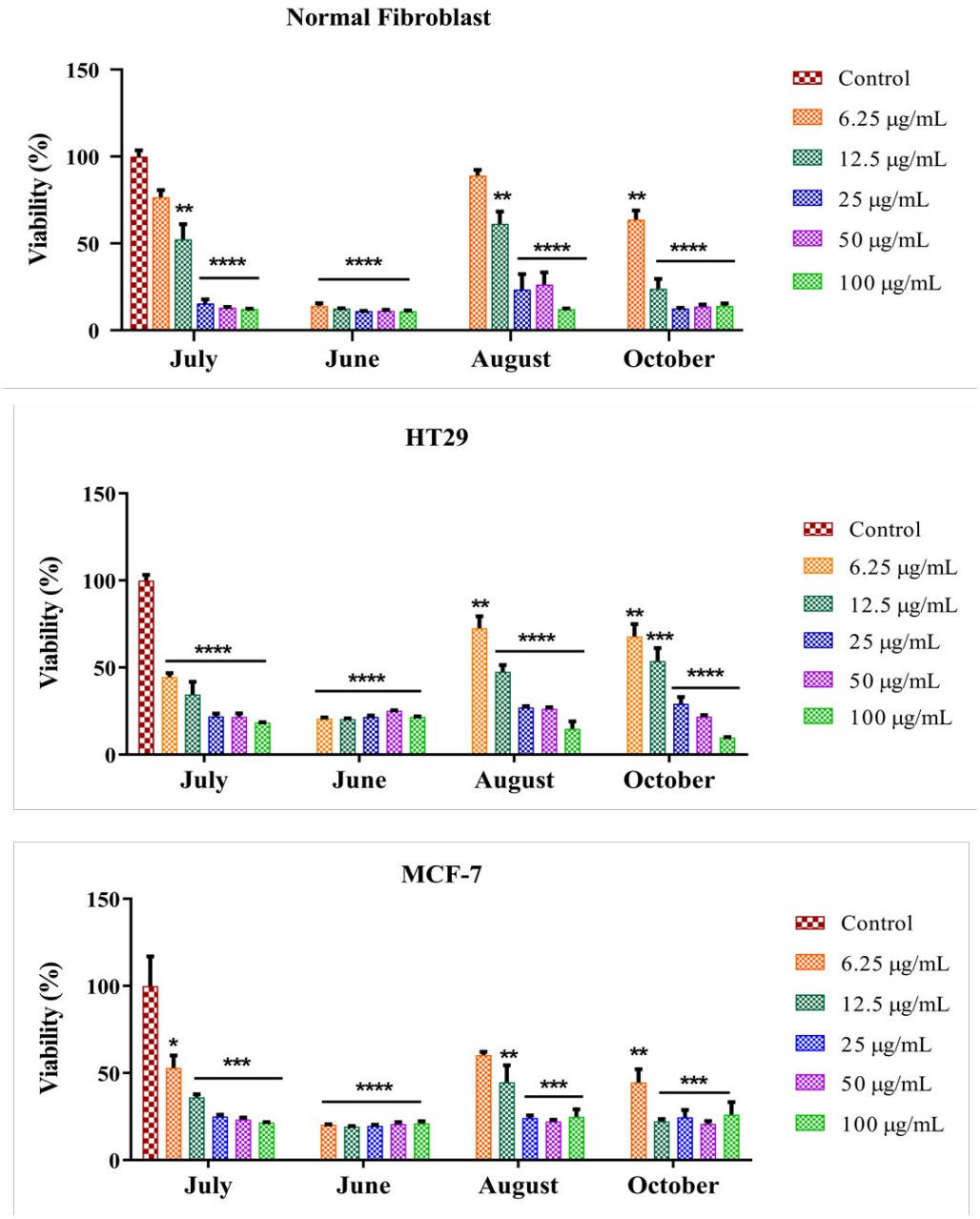


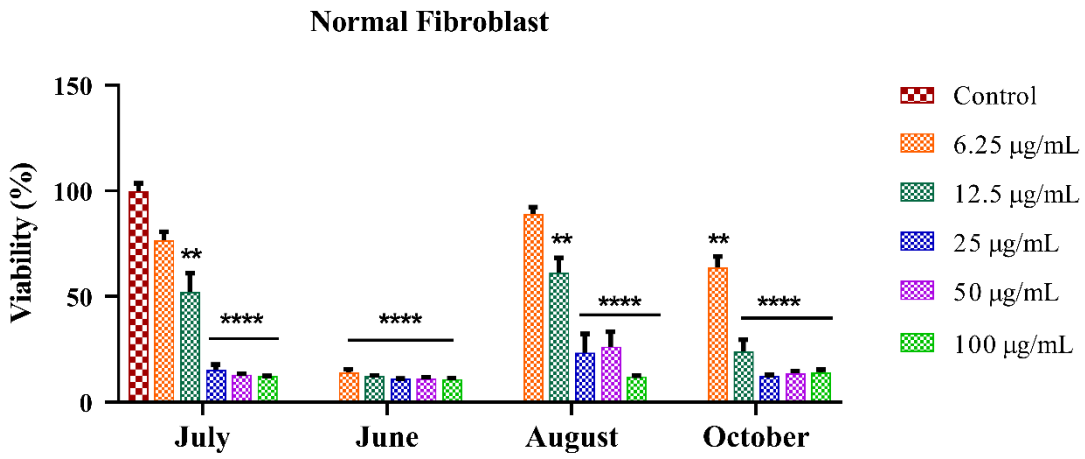

Fig. 3. The growth inhibition activity of various concentrations of *A. biennis* EOs on human normal fibroblast, HT29, and MCF-7 cancer cell lines. Values were mean±SEM of at least three independent experiments (n=3). **** $p<0.05$ compared to the control group.

4. A normal cell line must be added to the article to make a better conclusion.

**Response**;

The cytotoxic effect of essential oils on fibroblasts, as a model of the normal cell line, has been evaluated. The obtained result has been added to the manuscript.

According to the data presented in Table 2, the most significant reduction in cell viability across all concentrations was observed with the essential oil extracted in June, demonstrating an $IC_{50}$ of 2.99 ± 0.23, 2.41 ± 0.2, and 2.84 ± 0.15 µg/ml against normal fibroblast, HT-29, and MCF-7 cells, respectively (**** $p<0.05$). Interestingly, the $IC_{50}$ for the normal human fibroblast across four months was higher than any of the above cancer cells, suggesting the selectivity of the *A. biennis* EOs compounds to inhibit cancer cells. Similar results were shown the J774 normal cells and normal human Skin cells have not been affected significantly by dichloromethane extract from *A. biennis* (47) and *Artemisia vulgaris* Eos (48).



6. Add the following references;

- Cytotoxic and apoptotic effects of different extracts of *Artemisia biennis* Willd. on K562 and HL-60 cell lines

- Preparation and characterization of pullulan-soy protein concentrate blended film incorporated with *Zataria multiflora* and *Artemisia biennis* essential oils

**Response**;

The aforementioned references were added to the text;

Similar results were shown the J774 normal cells and normal human Skin cells have not been affected significantly by dichloromethane extract from *A. biennis* (47) and *Artemisia vulgaris* Eos (48).

The EOs of *A. biennis* were found to have antibacterial activity against *Staphylococcus aureus*, *Lactobacillus plantarum*, and *Escherichia coli* (8).

## 2 Revision 1

1 comment · 20 May 2024

**Referee 1** Reviewer

REJECT 20 May 2024

Dear Editor
Have a nice day.
The addressed issues and concerns have not been effectively resolved.
In my opinion, this manuscript cannot be considered for publication.

3

# Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

**Jundishapur Journal of Natural Pharmaceutical Products**

Manuscript ID: **147988**

Respected Editors and Reviewers,

Thank you very much for your comments and suggestions on our manuscript entitled "**Cytotoxic Activity and Phytochemical Analysis of *Artemisia biennis* Essential Oils in Different Growth Stages**". Those comments are all valuable and very helpful for revising and improving our paper. We investigated all comments carefully and have made corrections which we hope meet with approval. Revised portions are marked by text highlights in the revised version of the manuscript. We have tried our best to revise the manuscript according to the comments. According to the editor's opinion, the previous comments were re-revised and answered point by point and highlighted in blue in the text. Also, the answers to current comments are highlighted in yellow in the text and the detailed corrections are listed below;

## Previous comments

## Reviewer 1#

1.

**Response**;

This comment was somewhat ambiguous.

1. While determination of components and biological effects of the essential oils obtained from different plant species in the genus *Artemisia* has been regarded as the subject of several studies (Studies on pharmaceutical ethnobotany in the region of Turkmen Sahra, north of Iran (Part 1): General results), research specifically

focusing on the effect of different growth stages on the contents of the essential oil and its toxicity performance is relatively limited in this genus.

Refer to page 3, Objectives section.

2. It is necessary to specify the number of repetitions of experiments.

**Response**;

The number of repetitions of experiments was added to the text.

3. There are many problems in the cytotoxicity test steps.

**Response**;

The cytotoxicity test section was revised totally.

4. Report the SD value for all quantitative data.

**Response**;

Quantitative data of cytotoxicity results and EOs yields within text were modified.

-refer to page 1, the abstract section.

-refer to page 5, the Statistical Analysis and phytochemical analysis sections.

-refer to page 14, the Cytotoxicity results section.

-refer to table 1, the EOs yields results.

-refer to table 2, the $IC_{50}$ results.

5. It is necessary to mention the source of the materials used in a separate section.

**Response**;

Corrections were made and highlighted in the relevant section.

Refer to page 3, material section.

6. The reviewed methods are not based on valid references.

**Response**;

Valid references were added to essential oil preparation (ref 14), GC/MS analysis (ref 15 and 16), and MTT assay (ref 17 and 18).

## Reviewer 2#

1. Why did you extract essential oil from the aerial part of the plant in all cases? (Naturally, at different times, according to the presence of flowers or fruits, the composition of aerial organs is different).

**Response**;

- Most of the previous studies on the *Artemisia* genus were on the aerial parts of the plant (21).
- Furthermore in traditional Iranian medicine, the aerial parts of this genus are used for therapeutic purposes (9, 20).
- Another reason for choosing the aerial part is that the aerial parts of *A. biennis* contain a higher concentration of essential oils compared to other parts of the plant such as the roots. Also, in this study, because the aerial parts were harvested in different months, the flowers and fruits of the plant are also included. For example, the sample harvested in August and October contained flowers and fruits, respectively. Choosing four different harvest times includes all the different stages of growth of this plant.

-refer to page 6, the phytochemical analysis section.

2. Explain the reason for choosing these cell lines.

**Response**;

Corrections were made and highlighted in the related section.

-refer to page 13 and 14, the Cytotoxicity Results section.

3. Mention the herbarium number of the plant.

**Response**;

Herbarium number were added to the Preparation of EO section and highlighted.

-Refer to page 4, Preparation of Eos section.

4. 2 figures are mentioned in the article, but these figures are not available in the article.

**Response**;

Figures were added to the article.

3. A normal cell line must be added to the article to make a better conclusion.

**Response**;

A normal cell line was added to the article.

6. Add the following references;

- Cytotoxic and apoptotic effects of different extracts of *Artemisia biennis* Willd. On K562 and HL-60 cell lines

- Preparation and characterization of pullulan-soy protein concentrate blended film incorporated with *Zataria multiflora* and *Artemisia biennis* essential oils

**Response**;

The suggested references were added to the text with ref 50 and 11.

## Current comments

## Reviewer #1

Dear Editor

Have a nice day.

The addressed issues and concerns have not been effectively resolved. In my opinion, this manuscript cannot be considered for publication.

We appreciate all the time that was taken by reviewer to read the manuscript and provide us with his/her comments.

## Reviewer #2

The manuscript was reviewed. Despite having some interesting results suitable for publication in this journal, the text still suffers from severe problems that require a major revision:

We are grateful for the reviewer diligent review of the paper and the valuable suggestions provided. The manuscript was completely revised again and major corrections were made.

1. The English is not of appropriate standard and contains numerous errors. An edited copy is attached which shows errors just as examples. The whole text needs severe revision and attention.

**Response**;

> The entire text was rewritten. Text was revised based on journal guidelines and the grammar and language checked by Grammarly.

2. References cited in the text do not match the references at the end of the manuscript. For example, see reference 58.

**Response**;

Thank you for your attention. References were checked and replaced with appropriate references.

2. I have proposed some better related references that are not used by authors.

**Response;**

Suggested references have been used in manuscript.

1. Iran J Pharm Res. 2014 spring; 13(2): 551–559.PMCID: PMC4157030 PMID: 25237350. Total Phenolic Contents and Antioxidant Activities of Different Extracts and Fractions from the Aerial Parts of Artemisia biennis Willd

Refer to ref 2

2. Analysis of the Essential Oils of Five Artemisia Species and Evaluation of their Cytotoxic and Proapoptotic Effects

Refer to ref 4

3. Comparative Phytochemical Analysis of Essential Oils from Different Biological Parts of Artemisia herba alba and Their Cytotoxic Effect on Cancer Cells Mounir Tilaoui,Hassan Ait Mouse,Abdeslam Jaafari,Abdelmajid Zyad Published: July 21, 2015 https://doi.org/10.1371/journal.pone.0131799

Refer to ref 6

4. Comparison of the cytotoxic effects of different fractions of Artemisia ciniformis and Artemisia biennis on B16/F10, PC3 and MCF7 Cells Ramazani, Elham1,2; Tayarani-Najaran, Zahra3; Shokoohinia, Yalda4,5; Mojarrab, Mahdi4,6, Author Information Research in Pharmaceutical Sciences 15(3):p 273-280, June 2020. | DOI: 10.4103/1735-5362.288434

Refer to ref 7

4. Not all of the changes claimed by authors (in the response letter of authors) are addressed in the revised manuscript.

The previous comments were re-revised and answered point by point and highlighted in blue in the text. Additionally, the answers to current comments are highlighted in yellow in the text.

## 3 Revision 2

1 comment · 30 Jul 2024

### Associate Editor

ACCEPT 30 Jul 2024

Thank you for submitting your valuable manuscript to JJNPP.
I am glad to inform you that your paper has been accepted for publication in this journal.

We hope the authors will consider JJNPP as a potential journal for future publications and cooperate as reviewers for this association.

Best regards