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In Vitro Antimicrobial and Cytotoxic Activities of Verbena officinalis L. Extracts: Preliminary Screening and Dose–Response Evaluation in MCF-7 Breast Cancer Cells

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Abstract

Background: *Verbena officinalis L.* is a medicinal plant with a variety of secondary metabolites and described biological activities. Although previous investigations have demonstrated antimicrobial and antiproliferative properties of preparations derived from *V. officinalis*, biological responses may vary considerably depending on polarity of the solvent, extraction conditions and experimental models. Further evaluation of various plant extracts could help to understand their possible biological activities. **Objective:** The objective of this study was to evaluate concentration dependent cytotoxic responses of MCF-7 human breast cancer cells after exposure to hexane and ethyl acetate fractions and to study the antimicrobial activity of aqueous and methanolic extracts of *V. officinalis* against selected microorganisms. **Materials and Methods:** Aqueous and methanolic extracts were prepared from the leaves and stems of *V. officinalis*. Antimicrobial activity was assessed using the agar-well diffusion method against *Staphylococcus aureus*, *Streptococcus sobrinus*, *Klebsiella pneumoniae*, and *Candida albicans* at nominal extract concentrations of 12.5%, 25%, 50%, and 100%. The cytotoxic responses of MCF-7 cells were evaluated using the MTT assay following exposure to hexane and ethyl acetate fractions at concentrations ranging from 10 to 300 µg/mL, with vincristine included as a reference treatment. The available data were evaluated using descriptive statistics and exploratory concentration–response modeling. **Results:** The aqueous extract produced inhibition zones of 13–14 mm against *S. aureus* and 13–15 mm against *S. sobrinus* at the two highest tested concentrations. The methanolic extract produced inhibition zones of 13–15 mm against

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S. sobrinus. No measurable inhibition was reported against *K. pneumoniae* or *C. albicans*. In the MTT assay, both plant fractions demonstrated concentration-associated inhibition of MCF-7 cellular metabolic activity. At 300 µg/mL, mean inhibition reached 63.6% for the hexane fraction and 49.6% for the ethyl acetate fraction, compared with 93.9% for vincristine. Calculated IC50 values IC50 values were 234.99 µg/mL for hexane, 274.10 µg/mL for ethyl acetate, and 21.25 µg/mL for vincristine. Exploratory reanalysis yielded different apparent IC50 estimates, particularly for the ethyl acetate fraction. **Conclusion:** It was shown that under the conditions of the experiment the preparations of verbena officinalis had a selective antimicrobial action and inhibited the metabolic activity of MCF-7 cells depending on the concentration. Overall, the hexane fraction showed more inhibition than the ethyl acetate fraction. These preliminary results are an incentive for further standardized studies to assess the biological significance and repeatability of the responses found.

Keywords: Antimicrobial activity; medicinal plants; MCF-7; *Verbena officinalis*; MTT assay; dose–response; breast cancer; cytotoxicity; IC50.

Introduction

Medicinal plants have played an important role in the discovery and development of pharmacologically active substances and continue to be important sources of structurally diverse natural products. Biological properties are often associated with secondary metabolites, such as phenolic compounds, flavonoids, terpenoids, alkaloids and glycosides. These compounds have been studied by scientists because they have been reported to have antioxidant, antimicrobial, anti-inflammatory and antiproliferative properties. The biological efficacy of the plant-derived preparations, however, depends on several factors like the biological models used for evaluation, the extraction methods, the chemical composition, the experimental conditions and the botanical origin. The continuous quest for compounds that affect the growth and survival of cancer cells and the increasing problem of antimicrobial resistance have promoted further research on medicinal plants as a source of bioactive molecules. Screening of natural products is an essential first step to reveal biological activities that may need further chemical and mechanistic investigations. However, laboratory screening assays should not be taken as an indication of clinical efficacy, as further investigations to address selectivity, safety, pharmacological mechanisms and reproducibility are necessary for the translation of experimental observations into

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therapeutic applications. *Verbena officinalis L*. is a perennial herb of the *Verbenaceae* family. Common name: common vervain. It has been used traditionally in a number of herbal preparations and has attracted scientific interest due to its reported pharmacological properties. The description of antioxidant, anti-inflammatory, antimicrobial and other biological activities in *V. officinalis* [1] justifies continuing the investigation of the plant as a source of potential active natural constituents. *V. officinalis* contains phytochemicals belonging to different classes of secondary metabolites. The constituents most frequently reported are iridoid glycosides (verbenalin and hastatoside) and phenylethanoid glycosides (in particular verbascoside and isoverbascoside). Flavonoids and phenolic acids have also been identified in different preparations of the plant [1,2]. These are chemically diverse groups and may be responsible for the biological properties observed in the experimental studies. However, their relative abundance and presence can be influenced by the plant's geographical origin, environmental conditions, developmental stage, extraction solvent and processing methodology.

Previous phytochemical studies have shown that the chemical profiles of *V. officinalis* can be significantly affected by different extraction and cultivation conditions. Kubica et al. [2] investigated the phenylpropanoid glycosides and phenolic acids present in extracts of biomass obtained from different *V. officinalis* cultures and plants grown in soil, showing a variety of phytochemical composition and biological activity. These results underline the importance of considering the extraction conditions when interpreting the experimental responses and comparing the results among studies. Biological properties of medicinal plant extracts, including antimicrobial activity, are investigated. Plant-derived preparations may contain constituents that can affect microbial growth by various biochemical and cellular interactions. However, the antimicrobial responses are highly dependent on the physicochemical properties of the extract and the characteristics of the microorganism tested. The microbial responses may differ depending on the cell envelope structure, membrane permeability and susceptibility to certain compounds. Moreover, the results of the agar diffusion assays are affected by the solubility and diffusion properties of the tested preparations. Therefore, the preliminary antimicrobial screening is a useful tool to provide information of the presence or absence of measurable growth inhibition under predetermined experimental conditions, but it does not independently determine the therapeutic relevance or the antimicrobial potency. Further quantitative susceptibility

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testing and appropriate experimental controls are required to more accurately define the inhibitory effects of plant-derived preparations. Apart from anti-microbial studies, *V. officinalis* has been studied for the antiproliferative activity in experimental cancer models. Phenylethanoid glycosides from V. officinalis inhibited the proliferation of colon cancer cell lines, according to Encalada et al. [3]. This means that some constituents of plants may have an effect on cancer cells growth. Similarly, the in vitro antimelanoma activities of *V. officinalis* fractions were assessed by Nisar et al. [4]. This is further evidence for the significance of investigating the biological effects of various plant preparations on cancer cells.

These investigations provide a scientific basis for the study of *V. officinalis*. Each type of cancer has its own molecular characteristics, metabolic functions, and responses to biological agents. Hence, the effects of a particular plant extract or fraction may vary significantly between cellular models. Breast cancer is a significant field of research in experimental oncology and the biological effects of potential antiproliferative drugs are frequently evaluated using established human breast cancer cell lines. These models are commonly used in laboratory investigations as they provide a reproducible experimental system for assessing cellular responses to chemical and biological treatments, with MCF-7 cells being a popular choice among these models. The MTT assay is a colorimetric assay commonly used to determine the metabolic activity of cells. This is based on the reduction of the tetrazolium compound MTT to a colored formazan product by metabolically active cells. Changes in the measured signal after treatment can be indicative of altered cellular metabolic activity and viable cell number. However, the assay itself cannot distinguish between cytostatic effects, metabolic suppression and different cell death mechanisms.

The interpretation of MTT results therefore requires careful consideration of experimental controls, treatment concentrations, exposure conditions, and statistical methodology. In particular, concentration–response modeling may provide useful estimates of apparent inhibitory potency, but the reliability of such estimates depends on the quality and distribution of the experimental observations, the suitability of the selected model, and the availability of independent biological replicates. Despite the growing literature concerning the phytochemical composition and biological activities of *V. officinalis*, the responses of different experimental models to specific extract preparations remain an important area for investigation. Previous studies have examined antiproliferative activity in colon cancer and melanoma models [3,4],

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whereas the present investigation focuses on the reported responses of MCF-7 breast cancer cells to hexane and ethyl acetate fractions. In parallel, aqueous and methanolic extracts were evaluated against selected microorganisms to provide an additional preliminary assessment of biological activity.

This work is a contribution to science as it describes these experimental responses and attempts to study the changes in MCF-7 cell inhibition related to concentration. Methodological limitations are also discussed, focusing on the absence of experimental documentation and interpretation of the available IC50 estimates. Such an approach enables the presentation of the results according to the strength of evidence available while drawing attention to areas where further experimental validation is required. Therefore, the present study was designed to evaluate the preliminary antimicrobial activity of aqueous and methanolic extracts of *V. officinalis* against some bacterial and fungal organisms and to study the inhibition of MCF-7 cellular metabolic activity in a concentration-dependent manner when treated with hexane and ethyl acetate fractions. The study also aimed at evaluating the consistency of the available concentration–response estimates and at comparing the reported inhibitory responses with those of vincristine.

2. Materials and Methods

2.1. Study Design

The present study was an in vitro experimental study designed to evaluate the antimicrobial activity and cytotoxic potential of extracts and fractions of *Verbena officinalis L*. The experimental work was performed in two parts: concentration dependent effects of hexane and ethyl acetate fractions on MCF-7 human breast cancer cell line and screening of aqueous and methanolic extracts for antimicrobial activity against selected microorganisms. The antimicrobial component was determined by the agar-well diffusion method for the presence of the zone of inhibition of microbial growth at different concentrations of the extract. In the cytotoxicity part, the MTT colorimetric assay was used to determine any changes in cellular metabolic activity that occurred after exposure to the plant fractions with vincristine as the reference treatment. Descriptive statistical methods and exploratory concentration–response modeling were used to evaluate the experimental results. The analysis was performed to explain the biological responses observed, but not to infer those for which no experimental evidence was available.

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2.2. Plant Material Collection and Preparation

The botanical raw material used for the preparation of the extract was collected from wild-growing plants of leaves and stems of *Verbena officinalis L*. (family *Verbenaceae*). The plant material, after collection, was cleaned to remove visible impurities, dust and foreign material. The material was then ground into a fine powder to increase the surface area available for solvent extraction and to help recover the extractable plant constituents. The powdered plant material was used to prepare the methanolic and aqueous extracts.

2.3. Preparation of *Verbena officinalis* Extracts

2.3.1. Methanolic Extraction

The powdered leaves and stems of *V. officinalis* were used for the preparation of methanolic extract. The original experimental procedure involved mixing 400 mL methanol with approximately 100 g powdered plant material. The mixture was left for several days to permit extraction of the solvent soluble constituents which were processed with a GFL water bath. The mixture was filtered after the first extraction period to remove the liquid extract from the remaining plant material. The remaining residue was wrapped in filter paper and re-extracted hot in a special extractor with methanol. The recovered extracts were collected, dried and further subjected to biological evaluation.

2.3.2. Aqueous Extraction

For aqueous extraction, about 100 g of powdered *V. officinalis* plant material was mixed with water. The mixture was further treated in a GFL water bath to extract the water-soluble constituents. The mixture was filtered using standard laboratory filter paper and the aqueous extract was separated from the remaining plant residue after extraction. The extract was dried prior to antimicrobial testing. The original documentation did not mention the amount of water, temperature of extraction, time of extraction, concentration method or % yield. Hence, the aqueous extraction method is presented according to the currently available experimental data.

2.3.3. Hexane and Ethyl Acetate Fractions

Hexane and ethyl acetate fractions of *V. officinalis* were evaluated for their cytotoxic effects on MCF-7 breast cancer cells. In the original experimental data set these fractions were identified as separate treatment preparations, solvent-partitioning sequence, extraction ratios, fraction recovery or % yield. As such, throughout the study

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hexane and ethyl acetate preparations are referred to as fractions, based on their original experimental designation.

2.4. Antimicrobial Activity Assessment

2.4.1. Test Microorganisms

The antimicrobial activity of *V. officinalis* extracts was investigated against four microorganisms: *Staphylococcus aureus*, *Streptococcus sobrinus*, *Klebsiella pneumoniae* and *Candida albicans*. The selection of Gram-positive bacteria, a Gram-negative bacterium and a fungal species permitted a preliminary assessment of the inhibitory responses of different micro-organisms to the plant extracts**.** The microorganisms are reported according to the species names that were present in the original dataset.

2.4.2. Agar-Well Diffusion Assay

Antimicrobial activity was evaluated using the agar-well diffusion method, also described as the agar drilling method in the original experimental documentation. The aqueous and methanolic extracts were prepared at four nominal concentration levels: 12.5% , 25% , 50% and 100%. The antimicrobial activity of the extracts was determined against the selected microorganisms and the diameter of the inhibition zones formed around the wells was measured after incubation**.** The diameters of inhibition zones were measured in millimeters (mm)**.**

2.4.3. Antimicrobial Activity

The antimicrobial activity of each extract concentration was evaluated descriptively by the diameters of the inhibitory zones. A measurable zone of inhibition was considered as growth inhibition under the given assay conditions. By contrast, the absence of a measurable inhibition zone was taken as a recorded value of zero rather than as evidence that there was no biological activity or that the microbes were completely resistant.

2.5. In Vitro Cytotoxicity Assessment

2.5.1. MCF-7 Breast Cancer Cell Line

The human breast adenocarcinoma cell line MCF-7 was used as an experimental cellular model to evaluate the effects of *V. officinalis* fractions on the cellular metabolic activity.

2.5.2. MTT Cell Viability Assay

The cytotoxic responses of MCF-7 cells to *V. officinalis* fractions were assessed using the MTT assay. The assay relies on the cellular metabolic reduction of the yellow

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tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a colored formazan product. The resulting colorimetric signal is often used to evaluate the effect of experimental treatments on cell viability and is an indirect measure of cellular metabolic activity.MCF-7 cells were exposed to hexane and ethyl acetate fractions at the following concentrations: 10, 15, 20, 30, 40, 50, 100, 150, 200, and 300 µg/mL.Vincristine was used as reference drug at the same concentration range as previously reported. The original data provided three %-inhibition measurements for each concentration of treatment.

2.5.3. Calculation of Cellular Growth Inhibition

The original experimental data set reported each treatment and concentration as % inhibition values., the %-inhibition values in the original experimental dataset were preserved for the purpose of descriptive analysis.

2.5.4. Evaluation of Concentration-Dependent Responses

The concentration-dependent responses of MCF-7 cells were assessed using the reported inhibition %s for the hexane fraction, ethyl acetate fraction and vincristine. The inhibition response was evaluated for each treatment at concentrations from 10 to 300 µg/mL. Previously IC50 estimates were evaluated for consistency and changes in cellular metabolic inhibition were described using observed concentration–response patterns. The results were interpreted as inhibition of cellular metabolism rather than as direct evidence of a specific cytotoxic mechanism, as the MTT assay cannot independently distinguish between reduced metabolic activity, inhibition of cell proliferation and cell death.

2.6. Statistical Analysis

The available MTT data were analyzed using descriptive statistical methods. The arithmetic mean and sample standard deviation were calculated from the three inhibition measurements at each treatment concentration in the original dataset. Because the three measurements could not be shown to be independent, the standard deviations were treated as descriptive measures of the variation in reported values rather than as estimates of biological variation between experiments. An exploratory concentration–response analysis was conducted to determine the consistency of the IC50 estimates initially reported with the tabulated inhibition data. The mean inhibition values at the concentration level were analyzed using a logit-linear model. The model assumed fixed lower and upper response limits of 0% and 100%, respectively. The reported concentration–response relationships were only assessed by

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this modeling approach in an exploratory way. The concentration that produced 50% inhibition was the IC50 derived from the specified concentration–response model. The exploratory reanalysis values were compared descriptively with the original IC50 estimates. Differences between reported and refitted estimates were taken as indicators of the sensitivity of the model and of possible inconsistencies in available concentration–response documentation. Particular attention was paid to modeled IC50s above the maximum concentration tested experimentally because these estimates are based on extrapolation beyond the range of observed responses.

2.7. Phytochemical Context and Literature-Based Interpretation

To give a scientific background to the biological responses observed, phytochemical studies of *V. officinalis* were used. Special emphasis was placed on previously reported classes of secondary metabolites such as phenolic acids, flavonoids, iridoid glycosides and phenylethanoid glycosides. Contextual interpretation of potential biological activities and solvent-dependent variation in plant preparations was assessed through review of the literature.

2.8. Data Integrity and Reporting Considerations

The results of the experiments were presented according to the numerical values and the methodological information of the original documentation of the study. Original inhibition values and IC50 estimates reported in the sources were retained. The exploratory reanalysis was distinguished from the original experimental calculations. This was done to protect the integrity of the reported findings, maintain transparency and prevent unsupported statistical or mechanistic interpretations.

3. Results

3.1. Antimicrobial Activity of Verbena officinalis Extracts

The antimicrobial activity of *Verbena officinalis L.* extracts was evaluated against four microorganisms, including *Staphylococcus aureus*, *Streptococcus sobrinus*, *Klebsiella pneumoniae*, and *Candida albicans*, using the agar-well diffusion method. The aqueous and methanolic extracts were assessed at nominal concentrations of 12.5%, 25%, 50%, and 100%. The inhibition zones recorded indicated that the antimicrobial responses were selective and dependent on the microorganism, type of extract and concentration (Table 1). Inhibitory activity was detected in the aqueous extract against *S. aureus* and *S. sobrinus*. The microorganisms were inhibited at the concentration of 50% with the inhibition zones of 13 mm. The increase of the concentration to 100% resulted in inhibition zones of 14 mm against S. aureus and 15

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mm against *S. sobrinus* Methanolic extract produced inhibition zones of 13 and 15 mm against *S. sobrinus* at 50% and 100% concentration respectively. Measurable activity was also demonstrated. No inhibition zones were observed for the extract–organism combinations reported in the original data set at 12.5% and 25% concentrations. Furthermore, the extracts did not show any measurable inhibition against *K. pneumoniae* or *C. albicans* at any of the concentrations tested.

Table 1. Antimicrobial activity of Verbena officinalis extracts against selected microorganisms at different nominal concentrations.

Microorganism	Extract type	12.5%	25%	50%	100%
S. aureus	Aqueous	0	0	13	14
S. sobrinus	Aqueous	0	0	13	15
S. sobrinus	Methanolic	0	0	13	15
K. pneumoniae	Both extracts	0	0	0	0
C. albicans	Both extracts	0	0	0	0

Values represent inhibition-zone diameters in millimeters (mm). A value of zero means no inhibition zone was measured. For the original data set neither dispersion measures nor the number of independent experimental repetitions were given. Unlisted combinations should not be interpreted as negative results verified by experiment. The table includes the extract-organism combinations reported in the source. The observed antimicrobial responses were only at the higher concentrations of the extracts. The diameter of inhibition zone was 15 mm for *S. sobrinus*, which was the largest among the responsive microorganisms, in both aqueous and methanolic preparations at 100% concentration.

3.2. Concentration-Dependent Inhibition of MCF-7 Breast Cancer Cells

Hexane and ethyl acetate fractions of *V. officinalis* were found to be effective against MCF-7 human breast cancer cells determined by MTT assay at 10–300 µg/mL. Vincristine was included as a reference drug. The reported inhibition values showed a general increase in MCF-7 cellular metabolic inhibition with increasing concentration of both plant fractions (Table 2). The ethyl acetate fraction had a mean inhibition of 2.7 ± 0.6% at the lowest tested concentration of 10 µg/mL and the hexane fraction had a mean inhibition of 3.9 ± 0.2%. The hexane fraction showed increased inhibition up to 13.5 ± 0.9% at 50 µg/mL and the ethyl acetate fraction showed increased inhibition

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up to 8.5 ± 1.1%. Further increases were observed at 150 µg/mL, where mean inhibition reached 34.0 ± 3.0% and 25.5 ± 3.5% for the hexane and ethyl acetate fractions, respectively. At 300 µg/mL, the highest tested concentration, the hexane fraction produced 63.6 ± 2.3% inhibition, compared with 49.6 ± 6.1% for the ethyl acetate fraction. Vincristine demonstrated higher inhibition %s than both plant fractions across all investigated concentrations. Mean inhibition increased from 32.4 ± 2.1% at 10 µg/mL to 96.2 ± 1.3% at 200 µg/mL, followed by a recorded value of 93.9 ± 4.8% at 300 µg/mL.

Table 2. Concentration-dependent inhibition of MCF-7 cells following treatment with Verbena officinalis fractions and vincristine.

Concentration (µg/mL)	Hexane fraction (%)	Ethyl acetate fraction (%)	Vincristine (%)
10	3.9 ± 0.2	2.7 ± 0.6	32.4 ± 2.1
15	5.1 ± 0.2	3.0 ± 0.6	42.4 ± 1.1
20	5.0 ± 0.2	2.9 ± 0.6	51.3 ± 2.6
30	8.3 ± 0.4	4.8 ± 0.4	62.0 ± 1.5
40	11.8 ± 1.4	7.5 ± 0.8	69.8 ± 1.8
50	13.5 ± 0.9	8.5 ± 1.1	75.0 ± 1.5
100	21.7 ± 1.7	17.2 ± 1.0	83.7 ± 4.2
150	34.0 ± 3.0	25.5 ± 3.5	92.3 ± 2.7
200	42.0 ± 2.2	35.7 ± 3.1	96.2 ± 1.3
300	63.6 ± 2.3	49.6 ± 6.1	93.9 ± 4.8

Values are mean ± standard deviation of 3 readings per concentration in the original data set. The documentation did not allow to determine whether these were technical or independent biological replicates. The hexane fraction showed higher mean inhibition %s than the ethyl acetate fraction at all concentrations studied. The largest absolute difference was 14.0 % points at 300 µg/mL and the largest difference of the average inhibition of the two fractions. However, the response increased with concentration, but some deviations from strictly monotonic pattern were observed at some concentrations. The answer was vincristine 200-300 µg/mL and both fractions 15-20 ug/mL. The results overall suggest a concentration-related response rather than a consistent increase in effect with each increasing concentration.

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3.3. Descriptive Comparison of the Plant Fractions

A descriptive comparison of the inhibitory responses recorded for the two plant fractions over the tested dose range showed differences. At each concentration tested the mean inhibition of the hexane fraction was higher than that of the ethyl acetate fraction. The hexane fraction had an average inhibition of 21.7% at 100 µg/mL, while the ethyl acetate fraction had an average inhibition of 17.2% (4.5 % points difference). At 200 µg/mL, the inhibition was enhanced to 42.0% and 35.7% respectively, with a difference of 6.3 % points. At 300 µg/mL, the difference increased to 14.0 % points.

Table 3. Descriptive comparison of MCF-7 inhibition by hexane and ethyl acetate fractions at selected concentrations.

Concentration (µg/mL)	Hexane inhibition (%)	Ethyl acetate inhibition (%)	Difference (% points)
50	13.5	8.5	5.0
100	21.7	17.2	4.5
150	34.0	25.5	8.5
200	42.0	35.7	6.3
300	63.6	49.6	14.0

The differences between mean inhibition in hexane and ethyl acetate fractions were determined by subtracting the mean inhibition of the ethyl acetate fraction from the mean inhibition of the hexane fraction. These are descriptive differences and not statistically significant treatment effects. The observed pattern suggests that the hexane fraction induces a more pronounced inhibition of the metabolic activity of MCF-7 cells under the experimental conditions reported.

3.4. Evaluation of Half-Maximal Inhibitory Concentrations (IC50)

The original experimental documentation recorded the IC50 values of the hexane fraction, ethyl acetate fraction and vincristine. The estimates were compared to apparent IC50 values derived from exploratory logit-linear concentration-response modeling. The source reports that the IC50 value of the hexane fraction was 234.99 µg/mL, that of the ethyl acetate fraction was 274.10 µg/mL and that of vincristine was 21.25 µg/mL. The exploratory model yielded the apparent IC50 estimates of 258.50, 373.40 and 19.25 µg/mL, respectively.

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Table 4. Comparison of **Calculated IC50 values IC50** and exploratory Recalculated IC50 values estimates for MCF-7 cells.

Treatment	Calculated IC50 values (µg/mL)	Recalculated IC50 values (µg/mL)	Re-fitted slope
Hexane fraction	234.99 ± 5.42	258.50	1.07
Ethyl acetate fraction	274.10 ± 2.25	373.40	1.11
Vincristine	21.25 ± 0.33	19.25	1.15

The original documentation did not define whether the ± values associated with the Calculated IC50 values IC50 estimates represented standard deviations, standard errors, or another measure of uncertainty. The re-fitted estimates were exploratory and were not accompanied by confidence intervals. The Calculated IC50 values estimates indicated a lower apparent IC50 for the hexane fraction than for the ethyl acetate fraction. The exploratory reanalysis preserved this ordering but produced different absolute estimates. For the hexane fraction, the Recalculated IC50 values was 23.51 µg/mL higher than the Calculated IC50 values value. For the ethyl acetate fraction, the Recalculated IC50 values exceeded the Calculated IC50 values estimate by 99.30 µg/mL. The re-fitted estimate for vincristine was 2.00 µg/mL lower than the Calculated IC50 values.

3.5. IC50 Estimates Within the Tested Concentration Range

The concentration–response data demonstrated that the hexane fraction exceeded 50% mean inhibition at the highest tested concentration of 300 µg/mL. At 200 µg/mL, mean inhibition was 42.0%, whereas at 300 µg/mL it reached 63.6%. Thus, the observed mean response crossed the 50% inhibition level between 200 and 300 µg/mL.

In contrast, a 50% mean inhibition was not observed within the concentration range tested for the ethyl acetate fraction. The highest mean inhibition was 49.6% at 300 µg/mL. The exploratory IC50 value of 373.40 µg/mL for the ethyl acetate fraction is thus a model-based extrapolation outside the highest concentration tested experimentally. The Calculated IC50 values ethyl acetate IC50 was 274.10 µg/mL, but the mean responses at each concentration level did not confirm 50% inhibition at that concentration or below. The available summary data were insufficient to resolve this discrepancy. The mean inhibition reported for Vincristine was 42.4% at 15 µg/mL and

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51.3% at 20 µg/mL. Hence, the mean response observed was above 50% inhibition for the concentrations 15-20 µg/mL. The source reported and exploratory IC50 estimates were similar, but their agreement with the observed means of concentration levels depends on the model used for estimation.

3.6. Concentration–Response Visualization

The concentration-dependent inhibitory responses of the hexane fraction, ethyl acetate fraction, and vincristine are summarized in Figure 1. The figure illustrates the overall increase in mean MCF-7 inhibition with increasing treatment concentration. The hexane fraction showed a greater inhibitory response than the ethyl acetate fraction throughout the investigated concentration range. Vincristine demonstrated substantially higher inhibition %s, particularly at lower and intermediate concentrations. Mean inhibition values from the source dataset; concentrations are displayed on a categorical axis.

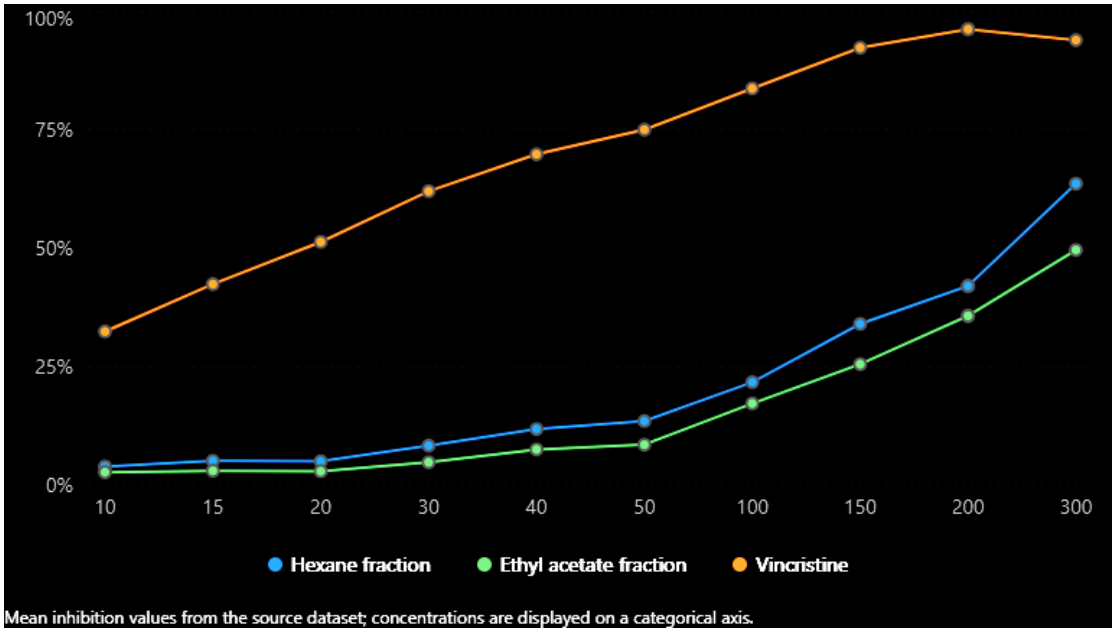


Figure 1. The exposure to Verbena officinalis fractions and vincristine inhibits the metabolic activity of MCF-7 cells in a concentration-dependent manner.

Points are the means of reported concentration levels. Visual guides such as connecting lines are not indicative of fitted concentration–response models. The observed data support a descriptive concentration-dependent inhibitory pattern for both of the plant fractions.

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3.7. Statistical Interpretation and Data Consistency

The available experimental data allowed for the descriptive statistical characterization of the inhibition responses in the MCF-7 cell line but did not contain sufficient information to allow for formal inferential analysis. The data reported were three measurements of inhibition for each concentration and treatment. However, the original paper did not specify whether these measurements were from independent biological experiments or repeated technical measurements. The differences between the Calculated IC50 values and the exploratory Recalculated IC50 values estimates also further illustrate the sensitivity of the results to the analytical approach. The ethyl acetate fraction in particular showed the greatest difference, and the IC50 that was back-calculated was higher than the range of concentrations that were tested. For this reason, the IC50 estimates should be considered as apparent concentration-response parameters, and not as definitive measures of comparative cytotoxic potency.

4. Discussion

4.1. Principal Findings and Their Scientific Significance

This study evaluated the antimicrobial activity of the aqueous and methanolic extracts of *Verbena officinalis L*. against some microorganisms, and the concentration-dependent effects of hexane and ethyl acetate fractions on the MCF-7 cell line of human breast cancer. Results showed selective inhibition of microbial growth and concentration related overall decrease of metabolic activity of MCF-7 cells. These observations are consistent with preliminary evidence of biological activity in the preparations tested, but their interpretation must take into account the variability associated with the extraction. The biological relevance of *V. officinalis* has been extensively discussed in phytochemical and pharmacological literature. Kubica et al. [1] identified iridoid glycosides, phenylpropanoid glycosides, phenolic acids, flavonoids, and terpenoids among the principal chemical groups associated with this species. These compounds provide a biochemical background for studying their anti-proliferative properties and anti-microbial. The aqueous and methanolic extracts were tested on micro-organisms and the hexane and ethyl acetate fractions on MCF-7 cells.

4.2. Antimicrobial Activity and Microorganism-Dependent Responses

The antimicrobial screening demonstrated measurable inhibition against *Staphylococcus aureus* and *Streptococcus sobrinus*, while no inhibition zones were recorded against Klebsiella pneumoniae or Candida albicans under the investigated conditions. This pattern indicates that the observed inhibitory responses depended on

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the tested microorganism and extract preparation. The aqueous extract produced inhibition zones of 13–14 mm against *S. aureus* and 13–15 mm against *S. sobrinus* at the two highest nominal concentrations. Similarly, the methanolic extract produced inhibition zones of 13–15 mm against *S. sobrinus*. The lack of measurable inhibition at lower concentrations indicates that the observed responses were linked to higher nominal extract concentrations. Differences in susceptibility among microbial species may be influenced by cell-envelope characteristics, membrane permeability, intrinsic defense mechanisms, and interactions with individual chemical constituents. Gram-negative bacteria possess an outer membrane that can restrict the penetration of certain antimicrobial compounds. This structural characteristic provides one possible explanation for differences between Gram-positive and Gram-negative organisms, although it cannot independently explain the absence of measurable inhibition against *K. pneumoniae* in the present experiment.

The fungal organism C. albicans is different from bacteria in its cellular organization, membrane composition and biological responses to antimicrobial substances. The present results do not suggest complete absence of antifungal activity or fungal resistance. It has been shown in methodological studies that physicochemical characteristics of plant extracts have an important effect on agar diffusion assays . In particular, compounds that are not very soluble in water may not diffuse well through agar, resulting in little or no zone of inhibition even when they are biologically active in other experimental systems. In particular, diffusion-based methods may give inaccurate estimates of the antimicrobial activity of plant extracts when chemically complex preparations contain constituents with different polarities as highlighted by Eloff [6]. Similarly, comparative methodological studies have demonstrated that broth microdilution provides more consistent quantitative susceptibility measures than diffusion-based methods for some plant extracts and phenolic compounds [7]. Therefore, these zones of inhibition observed in this study are best interpreted as initial evidence of inhibition of microbial growth and not as conclusive proof of antimicrobial activity.

4.3. Phytochemical Diversity and Solvent-Dependent Biological Activity

The chemical makeup of extracts from medicinal plants is tightly linked to their biological attributes, which are affected by the solvent properties and the extraction technique. Different polarity solvents will likely recover different groups of secondary metabolites, which may result in preparations with different biological responses.

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Previous investigations have demonstrated the substantial phytochemical diversity in *V. officinalis*. Kubica et al. [2] investigated the presence of phenolic acids and phenylpropanoid glycosides in extracts from soil-grown plants and microshoot cultures of various plants. The biological properties and chemical profiles of the plant preparations were found to be different, which emphasizes the importance of extraction and cultivation conditions in phytochemical research. In our current study, selective antimicrobial responses of the aqueous and methanolic extracts and the distinct inhibitory profiles of the hexane and ethyl acetate fractions within MCF-7 cells were revealed. These differences are in accordance with the general observation that plant extracts prepared with different solvents may have different biological activities.

4.4. Concentration-Dependent Inhibition of MCF-7 Breast Cancer Cells

The MTT assay showed an overall increase in metabolic inhibition in the MCF-7 cells in a concentration-dependent manner following exposure to both plant fractions. The average inhibition % values of the hexane fraction were always higher than those of the ethyl acetate fraction in the whole range of concentrations studied. Hexane fraction showed a mean inhibition of 63.6% at the highest concentration tested (300 µg/mL) and ethyl acetate fraction showed a mean inhibition of 49.6%. These results suggest that the hexane preparation produced a more marked inhibitory response under the conditions of the experiments reported. The observed differences may reflect a difference in the chemical composition of the fractions, which may include the possible enrichment of components with different physicochemical and biological properties. This is, however, a speculative explanation without chemical profiling or compound-specific testing. Previous studies provide relevant evidence to justify the investigation of *V. officinalis*-derived preparations in cancer-cell models. Encalada et al. [3] investigated phenylethanoid glycosides from *V. officinalis* and observed antiproliferative activity against colon cancer cell lines. Their study showed that the biological responses of phenylethanoid glycosides depend on the structural differences, which indicates that the activity of plant-derived preparations may be greatly affected by their chemical composition.

In another study, Nisar et al. [4] evaluated the antiproliferation activity of *V. officinalis* fractions against melanoma cell lines and identified preparations with high antiproliferation activity. Furthermore, the authors performed mass spectrometric analysis to identify the chemical components present in each fraction. These results are in agreement with the general hypothesis that *V. officinalis* contains compounds that

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can modulate the response of cancer cells. However, results obtained from colon cancer and melanoma models cannot be directly transferred to MCF-7 breast cancer cells. Large differences in observed responses are observed with differences in cellular phenotype, metabolic characteristics, experimental conditions, treatment duration and extract composition. Therefore, the present findings should be considered as preliminary evidence for concentration-dependent inhibition in the MCF-7 model and not as confirmation of a generalized anticancer effect.

4.5. Comparison Between Hexane and Ethyl Acetate Fractions

At all concentrations studied the hexane fraction exhibited a higher mean inhibition than the ethyl acetate fraction. This pattern suggested that the two fractions had a different biological activity under the conditions of the MTT assay. This distinction is important because solvent-dependent fractionation can affect the relative abundance of compounds with different biological properties and polarities. Fractionation of *V. officinalis* preparations may provide fractions with different antiproliferative activities against melanoma cells as shown by Nisar et al. [4]. In addition, their study revealed that some fractions were composed of various chemical constituents including compounds tentatively identified by mass spectrometric analysis

However, the results of that study cannot be extrapolated to the composition of the hexane or ethyl acetate fractions studied in this study. Differences in solubility, stability, cellular uptake and interactions with the MTT assay system could also influence the relative activity of the fractions. The underlying mechanisms of the observed differences are not resolved due to a lack of direct chemical analysis and complementary cellular assays. Moreover, no independent biological replicates were reported, and hence it is not possible to evaluate the statistical significance of these differences, although the hexane fraction appeared to have consistently higher mean % of inhibition. The observed ordering is to be interpreted as a descriptive difference in the apparent inhibitory response rather than a statistically demonstrated superiority of one fraction over the other.

4.6. Comparison with Vincristine

Vincristine, the reference drug, showed significantly higher %s of inhibition of MCF-7 than either plant fraction at the range of concentrations studied. The IC50 of vincristine was reported as 21.25 µg/mL. While the IC50 values of hexane and ethyl acetate fractions were 234.99 µg/mL and 274.10 µg/mL, respectively. These values are indicative of a higher level of apparent inhibitory activity of the

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reference treatment in the experimental conditions reported. However, care should be exercised in interpreting this comparison. The plant fractions are chemically complex preparations of incompletely characterized composition whereas vincristine is a defined pharmaceutical compound. Additionally, the lack of information about the duration of treatments, the solvent controls, the number of replicates and the raw absorbance values limits the reliability of the quantitative comparison of potencies. The greater inhibitory response of vincristine does not exclude the biological relevance of the plant fractions. Instead, the results show that the fractions produced quantifiable responses at relatively higher concentrations.

4.7. IC50 Estimates and Concentration–Response Modeling

In experimental pharmacology concentration dependent inhibition is often described by IC50 values. However, the reliability depends on the quality of experimental measurements, the appropriateness of the concentration-response model and the adequate representation of the range of response. The source's IC50 values were compared to those derived from exploratory logit-linear modeling in the present study. The first estimated values were 234.99 µg/mL for hexane fraction, 274.10µg/mL for ethyl acetate fraction and 21.25 µg/mL for vincristine. The exploratory reanalysis yielded the apparent IC50 estimates of 258.50, 373.40 and 19.25 µg/mL, respectively. The relative order of treatments was maintained, although absolute estimates were discrepant, especially for the ethyl acetate fraction. Interpretation of the response to ethyl acetate is especially important because the greatest mean inhibition observed was 49.6% at 300 µg/mL. So, the means at the concentration level did not reveal 50% inhibition in the experimentally explored range. Hence, the exploratory IC50 estimate of 373.40 µg/mL is an extrapolation beyond the highest concentration tested. The source's reported estimate of 274.10 µg/mL is not supported directly by an observed mean inhibition of at least 50% within the available concentration-level data. However, the fitting procedures and the raw experimental measurements must be inspected so as to understand the precise causes of these problems. Another thing you might want to consider is the vincristine data. The source reported IC50 as 21.25 µg/mL and mean inhibition as >50% at 20 µg/mL. This discrepancy indicates that the concentration interval suggested by adjacent observed means does not necessarily coincide exactly with the fitted IC50 estimates, particularly when models impose some assumptions on the response curve. Thus, IC50 values presented here are to be treated as approximate values, not quantitative measures of treatment potency.

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4.8. Methodological Interpretation of the MTT Assay

The MTT assay is a classical method in experimental oncology to assess the cellular response to biological and pharmacological agents. Because the signal recorded is a measure of the reduction of tetrazolium reagent, not a direct measure of a specific cell-death mechanism. Ghasemi et al. [5] performed a critical review of the MTT assay and found that the measurements can be influenced by a range of experimental parameters such as cell density, metabolic state, culture conditions, reagent concentration, incubation time and chemical interference. The limitations and utility of the assay were critically evaluated in this study. These results underscore the importance of assay optimization and proper controls in the interpretation of cellular viability and toxicity data. The reductions in MTT-derived cellular activity observed in this study were taken as evidence of metabolic inhibition associated with treatment. However, these findings do not necessarily imply apoptosis, necrosis, cell-cycle arrest or any other specific biological mechanism. Also, plant preparations may contain constituents that affect cellular metabolism or colorimetric assays independent of cell number. Hence, the term “cytotoxic activity” used in this study should be interpreted as an operational description of the MTT screening response rather than a proof of treatment-induced cell death.

4.9. Biological Relevance and Implications for Natural-Product Research

The results of the present study provide preliminary information on biological responses to specific *V. officinalis* formulations. The MCF-7 experiments showed a concentration-dependent inhibition of cellular metabolism and the antimicrobial data showed a measurable inhibition of growth of selected microorganisms. These results are consistent with the general literature describing the biological activities of preparations obtained from V. officinalis [1–4]. However, the present study is different from those studies that have used purified compounds, phytochemical profiling or multiple experimental models. The main contribution of the present dataset is in characterizing reported screening responses, not in identifying active compounds or confirming pharmacological mechanisms. These results also highlight the need to differentiate between experimentally proven therapeutic potential and initial biological activity. The hexane fraction showed higher apparent inhibition than the ethyl acetate fraction, but the available data do not show selectivity for malignant cells. Moreover, the antimicrobial inhibition zones do not show clinically relevant antimicrobial efficacy.

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4.10. Strengths and Limitations of the Present Findings

The present study deals with evaluation of *V. officinalis* preparations in two different biological screening systems. This is important as it gives descriptive information on the antimicrobial and MCF-7 cellular responses. The use of multiple treatment concentrations rather than a single exposure level also allowed examination of concentration-associated patterns. Furthermore, the MCF-7 assay served as a reference response due to the presence of vincristine. However, there are several constraints on interpretation. No direct phytochemical characterization was performed, thus the constituents responsible for the observed responses were not identified. Incomplete documentation of the extraction and fractionation processes hampered reproducibility and comparison with previous studies. Antimicrobial discovery was limited to agar-well diffusion measurements, and no quantitative susceptibility testing or proper reference controls were documented. The MTT data were missing experimental controls, raw absorbance values and independent biological replication. Additionally, interpretation of quantitative potency was confounded by discrepancies between Calculated IC50 values and exploratory Recalculated IC50 values estimates.

Collectively, the present results suggest that specific *V. officinalis* preparations were associated with concentration-dependent suppression of MCF-7 cellular metabolic activity and measurable antimicrobial inhibition. Antimicrobial responses were microorganism-dependent. MCF-7 results showed consistently higher mean inhibition in hexane fraction than ethyl acetate fraction. The results are in general agreement with data on the biological activities and chemical diversity of V. officinalis [1–4]. But, owing to the lack of chemical characterization, incomplete experimental documentation and uncertainty in IC50 estimation, definitive conclusions regarding comparative potency, active constituents or mechanisms cannot be drawn. Hence, the present study is to be viewed as a preliminary in vitro screening study providing descriptive evidence for further evaluation of *V. officinalis* preparations rather than as a confirmation of antimicrobial or anticancer therapeutic efficacy.

5. Study Limitations and Future Research Directions

5.1. Study Limitations

The present study provides preliminary evidence of antimicrobial activity and concentration-associated inhibition of MCF-7 cellular metabolic activity by selected Verbena officinalis L. preparations. However, several methodological and analytical limitations should be considered when interpreting the findings.First, incomplete

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documentation of plant collection, botanical authentication, extraction conditions, and fractionation procedures limits the reproducibility of the experimental methods. Information concerning the geographical origin of the plant material, voucher specimen, extraction temperature, solvent ratios, and extraction yields was not sufficiently documented. These factors may influence the chemical composition and biological properties of medicinal plant preparations.Second, the extracts and fractions were not subjected to direct phytochemical characterization. Although previous investigations have identified iridoid glycosides, phenylethanoid glycosides, phenolic acids, and flavonoids in V. officinalis, the presence and concentrations of these constituents in the preparations evaluated in the present study remain unknown. Consequently, the observed biological responses cannot be attributed to specific phytochemical compounds or classes. Third, antimicrobial activity was evaluated using the agar-well diffusion method without documented minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), or minimum fungicidal concentration (MFC) measurements. Furthermore, incomplete information concerning microbial strain identification, inoculum standardization, experimental replication, and reference controls restricts the interpretation and comparability of the inhibition-zone measurements.Fourth, the MTT assay was conducted using a single cancer-cell model, MCF-7.

Although this model is widely employed in experimental breast cancer research, the findings cannot be generalized to other breast cancer subtypes or malignancies. The absence of a nonmalignant reference cell line also prevented evaluation of cancer-cell selectivity. Fifth, the available MTT documentation did not establish whether the three reported measurements represented independent biological replicates or technical replicates. In addition, raw absorbance readings, complete control measurements, and several procedural parameters were unavailable. These limitations prevented independent verification of the inhibition calculations and reliable inferential statistical comparisons. Sixth, discrepancies were identified between the original IC50 estimates and those obtained through exploratory concentration–response modeling. This issue was particularly relevant to the ethyl acetate fraction, for which the maximum observed mean inhibition remained below 50% at the highest tested concentration. Consequently, the corresponding IC50 estimate was not directly established within the measured concentration range. Finally, the MTT assay measures cellular metabolic activity and does not independently establish apoptosis, necrosis, cell-cycle arrest, or other

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mechanisms of cell death. Therefore, the observed inhibitory responses should not be interpreted as definitive evidence of a specific anticancer mechanism or therapeutic efficacy.

5.2. Future Research Directions

Future investigations should prioritize the standardization and comprehensive documentation of plant collection, botanical authentication, extraction procedures, and fractionation conditions to improve experimental reproducibility. Phytochemical characterization using appropriate chromatographic and spectrometric techniques, including high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS), would help identify the principal constituents of the biologically active preparations and explore potential relationships between chemical composition and biological responses. Further antimicrobial investigations should incorporate standardized susceptibility-testing procedures, appropriate positive and negative controls, independently replicated experiments, and quantitative MIC and MBC or MFC measurements, as applicable. For the cellular investigations, additional experiments using independently replicated concentration–response assays and an expanded concentration range would improve the reliability of IC50 estimation. In particular, additional concentrations would be necessary to establish the response profile of the ethyl acetate fraction beyond the currently observed range. Evaluation in additional breast cancer cell lines and nonmalignant mammary epithelial cells would provide information regarding the reproducibility and selectivity of the observed inhibitory responses. Complementary assays assessing apoptosis, cell-cycle distribution, and other relevant cellular processes could subsequently clarify the mechanisms underlying the observed changes in MCF-7 metabolic activity. These investigations would help determine whether the preliminary responses observed in the present study warrant further pharmacological development.

6. Conclusions

The present study demonstrated preliminary antimicrobial activity and concentration-associated inhibition of MCF-7 cellular metabolic activity by selected *Verbena officinalis L.* preparations. The aqueous extract exhibited measurable antimicrobial activity against *Staphylococcus aureus* and *Streptococcus sobrinus*, while the methanolic extract showed measurable inhibition against *S. sobrinus*. No inhibition zones were recorded against *Klebsiella pneumoniae* or *Candida albicans* under the reported experimental conditions. Both the hexane and ethyl acetate fractions produced

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concentration-associated inhibition of MCF-7 cellular metabolic activity, with the hexane fraction consistently exhibiting higher mean inhibition %s. At 300 µg/mL, the hexane and ethyl acetate fractions produced 63.6% and 49.6% mean inhibition, respectively, compared with 93.9% for vincristine. Although the original IC50 estimates suggested greater apparent inhibitory activity of the hexane fraction, exploratory reanalysis identified discrepancies in the estimated values, particularly for the ethyl acetate fraction, whose observed mean inhibition did not reach 50% within the tested concentration range.

Overall, these findings suggest that selected *V. officinalis* preparations possess measurable biological activity under the investigated in vitro conditions. However, incomplete methodological documentation, the absence of direct phytochemical characterization, and limitations in experimental replication and concentration–response analysis prevent definitive conclusions regarding antimicrobial potency, cancer-cell selectivity, or underlying mechanisms. The present results therefore provide a preliminary experimental basis for further standardized investigations rather than evidence of established therapeutic efficacy.

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