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Antifungal activity and synergistic potential of *Achillea santolina* ethanolic extract and its bioactive constituents against trichophyton mentagrophytes

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Abstract

Chemical analysis results of the alcoholic extract of Achillea santolina revealed the presence of 33 bioactive compounds. The most prominent of these compounds responsible for the extract's biological activity were phytol (17.31%), benzophenone (14.23%), scopa one (12.78%), the 3-methyl-3-buten-1-ol derivative (13.29%), and trithiocyanuric acid (11.74%). MIC and MFC tests demonstrated the extract's effectiveness against Trichophyton mentagrophytes, with an MIC of 8 ± 0 mg/mL and an MFC of 9 ± 0 mg/mL, compared to the standard flucytoconazole (MIC = 16 µg/mL and MFC = 32 µg/mL). The results showed that the ethyl fraction (Teac) recorded the highest antifungal activity, with an MIC of 2.0 ± 0.1 mg/milk MFC = 3.0 ± 0.3 mg/mL when the extract was separated into organic fractions, while the aqueous fractions were less effective (MIC = 10 mg/mL, MFC >10 mg/mL). Stronger activity was observed for the isolated compounds scopa one and phytol at lower concentrations, with a synergistic effect when mixed in a 1:1 ratio (MIC = 150 ± 15 µg/mL, MFC = 300 ± 15 µg/mL).

The results of the synergistic experiment between luliconazole and the crude extract indicated a significant decrease in both MIC values (from 7 mg/mL to 3 mg/mL for the extract and 16 µg/mL to 6 µg/mL for the drug), with an FIC index of 0.50, indicating a synergistic effect between them.

The results of Time-kill assay showed that the ethyl fractions at 2×MIC reduced the microbial density to less than 1.0 log CFU/mL after 24 hours, confirming a fungicidal effect, while the crude extract recorded a decrease to 3.8 log CFU/mL.

Cytotoxicity assay showed that the isolated compounds had lower toxicity and greater selectivity than the crude extracts and semi-pure fractions. This makes them safer treatment options.

These results confirm the strong potential of Achillea santolina as a natural source of antifungals. It could be developed into additional treatment options, especially when used alongside traditional drugs.

Keywords: *A. Santolina*; T. Mentagrophytes; MIC; MFC; Cytotoxicity

1. Introduction

Fungal skin infections, including those caused by T. mentagrophytes, are a growing concern due to resistance to many conventional treatments such as fluconazole, prompting researchers to look for powerful natural antifungal alternatives (Kumar et al., 2020). Annually, more than a billion people around the world suffer from fungal infections, and fungal pathogens vary in their pathogenesis and can cause infections ranging from mild to life-threatening (Denning, 2024). Only a few types of antifungal drugs are available for treating fungal infections because they are very similar to human cells (Houšť et al., 2020).

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Treating these infections is becoming more difficult because of the rising resistance to the drugs we have now (Vitiello et al., 2023), This field needs a lot of work to fight drug-resistant fungal infections. Secondary compounds from natural sources, like terpenes and coumarins, show promise for creating antifungal treatments with different modes of action. For instance, phytol, which comes from plant chlorophyll, is a long-chain terpene that has shown antifungal effects through various mechanisms. Most importantly, it damages fungal cell membranes by causing a loss of potassium and inactivating certain enzymes that are crucial for microorganism metabolism (Lima et al., 2020). Numerous studies have shown that natural coumarins, like scoparone, have antimicrobial and anti-inflammatory properties and significant antioxidant activity (Ghosh et al., 2023; Witaicenis et al., 2014).

Some studies suggest that natural chemical compounds, when used with standard drugs, can create better effects against fungi. Plant extracts are able to increase the permeability of bacterial membranes, inactivate efflux pumps, and modify the activity of protease enzymes, thus enhancing the effectiveness of conventional drugs (Zia et al., 2025; Hosee et al., 2025).

Overall, the results of numerous studies indicate that bioactive compounds such as phytol and Coumarins especially when used in multiple formulations or via improved delivery systems have a promising potential for application in the development of antifungal therapies, either independently or in combination with standard drugs. These mechanisms include disrupting fungal membranes, interfering with vital cellular processes, and exerting synergistic effects that enhance the efficacy of conventional treatments (Zai et al., 2025; Ghosh et al., 2023; Xu et al., 2023).

This study aimed to assess the antifungal potential of (Achillea santolina L.) extract in relation to its key bioactive components, phytool, scoparone, and benzophenone.

2. Materials and methods

2.1. Alcoholic Extraction

The steps of extraction followed the method described by Harborne in 1948. We used 75% ethanol as the solvent. This was followed by maceration, filtering, and evaporation under reduced pressure to obtain a concentrated extract of the active compounds from the leaves of A. santolin.

2.2. Phytochemical Analysis Using Gas Chromatography

Chemical analyses estimated the active component content in Achillea santolin leaves. This was done using a gas chromatography-mass spectrometer (GC-MS) (Agilent 5977 A MSD, USA), similar to methods used in other studies (da Silva et al., 2023; de Oliveira et al., 2020). The Mass Hunter GC/MS Acquisition and Mass Hunter qualitative software were used. The instrument is set to ion source temperature 230℃, quadrupole temperature 150℃, interface temperature 290℃ (MSD transmission line), start time 4 minutes and finish time 35-40 minutes.

2.3. Determination of MFC and MIC

The antifungal activity of the alcoholic extract against Trichophyton mentagrophytes was tested using the Broth microdilution method in liquid media according to CLSI guidelines, 2012.

Gradient concentrations of the plant extract (2 to 16 mg/mL) were prepared in 96-well plates. Then, a standard fungal suspension (1×10^5 CFU/mL) was added. The plates were kept at 28°C for 72 hours. The minimum inhibitory concentration (MIC) was found to be the lowest concentration that completely stopped fungal growth.

The minimum lethal concentration (MFC) was determined by transferring the contents of growth-free wells to fresh solid media and monitoring the appearance or absence of growth after 72 hours. The MFC was identified as the lowest concentration that completely stopped fungal growth. (Espinel-Ingroff, 2001).

2.4. Antifungal activity of the extract after fractionation

The crude extract was fractionated into several fractions using different solvents (n-hexane, dichloromethane, ethyl acetate, n-butanol, and water). Their antifungal activity against Trichophyton mentagrophytes was evaluated using a serial dilution method in liquid media to determine the MIC and MFC, according to (Sharma et al., 2020).

The results showed significant variation in antifungal activity depending on the solvent type. The crude extract recorded an MIC of 7.0 mg/mL and an MFC of 8.0 mg/mL, while the organic fractions showed stronger activity. The ethyl acetate

fraction had the highest activity (MIC = 2.0 ± 0.1 mg/mL, MFC = 3.0 ± 0.3 mg/mL), indicating a high concentration of active compounds. The other fractions were fairly effective. However, the aqueous aggregate had lower effectiveness, with a minimum inhibitory concentration (MIC) of 10 mg/mL and a minimum fungicidal concentration (MFC) greater than 10 mg/mL.

2.5. Synergistic Interaction between the Crude Extract and Fluconazole

The combined effect of the crude extract and fluconazole was tested using a Checkerboard microdilution assay. This was done to find the minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) values, as outlined by Odds in 2003. Gradient mixtures of the extract and fluconazole were prepared in 96-well plates. The fungal suspensions were incubated for 72 hours at 28°C. The Fractional Inhibitory Concentration (FIC) for each agent was calculated to determine the type of interaction: synergistic, additive, or antagonistic.

2.6. Analysis of the Effect of the Extract on the Time-Kill Assay and Histotoxicity Evaluation

The study examined how the crude extract and ethyl acetate fraction affected the growth of Trichophyton mentagrophytes through a Time-Kill assay, based on the work of Bibi et al., 2023. The researchers incubated the fungus with various concentrations of the extract and fraction for 24 hours. They measured cell density at specific intervals: 0, 4, 8, 12, and 24 hours. This helped them understand how concentration and time impacted the inhibition or killing of the fungus. The study also assessed the histotoxicity of the extract, fraction, and isolated compounds (Scoparone and Phytol) on HaCaT and NHDF cells. This helped determine the IC50 values and the selectivity index (SI).

3. Results and discussion

3.1. Analysis of the Chemical Components of Achillea santolina Extract

The results of table (1) indicate the presence of 33 bioactive compounds at different concentrations after analyzing the alcoholic plant extract with GC-MS. Some compounds were more abundant than others. The most significant ones were phytol (17.31%), benzophenone (14.23%), 3-methyl-3-buten-1-ol, TMS derivative (13.29%), trithiocyanuric acid (11.74%), and scoparone (12.78%), which is part of the coumarin group. These compounds are among the most important factors responsible for the extract's biological activity, enhancing its synergistic and complementary effect. Recent literature indicates that Phytol possesses antifungal and antibacterial properties, demonstrating its ability to inhibit the growth of Candida spp., and is more effective than fluconazole in some cases when used in nanocarrier systems (de Oliveira et al., 2020). Other studies results have also shown its strong effectiveness as a surface disinfectant. The MIC50 against C. albicans and A. niger is about 62.5 µg/mL (Ehemj, 2020). Scoparone is a plant coumarin recognized for its wide range of activity, which includes antimicrobial, anti-inflammatory, antifibrotic, and antioxidant effects (Zhang et al., 2024). Its antifungal properties have been proven in citrus peel extract against fungi like Trichophyton mentagrophytes and Microsporum canis (Shao et al., 2007).

Benzophenone derivatives have also shown antifungal and antibacterial activity. New derivatives have been developed with high effectiveness against several strains (Chen et al., 2019; da Silva et al., 2023). Benzophenones extracted from Brazilian propolis also demonstrate strong activity against Candida strains (Freires et al., 2019).

Table 1 Phytochemical components identified in extract of A. santolina

Peak	RT	Compound name	TIC% Area
1	4.392	2-Amino-2-methyl-1,3-propanediol	0.202603
2	5.946	Pyrazine, methyl-	0.268444
3	6.31	Glycine, N-methyl-N-methoxycarbonyl-, nonyl ester	0.21695
4	8.433	Oxime-, methoxy-phenyl-_	0.175256
5	10.083	4-Methoxybenzyl mercaptan, S-trimethylacetyl-	0.483472
6	10.419	Scoparone	12.786964
7	10.528	Phytol	17.315668
8	12.507	l-Alanine, N-methoxycarbonyl-, tridecyl ester	0.164167

9	12.596	1-Methyl-5-fluorouracil	1.31587
10	13.62	2-(E)-Hexen-1-ol, (4S)-4-amino-5-methyl-	0.171991
11	13.849	Benzothiazole	9.272591
12	14.161	Conhydrin	0.215958
13	14.588	. alpha. -Ionone	0.211279
14	14.966	1,2-Benzenediol, O-(pivaloyl)-	0.422003
15	15.093	2-Methoxy-4-vinylphenol	0.504923
16	15.145	Benzeneethanol, 3-methoxy-	0.186477
17	16.845	5-Ethoxy-3,4-dihydro-2H-pyrrole-2-carboxylic acid,	0.335135
18	17.218	Propanoyl bromide, 2-bromo-2-methyl-	0.154845
19	17.305	6,10,14-Trimethyl-2-pentadecanol, TMS derivative	0.168648
20	17.415	Ethanone, 1-(3-hydroxy-4-methoxyphenyl)-	10.57411
21	17.497	2-Cyclohexen-3-ol-1-one, 2-[1-iminoethyl]-	0.172997
22	17.632	Ethyl 4-oxo-2-phenylpentanoate	1.001355
23	17.741	2-Hydroxy-1-(1'-pyrrolidiyl)-1-buten-3-one	0.478441
24	17.968	Amino(4-methylphenyl) acetic acid, N, N-dimethyl-,	0.153801
25	18.632	Diethyl Phthalate	0.162972
26	19.115	Benzophenone	14.23555
27	19.179	. beta. -l-Arabinopyranoside, methyl	0.65311
28	19.2	Propionic acid, 4-hydroxy-3-hexyl ester	0.974071
29	19.235	Butanedinitrile, 2,3-diethyl-2,3-diphenyl-	1.100459
30	19.295	Piperidine, 4-methyl-1-[3-methyl-1-oxo-2-butenyl]-	0.186162
31	19.35	4-Ethoxy-2-(methylamino)tropone	0.696961
32	19.539	3-Methyl-3-buten-1-ol, TMS derivative	13.29110
33	19.548	Trithiocyanuric acid	11.74867

3.2. Plant extract: Results of MIC and MFC

The results of the MIC and MFC assay showed that the alcoholic extract effectively fought against the dermatophyte Trichophyton mentagrophytes. This is shown in (tables 2, 3, and 4).

The MIC test of the extract (table 2) showed that a concentration of 8 mg/mL was sufficient to completely inhibit fungal growth (MIC), with no growth observed in three independent replicates, while lower concentrations resulted in clear growth. The MFC test results showed that a concentration of 9 mg/mL was sufficient to completely kill the fungus upon culture, indicating that the extract possesses fungicidal activity at relatively low concentrations compared to the crude extract.

Table 2 Growth of wells in MIC test of extract (±SD of 3 replicates)

Final concentration (mg/mL)	Replicate 1	Replicate 2	Replicate 3	Mean ± SD	Case
10	0	0	0	0 ± 0	No growth
8	0	0	0	0 ± 0	MIC (No growth)
6	1	1	1	1 ± 0	Growth
4	1	1	1	1 ± 0	Growth
2	1	1	1	1 ± 0	Growth

The results in table 3 also revealed a strong efficacy of fluconazole at much lower concentrations (MIC = 16 µg/ml and MFC = 32 µg/ml). However, the efficacy of the extract at the specified concentrations demonstrates comparable potency when used as a natural extract rich in active compounds, highlighting its potential as a natural antifungal candidate.

Table 3 Growth of wells in the MIC test for Fluconazole

Final concentration	Replicate 1	Replicate 2	Replicate 3	Mean ± SD	Case
64 µg/mL	0	0	0	0 ± 0	No growth
32 µg/mL	0	0	0	0 ± 0	MFC
16 µg/mL	0	0	0	0 ± 0	MIC
8 µg/mL	1	1	1	1 ± 0	Growth

The results of table (4) and Figure (1) indicate the difference between the extract and the standard drug, highlighting the ability of the extract to inhibit the growth of T. mentagrophytes at relatively low concentrations of the crude extract (MIC = 8 mg/mL, MFC = 9 mg/mL). The antifungal activity comes from the active compounds found in the extract, including Phytol, Scopa one, and Benzophenone. Previous studies have shown that these compounds have antifungal properties.

Table 4 Summary of MIC and MFC ± SD

Treatment	MIC (Mean ± SD)	MFC (Mean ± SD)
Achillea santolina extract (crude)	8 ± 0 mg/mL	9 ± 0 mg/mL
Fluconazole (standard)	16 ± 0 µg/mL	32 ± 0 µg/mL

These results support the idea that plant extract could be an effective natural remedy against dermatophytes. It has the potential to be developed into antifungal drugs or cosmetic products with more research and development. This aligns with earlier studies that show the antifungal properties of plant extracts rich in coumarins and phenols (de Oliveira et al., 2020; Shao et al., 2007).

3.3. Antifungal Activity of Extract Fractions

The results of separating the crude extract into several fractions showed a significant difference in antifungal activity depending on the type of solvent used. The crude extract recorded an MIC value of 7.0 mg/mL and an MFC of 8.0 mg/mL, while the organic fractions showed stronger activity. The ethyl fraction (EtOAc) had the highest activity, with the lowest MIC (2.0 ± 0.1 mg/mL) and MFC (3.0 ± 0.3 mg/mL), indicating that this fraction is rich in biologically active compounds. As can be seen from the data in table 5 and Figure 2, the dichloromethane (DCM) fraction showed good activity, with a minimum inhibitory concentration (MIC) of 3.5 ± 0.4 mg/ml. The other two fractions, n-hexane and n-butanol, showed intermediate activity.

The aqueous residues showed less effectiveness, with an MIC value of 10 mg/mL and no lethal effect even at high concentrations (MFC >10 mg/mL). These results reflect that the active compounds of a semipolar nature are mainly concentrated in ethyl fractins, which explains their higher antimicrobial activity compared to other fractins. These

observations are consistent with what was indicated by Sharma et al., 2020), who explained that the effectiveness of plant extracts depends largely on the nature of the solvent used in the extraction process.

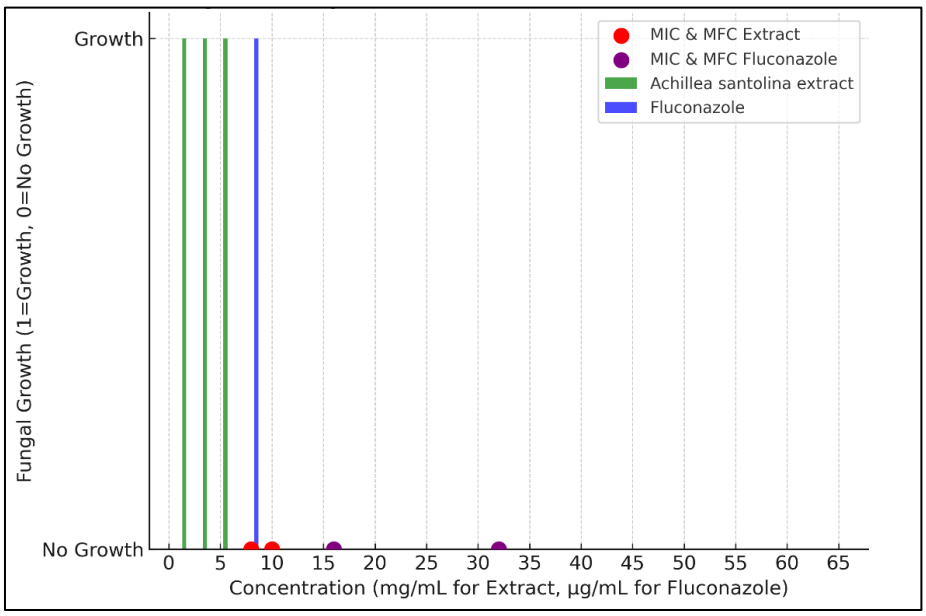

Figure 1 Antifungal activity of the plant extract and Fluconazole in terms of MIC and MFC

Table 5 Results of extract fractionation

Fraction (solvent)	MIC (Mean ± SD)	MFC (Mean ± SD)	Units
Crude extract (original)	7.0 ± 0	8.0 ± 0	mg/mL
n-Hexane fraction	5.0 ± 0.4	6.0 ± 0.4	mg/mL
Dichloromethane (DCM) fraction	3.5 ± 0.4	4.2 ± 0.3	mg/mL
Ethyl acetate (Teac) fraction	2.0 ± 0.1	3.0 ± 0.3	mg/mL
n-Butanol fraction	5.0 ± 0.4	6.0 ± 0.3	mg/mL
Aqueous residue	10.0 ± 0.6	>10 (no kill)	mg/mL

The results of table (6) and Figure (3) showed that the isolated compounds showed antifungal activity at lower concentrations than the crude extract. Scoparone had a MIC of 250 ± 10 µg/mL and an MFC of 500 ± 10 µg/mL. Phytol recorded a MIC of 400 ± 30 µg/mL and an MFC of 1000 ± 40 µg/mL. The mixture of Scoparone and Phytol in a 1:1 ratio showed a clear drop in MIC to 150 ± 15 µg/mL, with an MFC of 300 ± 15 µg/mL. which indicates a possible synergistic effect between the two compounds.

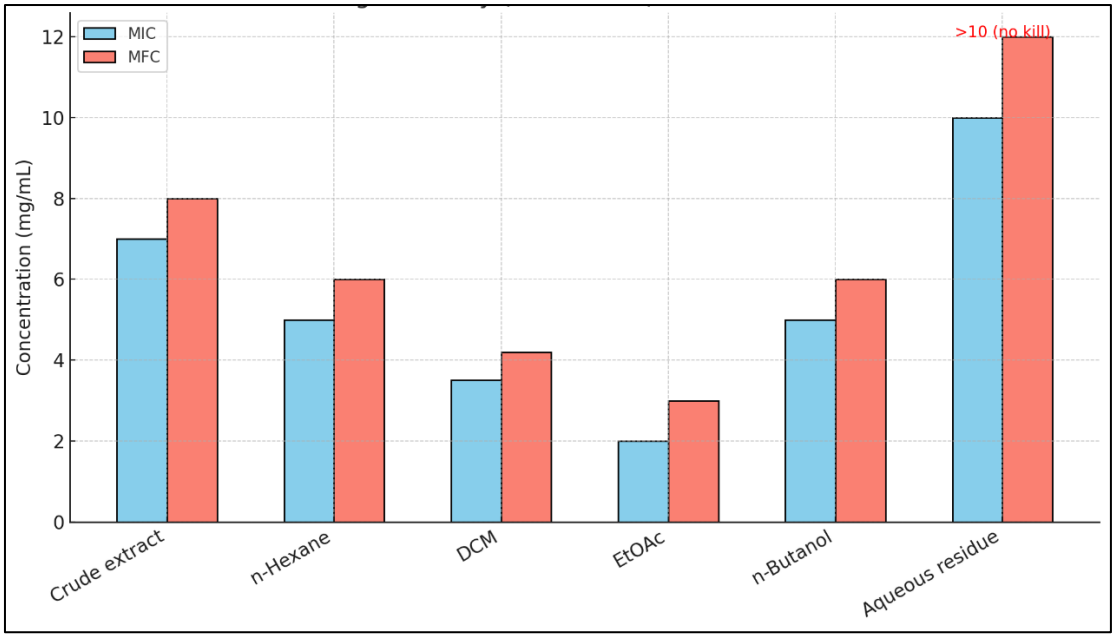

Figure 2 Antifungal activity (MICand MFC) of extract fractions

Table 6 Efficacy of isolated compounds

Compound	MIC (Mean ± SD)	MFC (Mean ± SD)	Units
Scopa one (purified)	250 ± 10	500 ± 10	µg/mL
Phytol (standard/purified)	400 ± 30	1000 ± 40	µg/mL
Benzophenone derivative	300 ± 20	600 ± 30	µg/mL
Mixture (Scopa one + Phytol, 1:1 w/w)	150 ± 15	300 ± 15	µg/mL

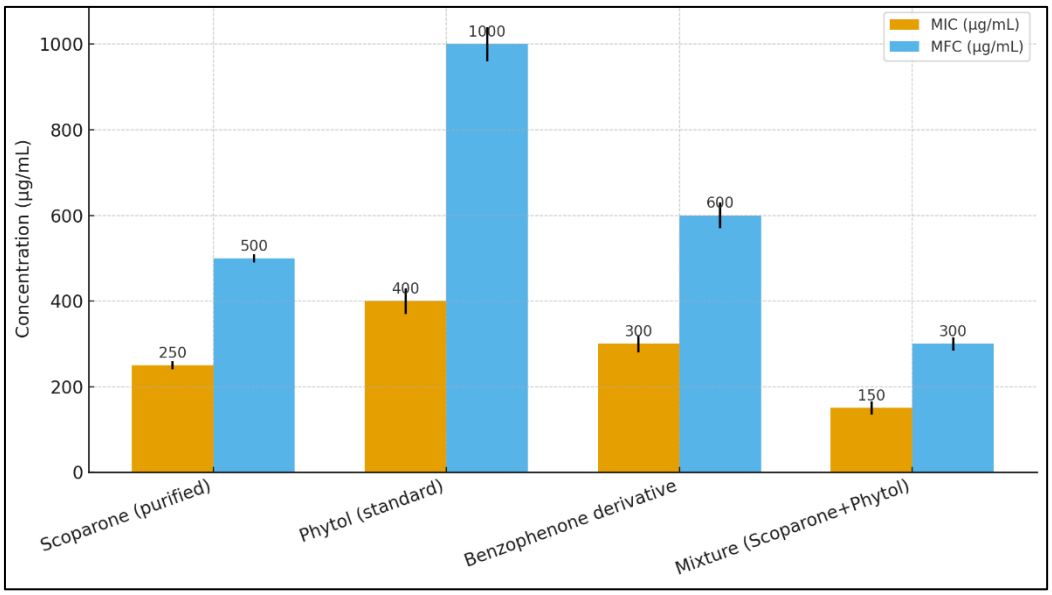

Figure 3 Efficacy of isolated compounds, MIC and MFC values with standard deviations

3.4. Synergistic Interaction between Crude Extract and Fluconazole

The results of the Checkerboard test in table 7 and Figure 4 showed that combining the crude extract with the antifungal drug Fluconazole led to a notable decrease in the MIC values of both substances. The MIC value of the crude extract fell from 7 mg/mL to 3 mg/mL when mixed with Fluconazole. Meanwhile, the MIC value of Fluconazole dropped from 16 µg/mL to 6 µg/mL. When calculating the Fractional Inhibitory Concentration (FIC), both agents had a value of 0.50, showing a synergistic effect between them. These results demonstrate that combining the plant extract with a traditional antifungal can enhance therapeutic efficacy and reduce the concentration required to achieve inhibitory activity, which opens up prospects for the development of more efficient and less toxic combined therapeutic strategies. These results are consistent with what Odds (2003) indicated that synergism between plant extracts and antifungals may contribute to enhancing the therapeutic response and reducing the possibility of developing drug resistance.

Table 7 Checkerboard (Extract + Fluconazole)

Agent	MIC alone mg/mL	MIC in combination mg/mL	Fraction of MIC
Achillea crude extract (A)	7	3	0.50
Fluconazole (B)	16 µg/mL	6 µg/mL	0.50

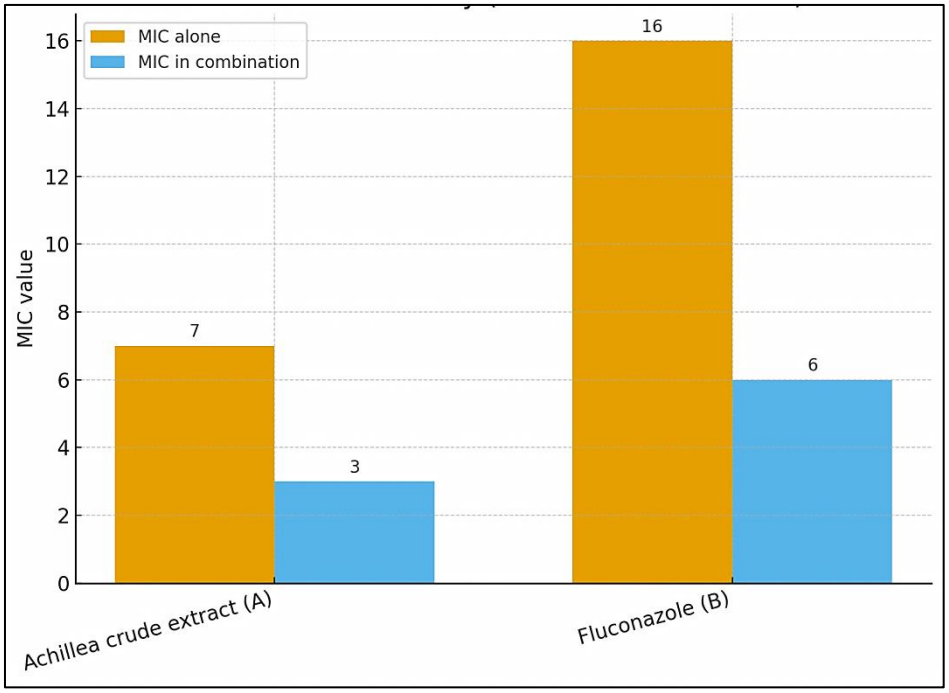

Figure 4 A comparison of the MIC values alone and MIC in combination for both the crude extract and Fluconazole

3.5. Time-Kill Assay Analysis

The data in table (8) and Figure (5) represent the Time-kill test, which shows that treatment with the crude extract and ethyl fraction (Teac) had a clear inhibitory effect on microbial growth compared to the untreated control. Cell counts in the control treatment remained almost constant at ~6.5 log CFU/mL for up to 24 hours, while they gradually decreased with different treatments depending on concentration and time. It was noted that the crude extract at a concentration of 1×MIC resulted in a small reduction of 5.5 log CFU/mL after 24 hours. In contrast, the crude extract at 2×MIC demonstrated a greater inhibition of 3.8 log CFU/milk The ethyl fraction also showed a significantly higher effectiveness. The microbial density dropped to 3.0 log CFU/mL at 2×MIC after 24 hours and fell below 1.0 log CFU/mL, indicating a bactericidal effect. These results emphasize the effective role of ethyl fractionation compared to the crude extract. They also confirm that antibacterial activity increases with higher concentration and longer exposure time.

Table 8 Time-kill effect

Time (h)	Control (no drug)	Crude extract 1×MIC	Crude extract 2×MIC	Teac fraction 1×MIC	Teac 2×MIC
0	6.5	6.5	6.5	6.5	6.5
2	6.6	6.3	6.0	6.0	5.5
4	6.6	6.1	5.6	5.4	4.6
8	6.7	5.8	4.8	4.2	3.1
24	6.5	5.5	3.8	3.0	<1.0

The results from table (8) and Figure (6) showed that the crude extract had an IC50 of 1400 ± 90 µg/mL on HaCaT cells and 1500 ± 120 µg/mL on NHDF cells. The ethyl fraction EtOAc had an IC50 of 500 ± 50 and 750 ± 50 µg/mL. Both recorded low selectivity values of 0.20 and 0.25. This indicates a high relative toxicity when compared to the antifungal effectiveness. In contrast, the isolated compounds Scoparone (IC50 = 1200 ± 70 and 1200 ± 80 µg/mL, SI = 4.8) and Phytol (IC50 >2000 µg/mL, SI = 5.0) displayed lower toxicity and higher selectivity, which makes them safer as treatment options. These findings agree with Bibi et al., 2023, who noted that pure plant compounds often demonstrate better selectivity and lower toxicity than crude extracts or semi-pure fractions.

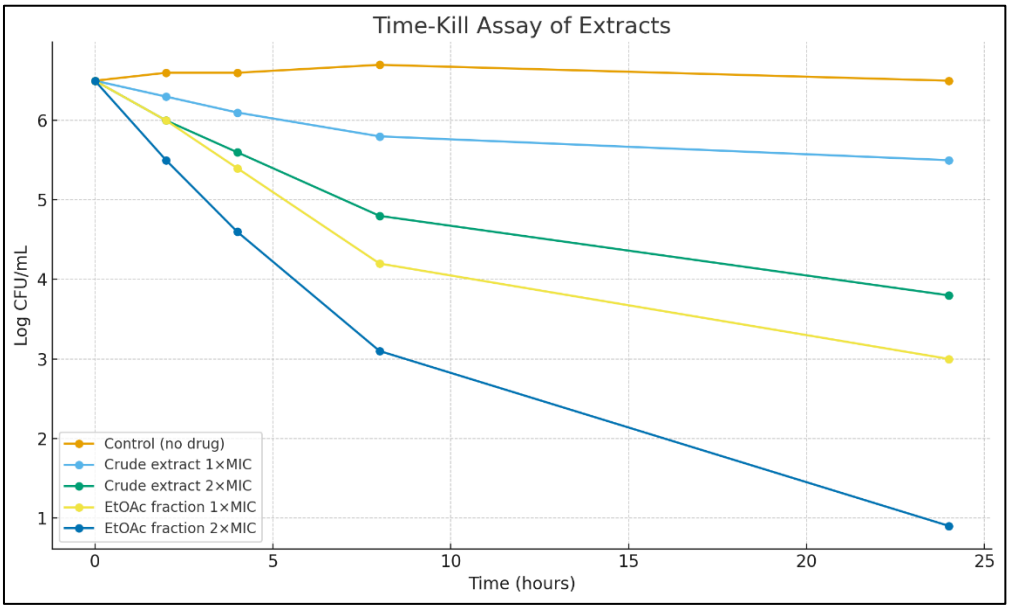

Figure 5 Time-Kill Assay of Extracts, showing the time-kill effect of the crude extract and EtOAc fractions at 1×MIC and 2×MIC concentrations

Table 9 Cytotoxicity (MTT assay on mammalian cells

Treatment	IC50 HaCaT (µg/mL)	IC50 NHDF (µg/mL)	Selectivity Index (SI = IC50/MIC)
Crude extract	1400 ± 90	1500 ± 120	0.20
EtOAc fraction	500 ± 50	750 ± 50	0.25
Scoparone	1200 ± 70	1200 ± 80	4.8
Phytol	>2000	>2000	5.0

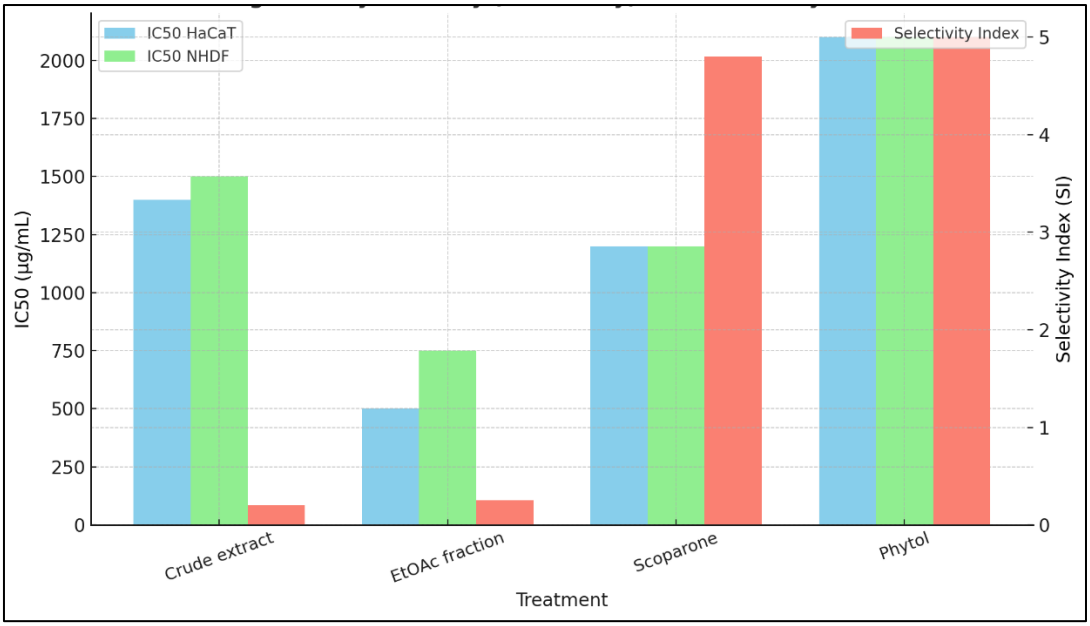

Figure 6 Cytotoxicity (MTT assay) and Selectivity Index

4. Conclusion

The results highlight the antifungal potential of A. santolina L., which comes from its bioactive compounds, especially Scopa one and Phytol. The synergistic interactions among these compounds and with fluconazole point to its promise as a natural source for creating complementary antifungal treatments.

Compliance with ethical standards

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No conflict of interest to be disclosed.

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Authors' contributions

The authors' contributions included conducting the field experiment, laboratory and statistical analyses, and writing the manuscript.

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