Journal of King Saud University - Science 36 (2024) 103241

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Journal of King Saud University - Science

journal homepage: www.sciencedirect.com

The antiproliferative effects of *Marrubium vulgare, and* toxicity screening in zebrafish embryos

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ARTICLE INFO

Keywords:
Medicinal plants
Marrubium vulgare
Lamiaceae
Toxicity effects
Breast Cancer Cell

ABSTRACT

Consider the medicinal usefulness and ethnomedicinal uses referring to *Marrubium vulgare;* the CH_3OH extract of whole plant was investigated for the antiproliferative, effect and toxicological screening. Its antiproliferative effect was evaluated against A549 (lung), HepG2 (Liver), MDA-MB-231 (breast) and MCF-7 cancer cell-lines. Doxorubicin was used as a positive control. Toxicological evaluation of the *Marrubium vulgare* methanol extract (MVM) was carried out in zebrafish embryos. The MVM showed more effective inhibition against MCF-7and MDA-MB-231 breast-cancer cells. The IC_{50} value ranged from 43.5 to 223.5 μg/mL. In term of IC_{50} MVM showed a strong antiproliferative effect averse to MDA-MB-231 and MCF-7 in terms of IC_{50} values (43.5 and 46.5 μg/mL respectively). Lethal concentration of MVM in zebrafish embryos remained 5 μg/ml. The results obtained from zebrafish embryos tests indicates that the extract is toxic at some level and should be used carefully or avoid it during the pregnancy.

1. Introduction

Medicinal plants have developed and are developing as universal field of research, getting attraction and have impact on health system of the world. Herbal medicine has performed a vital part in the upkeep of the health protection structure of the people around the globe (Theodoridis et al., 2023). Herbal resources have persisted an essential part of mankind across the ages. World Health Organization (WHO) form an opinion that over eighty percent (80 %) of the developing nations occupants exercise conventional phytomedicines (Aziz et al., 2018).

Herbal medicinal products are exercise for the medicaments of varied infections. These herbs play a part as a motivating force for neoteric curative phytonutrient. The health-giving worth of medicinally important plants is attributable to the existence of a large array of natural products namely terpenoids, tannins, glycosides, alkaloids and volatile oil. Health-giving plants and its extracts depict a bountiful foundation of crude drugs that blessed with medicative healthful (Alqahtani et al., 2022). *Marrubium vulgare*, habitually familiar as white horehound, is a perennial herbaceous plant be included to the "family Lamiaceae". It is native to Europe, northern Africa, and western Asia. This plant is well-familiar for its historical uses in folk medicine, particularly for its potential medicinal properties. *Marrubium vulgare* has an extensive history of usage in herbal medicine. Some of its traditional and potential medicinal uses include Respiratory Health: It has been used to alleviate respiratory conditions, such as coughs, colds, and bronchitis, due to its expectorant and cough-suppressant properties. White horehound has been employed to treat digestive issues like indigestion and flatulence. It may have mild anti-inflammatory effects and has been used to ease mild inflammation. *Marrubium vulgare* is customarily a considerable footing for the industries of food and curative. To give you an idea, only in India, there exists thirty-three enlisted botanical products comprehend of white horehound. This plant is worth source of phytoconstituents. (Aćimović et al., 2020; Ullah and Alqahtani, 2022). White horehound has a bitter taste, which is believed to stimulate the production of digestive juices and may contribute to its digestive benefits (Chaachouay et al., 2019). Besides this, *Marrubium vulgare* is named očajnica in Serbian language, which means "desperate woman" its tea hold to be best medication for the women impotent to conceive. In addition to its

Peer review under responsibility of King Saud University

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https://doi.org/10.1016/j.jksus.2024.103241
Received 21 September 2023; Received in revised form 1 May 2024; Accepted 6 May 2024
Available online 7 May 2024

medicinal uses, *Marrubium vulgare* has been used in traditional herbal remedies for various ailments, including diabetes, menstrual problems, and skin conditions. Its decoction with honey provides relief in the respiratory problem like cough and bronchitis. (Aćimović et al., 2020; Steinmetz, 1954). *Marrubium vulgare* is a rich source of different classes of natural compounds like Terpenoids (Marrubic acid, Sacranoside A, Vulgarin, Marrubiin, 12(S)-hydroxymarrubiin3-Deoxo-15(S)-methoxy-velutine, 11-Oxomarrubiin, Vulgarol, Dihydroperegrinin, Marrulibaacetal, Premarrubiin, Cyllenin A, Peregrinol, Polyodonine, Preleosibirin, Marrubenol, Oleanolic acid, Vulgarcoside A, Deacetylvitexilactone, β-sitosterol, Peregrinin, Deacetylforskolin, Carnosol, Lupeol, Coumarins (Aesculin, Umbelliferone), Phenolic Compounds (Gallic acid, Protocatechuic, Gentisic, p-Hydroxybenzoic, Syringic Ferulic, Caffeic-p-Coumaric, o-Coumaric, Sinapic, Rosmarinic, Caffeoylmalic and Chlorogenic acids), glycosides (Acteoside, Alyssonoside, Arenarioside, Ballotetroside, Forsythoside B, Leucosceptoside A, Marruboside, Samioside), Flavonoids (Naringenin, Naringin, Acacetin, Apigenin, Chrysoeriol, Diosmetin, Ladanein, Luteolin, Kaempferol, Quercetin, Galangin, Apigenin, Luteolin etc (Aćimović et al., 2020). A good many medicative possessions of *Marrubium vulgare*; extracts have been scrutinized. As a favorable antioxidant source, *Marrubium vulgare*, manifest to be applicable in medicament of liver diseases, cancer, and diabetes mellitus. Along with, a large number of researches designated that this plant owns hemostatic and anti-inflammatory potential, as well as sedative, antihypertensive and antimicrobial properties. These activities may be referred to its rich phytoconstituents nature (Aćimović et al., 2020).

The zebrafish (Danio rerio) is an appropriate model for analysis of drugs suitability and its usages to treat human diseases based on phylogenetic examination of fish and human genomes, which displays alike physiology and morphology of the nervous, cardiovascular, and digestive systems (Han et al., 2018). Zebrafish also provide a significant data file for vertebrate animals that enables researchers to anchor biochemical, genetic, and cellular hypotheses to high-performance observations at structural, functional, and behavioral levels (Garcia et al., 2016). The zebrafish embryotoxicity test, or fish embryotoxicity test (FET), is gaining popularity because it provides a total and well-defined developmental duration for a vertebrate embryo and allows the study of its early life stages (Hermsen et al., 2011). Zebrafish toxicity studies range from assessing the toxicity of bioactive compounds or crude extracts from plants to determining the optimal process. Most of the studied extracts were polar, such as ethanol, methanol, and aqueous solutions, which were used to detect the toxicity and bioactivity (Modarresi Chahardehi et al., 2020).

Keeping in mind the importance of *M. vulgare* this study was sketch to scrutinize the teratogenicity of varied extracts formulated from the leaves of *Marrubium vulgare* in the embryos of zebrafish together with its apoptotic effectiveness averse to the most perceptive cancer cells.

2. Material and methods

2.1. Plants material

The plant *Marrubium vulgare* were gather from the Hawdaf Sudayeer Dam on 18-March 2017 and was recognized by a taxonomist Rifayat Ullah, at College of Pharmacy; King Saud University Saudi Arabia. "The specimen vouchers were deposited in the Herbarium of the Medicinal Aromatic and Poisonous Plants Research Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, with voucher numbers MV-2019".

2.2. Extraction and fractionation

The total plant material (1 kg) was made dry in shad and pulverize into crumb with the help of grinder machine and at last sank in CH_3-OH (5 L) for 10 days. It was agitated every bit and at last CH_3-OH was vaporized through rotary evaporator (Buchi, Switzerland); bath temperature 40–45 °C. The consequential CH_3-OH residue or extract (80 g) was fractionating-by-separating funnel into "*n*-hexane, chloroform, ethyl acetate and water fractions". The main methanol extract was gaged for Zebra fish and toxicological activities. The remaining fraction will be assessed in the next level of investigation.

2.3. MTT antiproliferative assay

A549 (lung, ACC 107), HepG2 (Liver, ACC 180), MCF-7 (breast, ACC 115) and MDA-MB-231 (breast, ACC 732) cancer cell-lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). The cells were grown in DMEM media (Gibco, USA) supplemented with 10 % FBS (Gibco, USA) and 100 U/mL penicillin/streptomycin (Gibco, USA). MTT assay (Thermo Fisher, Carlsbad, CA, M6494) was performed according to Nasr et al., (Nasr et al., 2020). In brief, cells with a density (50,000 cells / ml) were put on microplate (96 wells) and incubated for 24 h in the CO_2 incubator at 37 °C. Then, cells were exposed to various concentrations (50, 100, 150, 200, 250 μg/ mL) of extract as well 0.1 % DMSO (Sigma Aldrich, USA) which was served as a vehicle and further incubated for 48 h. After that, 10 μL of MTT (5 mg/ml) was-added was added in each well and incubated for 4 h. The formazan product of MTT reduction was dissolved in acidified isopropanol and reading of the results was done using Elisa reader (Bio-Tek, USA) at 570 nm wavelength. Doxorubicin was-used as a positive-control. The IC_{50} value was defined as the concentration of tested extract resulting in a 50 % reduction of cells growth. The IC_{50} values were determined by Origin Pro 8.5 software (Origin Pro software, USA). from a dose–response curve. Each experiment was performed in triplicate. The percentage of viable-cells was calculated-by-means of the-formula:

$$Cellviability(\%) = (Aoftreatedcells/Aofcontrolcells) \times 100$$

2.4. Animals husbandry and breeding

AB/Tuebingen TAB-14 (Wild type zebrafish strain) were got from international zebrafish main supply center and maintained at the "Department of Zoology, King Saud University, Riyadh, Kingdom of Saudi Arabia following guidelines of national and international Institutional Committees for the care and use of laboratory animals. The fertilized embryos were acquired by natural pairwise breeding of adult fish. The fertilized embryos were organized, dead embryos were removed and synchronous stage embryos were used for evaluation".

2.5. Ethical permission

In the current investigation the embryo toxicity of age up to 72 hrs involved and ethical permission was not compulsory for the study. Some studies reported less than five-day embryos do not required ethical permission. According to the US National Institutes of Health, zebrafish are treated live animals at hatching, which is approximately 72 h after fertilization (NIH Animal Program Director Guidelines for Zebrafish Larvae Incubators, n.d.; Gunatilake, 2019; Mendis et al., 2018; Strähle et al., 2012).

2.6. Preparing of stock solution

A stock solution of 25 mg/ml was made by dissolving the extracts in molecular biology grade DMSO (Sigma Aldrich).

2.7. Embryo treatment

Zebrafish embryos were exposed to serial dilution (0.5 1, 5, 15, 45, 150, and 300 μg/ml) of each plant extract. The embryos were remained exposed to the extracts for 3 days with replacement of embryo media

with fresh compound daily. Be noted that "the response of the embryos towards mortality, and embryonic toxicity (teratogenicity) was monitored once after 12 h and then after every 24 h" until the end of experiment. The experiment was repeated at least three times by using embryos obtained from different batch of breeding fish every time.

2.8. Statistical analysis

Data was analyzed for statistical significance using the student's t-test (Origin Pro software, USA). Values of each triplicate experiment are presented as mean ± SD.

3. Results and discussion

3.1. MTT antiproliferative assay

The extract showed a concentration dependent growth inhibition against all tested cancer cell lines (Fig. 1). The MVM showed more effective inhibition against MCF-7 and MDA-MB-231 breast cancer cells. As observed in (Table 1) IC_{50} ranged from 43.5 to 223.5 µg/mL. in terms of IC_{50} values, MVM showed a strong antiproliferative effect with IC_{50} values 43.5 and 46.5 against MCF-7and MDA-MB-231 respectively.

3.2. Zebrafish toxicity

3.2.1. MVM extract induced lethality in zebrafish embryos at concentration in micromolar range

Serial dilution of the extracts was exposed to Zebrafish embryos starting from 50 % epiboly stage (6 h post fertilization). The LD_{50} of MVM extract was calculated using probit analysis program and it was 5 µg/ml in wild type zebrafish embryos.

3.2.2. MVM extract induced teratogenic effect at sub-lethal concentration in zebrafish embryos

In order to investigate whether, MVM induce any abnormalities zebrafish embryonic development, zebrafish embryos were treated with sublethal concentration (a concentration at which maximum embryos survive) i.e 1 µg/ml. MVM induced various developmental defects and phenotypes in treated embryos. The developmental defects are depicted in Fig. 1. As it is evident from Fig. 2, the untreated (control) or mock (0.5 % DMSO) treated embryos did not show any developmentally delay or teratogenic phenotype and developed normally having normal

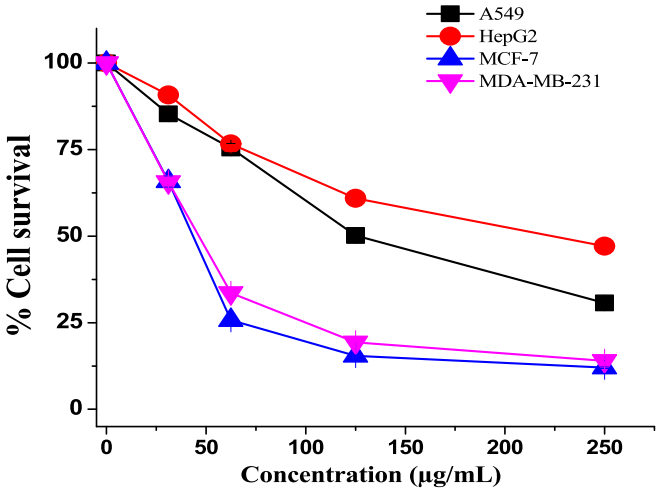

Fig. 1. Assessment of cell proliferation in A549, HepG2, MCF-7 and MDA-MB-231 cells treated with MVM by MTT assay. Cells were treated with different concentration of extract for 48 h.Values represents mean ± SD of three independent assays each with three replicates.

Table 1
The IC_{50} value of MVM extract as determined by MTT assay.

Sample	Cell lines and IC_{50} (µg/ mL)			
	A549	HepG2	MCF-7	MDA-MB-231
MVM	124.9 ± 0.6	223.5. ± 2.0	43.5 ± 1.0	46.5 ± 1.1
Doxorubicin	1.2 ± 0.2	1.3 ± 0.48	1.1 ± 0.2	1.2 ± 1.6

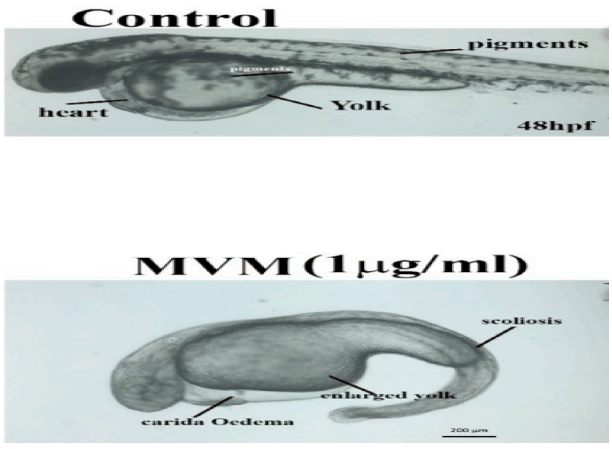

Fig. 2. Developmental defects induced by MVM extracts in zebrafish embryos.

pigmentation and yolk size. More than 90 % of mock treated embryos hatched around 48hpf same wise; the untreated control. Those embryos also had normal heart beat and circulation. The zebrafish embryos treated with methanol extract of MVM (1 µg/ml) did not show pigmentation and also possessed severe developmental delay (Fig. 2). The treated embryos kyphosis and scoliosis having curved bodies. The treated embryos had severe cardiac hypertrophy, absence of circulation and they died around or after 72hpf. The results obtained from zebrafish embryos screen indicates that these extracts are very toxic and should be practiced carefully during the pregnancy.

Plants residue or extract, herbal formulation and any drug which is planned to be practiced in individual essentially foremost be tried in appropriate experimental animals for the purpose to examined its wellbeing. Plants comprehend natural products that are very definite and occasionally awfully poisonous at high or low dose, and signify a captivating collection of bioactive phytochemicals with a wide-ranging activity in the state of human cells, parasites, fungi and bacteria. While white horehound has a history of use in herbal medicine, it's important to consult with a healthcare professional before using it for any medicinal purposes, as its safety and efficacy may vary from person to person, and it may interact with certain medications or have side effects in some individuals.

Marrubium vulgare is being utilized in countless traditional herbal formulations. Though there are not numerous toxicological researches, however, the existing data to date advises that *Marrubium vulgare* did not tempt toxicity in experimental animals. The current findings have revealed-strong- antiproliferative effects of *Marrubium vulgare* CH_3OH-extract on cancer cell-lines, B16-melanoma and U251-glioma, while exerting-minimal effects on primary-immune-cells (PBMCs). Although several studies revealed the ability of methanolic-extracts or essential-oils derived from plants belonging to the *Marrubium* genus to exert-antitumor effects against different tumor-cells (Hamedeyazdan et al., 2012; Hamedeyazdan et al., 2014; Paunovic et al., 2016). These results are strongly agreed with our results that The MVM showed more effective inhibition against MCF-7 and MDA-MB-231 breast cancer cells. Similarly, IC_{50} ranged from 43.5 to 223.5 µg/mL. in terms of IC_{50} values, MVM showed a strong antiproliferative effect with IC_{50} values 43.5 and 46.5 against MCF-7 and MDA-MB-231 respectively. Our previous study

reported 42 phytoconstituents in the methanol extract by GCMS-analysis of *Marrubium vulgare* methanol extract (MVM). Be noted that "the versatile and vast pharmacological effects of medicinal plants are completely dependent on their phytochemical constituents" (Ullah and Alqahtani, 2022; Kaushik et al., 2021). The presence of different phytochemicals in *Marrubium vulgare* plants in literature are reported (Aćimović et al., 2020) confirmed our study about the rich phytoconstituents nature of *Marrubium vulgare*. These are the plant metabolites responsible for different pharmacological potential. The numerous phyto compounds of diverse classes such as phenylpropanoids esters (ballotetroside; forsythoside B, acteoside and arenarioside); diterpene (marrubiin) have been isolated and recognized from the *Marrubium vulgare* extracts. Marrubiin is a natural chemical compound found in various plants, most notably in the plant *Marrubium vulgare*. Marrubiin is known for its potential medicinal properties and has been used in traditional herbal medicine for various purposes. Some of its reported uses and properties include: anti-microbial, hypotensive effect, cough and respiratory relief, antioxidants, anti-inflammatory and vasorelaxant. The additional reported diterpenes from the extract of *Marrubium vulgare* such as marrubenol and marrubinic acid also displayed antiedematogenic and analgesic effectiveness. Furthermore, the numerous phenylpropanoid esters of the *Marrubium vulgare* exhibited noteworthy anti-inflammatory effectiveness owing to the inhibition of thecyclooxygenase-2 (COX-2) enzyme (Singh et al., 2022). Other results specified that healing afterward the injury stood more noteworthy through the extract of plant as related to bioactive constituents alone. Such outcomes may well be owing to the occurrence of pharmacologically significant natural compounds counting marrubiin, in the extract of *Marrubium vulgare*. *Marrubiin* was described to block the L-type-calcium channel sand consequently reveal the ex-cytotoxicity and vasorelaxant effect of plant. Likewise, the inhibition of the section-channels possibly will lower the instigation of occupant microglial-cells and therefore lower the release of proinflammatory cytokines (IL- 1α, IL-6) (Schlemper et al., 1996; Kanyonga et al., 2011). Besides, the previous studies recognized that the diverse phytoconstituents such as ballotetroside, forsythoside B, acteoside renarioside, and as phenyl-propanoid-esters "existing in the extract can prevent the cyclooxygenase-2(COX-2) enzymes and thus are accountable for the anti-inflammatory" potential of plant (Singh et al., 2022). The current pharmacological effectiveness can be attributed or due to the antioxidant and antimicrobial potential of MVM (Ullah and Alqahtani, 2022). Our findings to the ability of MVM to inhibit proliferation, are in complete agreements with the results published by a team of researcher (Paunovic et al., 2016). However, we are reporting the developmental toxicity associated with methanolic extract of *Marrubium vulgare* for the first time which warrants further investigation to carefully analyze the *in vivo* developmental toxicity in suitable animal models and also selection of safe phytoconstituents to be used either as antiproliferative or folk medicine at least during pregnancy to avoid possible side effects in developing fetus.

4. Conclusion

By signifying the ability of MVM to inhibit-proliferation, our results suggest that this extract, could be a valuable source for promising constituents that could be used for cancer treatment. The *in vivo* toxicity screening in zebrafish embryos suggest further investigation are needed to analyze the *in vivo* developmental toxicity in suitable animal models and also selection of safe phytoconstituents to be used either as antiproliferative or folk medicine at least during pregnancy to avoid possible side effects in developing fetus.

CRediT authorship contribution statement

Riaz Ullah: Conceptualization, Project administration, Supervision. **Ali S. Alqahtani:** Data curation, Formal analysis, Funding acquisition. **Abdelaaty A. Shahat:** Investigation, Methodology, Validation, Visualization. **Fahd Nasr:** Formal analysis, Methodology, Writing – original draft. **Mohammad A. Wadaan:** Resources, Software, Supervision, Validation. **Muhammad Farooq:** Investigation, Methodology, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The Authors are thankful to the Researchers Supporting Project Number (RSP2024R132), at king Saud University Riyadh Saudi Arabia for financial support.

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