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INHIBITORY ACTIVITY, METABOLITE CONTENTS DETERMINATION, AND *IN SILICO* PREDICTION OF PARSLEY LEAVES FRACTION (*Petrocelinum crispum* MILL) AS ANTIFUNGAL AGENT OF *Malassezia furfur*

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ABSTRACT

Petrocelinum crispum leaves have Furocoumarin as an antimicrobial agent. In this present research, we examine the inhibitory activity of Parsley leaves fractions against *Malassezia furfur* by disc diffusion paper methods, determine the metabolite contents by LC-MS, and predict the biological activity and toxicity by in silico PASS Prediction. The inhibitory activity of both ethyl acetate and hexane fractions gave medium inhibition at concentrations of 0.15g/ml and 0.3g/ml, respectively. The metabolite contents were revealed by LC-MS, and the PASS prediction showed that the metabolite compounds identified have good biological activity and the xanthotoxin compound has shown non-toxic effects on the skin surface. Concluded that the ethyl acetate fraction showed better inhibition on the growth of Malassezia furfur by moderate category. The stigmastan-3,6-dione and aviprin metabolites were considered the most important metabolites on inhibitory activity predict by PASS prediction, and the xanthotoxin was considered important because of the non-toxic effects.

Keywords: *Petroselinum crispum* Mill, *invitro,* LCMS, *PASS Prediction, Malassezia furfur.*

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INTRODUCTION

Malassezia furfur is a fungus that resides on human skin whose life depends on lipids. This fungus is considered to be one of the causes of *Malassezia folliculitis* and *pityriasis versicolor*, which are associated with dandruff or seborrheic dermatitis, a contributing factor that aggravates other skin disorders such as atopic dermatitis, confluent, psoriasis and reticulate papillomatosis.[1,2] In some cases the prevalence of fungal infection by *Malassezia furfur* 2.1% was higher than by *Candida* 1.4%,[3] and 2.1% - 4.4% of the neonatal care unit depending on the diagnostic method.[4] The first-line therapy as an antifungal for *Malassezia* infection and used in the treatment of superficial mycoses is ketoconazole (KTZ). KTZ is a fungistatic imidazole group in the broad spectrum, whose mechanism is by inhibiting lanosterol 14α-demethylase. Lanosterol 14α-demethylase is an enzyme that regulates ergosterol synthesis by interfering with the biosynthesis of ergosterol, then will change the structure of the cell membrane which causes disruption of membrane integrity and permeability and then causes disruption of cell growth and reproduction.[5] Usually, azole antifungals such as ketoconazole in the form of a cream, shampoo, foam, or solution are used for the treatment of skin disorders related to *Malassezia* sp. But, ketoconazole can also cause urticaria and is reported to be toxic to mammalian cells.[6] Frequent use of these products can also lead to the development of resistance. Therefore we are interested in discovering and improving the treatment using *in silico* method in the early stages of testing. It is expected to provide data about the affinity prediction of compounds with receptors and the prediction of side effects of compounds contained

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in parsley leaves. Afterward, *in vitro* assay was performed to determine the inhibitory ability of the extract or fraction against the fungus In recent years the activity of conventional antibiotics has decreased due to increased resistance.[7] However, the interest in developing and finding plant compounds that have antimicrobial activity is also increasing every year.[8] The compounds found to act on the cell wall, protein synthesis, and DNA replication during the microbial division cycle.[9] Research institutes, the chemical, and pharmaceutical industries need techniques capable of identifying side effects in the early stages of product development example Computational Chemistry because this information comes rationally from in-vivo testing, the reduced demand for experimental animals, and the lack of important toxicity information. Computational chemistry that can be applied is the QSAR method (Quantitative Structure-Activity Relationship). The quantitative correlation between molecular structure and measured biological activity values was studied experimentally.[10] PASS Prediction is also used to predict the toxicity of the compound. This can help to get a prediction of toxicity before the experiment is carried out.[11] The ability of *Petrocelinum crispum* leaves as an antimicrobial agent proven to have antimicrobial activity against *Pseudomonas aeruginosa* using hot water extract of *Petrocelinum crispum* leaves (250 mg / mL).[12] Furocoumarins isolated from aqueous extract of *Petrocelinum crispum* leaves with 0.12–8.0% concentration exhibit inhibitory activity against *Erwinia carotovora, Listeria monocytogenes, Escherichia coli,* and *Listeria innocua*.[13] Whereas the antimicrobial test against *Malassezia furfur* has never been reported before.

EXPERIMENTAL

Preparation of Plant Extract

The material used was a Parsley leaf (*Petrocelinum crispum* Mill) sample which was obtained from UPT Materia Medika, Batu City, East Java Province. The plant identity was confirmed by a botanist at the Bandung Institute of Technology, Indonesia. The parsley leaves were dried out of direct sunlight and blended into a coarse powder. 96% ethanol extract was obtained by maceration for 72 hours and filtered using filter paper. Remaceration was carried out again 2 times. The solvent that had been collected was concentrated with a rotary evaporator at 45°C and then dried using a hair dryer.[14] The ethanol extract obtained was then partition liquid-liquid by means of graded fractionation using n-hexane and ethyl acetate solvents. 20 grams of ethanol extract was first dissolved with 500 ml of distilled water, then filled into a separating funnel and mixed with 500 ml of n-hexane, after being separated the two fractions were removed and separated in different containers. The water fraction was then fractionated again using ethyl acetate. The ethyl acetate and n-hexane fractions were collected, filtered, evaporated, dried, weighed, and ready to be used for antifungal testing. The extract and fraction were dissolved in dimethyl sulfoxide (DMSO) and diluted to give a concentration of 0.01 to 0.3 g/ml.[15]

Preparation of Media and Culture

The *Malassezia furfur* strain was purchased from the University of Indonesia. The fungal *Malassezia furfur* were grown on Potato dextrose agar (PDA) medium at 27˚C for 72 hours. The inhibition of bacterial growth by plant extract was evaluated using the disc diffusion method. Sterile Whatman filter paper was prepared and soaked separately in each extract for 5 minutes.[16] The extract and fraction concentrations were weighed and put into a sterile vial and each dissolved with DMSO. Glass utensils used in anti-fungal testing, such as petri disk, test tubes, and vials were sterilized using an oven with a temperature of 180˚C for 2 hours, while Potato Dextrose Agar (PDA) media as a growth medium and other plastic tools were sterilized using autoclave at 121˚C for 15 minutes.[17]

Antifungal Activity

The antifungal activity of fractions was determined using the disc diffusion method. A total of 15 ml of PDA media and 1 ml of the fungal suspension were put into a test tube, then homogenized and poured into a petri dish, allowed to solidify. After it was solidified, a disc paper with a diameter of 6 mm was attached containing the concentration of the ethanol extract, a fraction of n-hexane and ethyl acetate, ketoconazole, and DMSO onto PDA media that had solidified, and the Petri dishes were incubated for 72 hours at 27˚C ± 2˚C. The antimicrobial activity was measured by calculating the area of the clear zone that was formed around the disc paper is the indicator of antimicrobial activity.[17] All measurements were done with the naked eyes when looking at the back of the Petri dish. After incubation, measuring the

inhibition zone diameter with a caliper, the zone of growth inhibition was recorded (in millimeters) (Fig.-1).[18] The zone of inhibition can be grouped into several categories, e.g. weak, moderate, strong, and very strong (Table-1).[19]

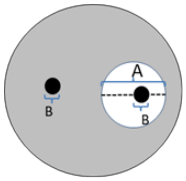


Fig.-1: (A.) Measurement Diameter of the Zone of Resistance, (B.) Diameter of the Disk Piper. The Gray Color Represents the Media Which is an Area of Bacterial Growth[20]

Table-1: Classification of Antifungal Inhibition Zones[19]

Inhibitory Strength	Zone of Inhibition
Weak	≤ 5 mm
Moderate	6 - 10 mm
Strong	11 - 20 mm
Very strong	>20 mm

LCMS

A column used was Intensity Solo 2 RP-18, 2.0 µm, 100 × 2.1 mm column (Bruker, Germany, Bremen,). In the LC-MS/MS preparation, the sample was dissolved in methanol and the vial was put into the autosampler. For elution, the concentration of 5 µL was injected and 0.250 mL/min flow rate was adjusted. Columns were stored at 35°C and 10°C for the sample. The mass spectrometer operated in negative mode with the following parameters: collision cell energy, 5.0 eV; nebulizer gas (N2) dry heater, 200 °C, –3.5 kV of ion spray voltage; dry gas (N2), 8.0 L.min−1; 2.0 bars and plate offset, 500 V. Internal calibration method is carried out with a solution consisting of 0.5 mL 1N NaOH solution, 250 mL H2O, 250 µL formic acid, 750 µL acetic acid, and 250 mL iPrOH on the HPC mode. The data obtained from the LC-MS analysis were processed using Data Analysis software 4.4™ (Bruker) to extract the mass spectral features of the raw data samples. Auto MS/MS mode is used to confirm fragment ions.[21]

Prediction of Biological Activity and Skin Toxicity

Prediction of biological activity and skin toxicity in silico was carried out using the PASS Prediction website by entering the MDL Mol file (*.mol) of the compound to be predicted. The prediction of antifungal activity shows the predictive probability of active (Pa) and inactive (Pi). The results obtained can help study the activity of *Malassezia furfur*. The Pa value higher than the Pi value indicates that the compound has the maximum possible activity.[22]

RESULTS AND DISCUSSION

***In vitro* Antifungal Assay**

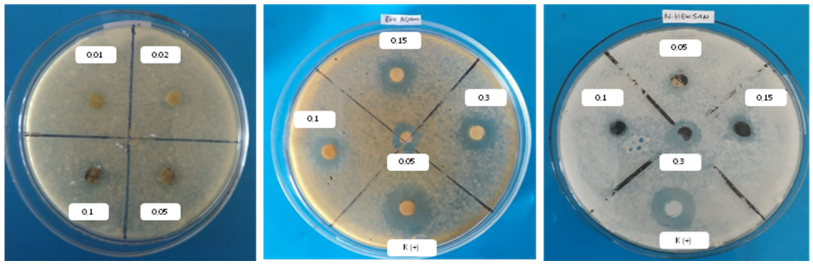
Antifungal activity against *Malassezia furfur* showed that the inhibition power began to form at a concentration of 0.05 g/ml. The test results using the disc diffusion method showed that both parsley leaf extract and fraction had antifungal activity against *Malassezia furfur* and the ethyl acetate fraction of parsley leaves showed a higher inhibition. The antifungal activity of the ethyl acetate fraction was followed by the n-hexane fraction and ethanol extract. Based on these results and compared with the inhibitory power of ketoconazole antibiotics which had the strongest inhibition category. The inhibitory activities of extracts, fractions, ketoconazole, and DMSO against *Malassezia furfur* are summarized in Table-2.

Table-2: Inhibitory Activity of Extract and Fraction of Parsley Leaves, Compared to Ketoconazole and DMSO Controls against *Malassezia Furfur*

Samples	Concentrations (g/ml)	Average (mm)	Inhibition category
Ethanol Extract	0.01	0	No fungicidal activity
	0.02	0	
	0.05	2.78±0.95	Weak

	0.1	3.11±0.69	
	0.15	3.45±0.69	
	0.3	4.44±1.89	
n-Hexane Fraction	0.05	2 ± 0.67	
	0.1	3.11 ± 2.8	Weak
	0.15	4.21 ± 3.6	
	0.3	6.89 ± 1.01	Moderate
Ethyl Acetate Fraction	0.05	4.22 ± 2.3	Weak
	0.1	5.33 ± 3.5	
	0.15	6.44 ± 1.3	Moderate
	0.3	7.89 ± 0.2	
Ketoconazole	0.01	28.89 ± 0.6	Very strong
DMSO		0	No fungicidal activity

The use of ketoconazole resulted in significant differences in the use of parsley leaf extract for the test concentration. These differences can be seen from the large diameter of the clear zone formed on the media (Fig.-2).



A **B** C

Fig.-2: The Results of Antifungal Activity Assay of, A. Ethanol Extract, B. Ethyl Acetate Fraction, and C. N-Hexane Fraction (*Petrocelinum Crispum* Mill) against *Malassezia Furfur*

It has been reported in a previous study, that the concentrations higher than 100 µg mL^{-1} of extracts with parsley essential oil, had antifungal activity by significantly inhibiting the radial growth of *C. acutatum*.[23] The inhibition zone is a clear area around the antimicrobial compound where the growth of the test microbes is inhibited. The formation of the inhibition zone is caused by the metabolite compounds contained in the extract and parsley leaf fraction diffusing into the agar medium so that the fungi that come in direct contact with the media the growth will be inhibited.[17] The positive control used was ketoconazole. ketoconazole is a synthetic antifungal that has a broad spectrum and fungistatic in nature. Ketoconazole works by inhibiting ergosterol biosynthesis by targeting the 14 α-demethylase enzymes from lanosterol. Ergosterol is a sterol found in fungi, responsible for membrane fluidity, stiffness, enzyme activity, and asymmetry.[24] Therapy the most active against *Malassezia furfur* that has been reported and has been circulating in the market is Ketoconazole (KTZ), with the diameter of the inhibition zone obtained from the diffusion test.[25] In this result, Ketoconazole also showed strong inhibitory activity against *Malassezia furfur*, almost the same as those reported in other studies.[25] Previous research mentioned that ethyl acetate is an effective semi-polar solvent for attracting alkaloid compounds, flavonoids, phenolic compounds, and tannins whereas n-hexane can attract alkaloid compounds, saponins, and steroids.[26] Flavonoids are strong antioxidants, anti-inflammatory, and antimicrobial against, microorganisms by inhibiting the bound membrane enzymes,[27] and in treating Seborrheic dermatitis, the quinoline alkaloid can be used as antifungal agents.[28] In this study, it was found that ethyl acetate fraction of *Petrocelinum crispum* Mill displayed the highest inhibitory effect at 0,3 g/ml, followed by n-hexane fraction and ethanol extract, although it was not as good as ketoconazole. The inhibitory activity of the n-hexane and ethyl acetate fractions from parsley stems and leaves has also been suggested by previous studies on the growth of the fungus *Colletotrichum acutatum.*[23]

LCMS Analysis

The LC-MS / MS data describe the differences in the compound content of the n-hexane fraction and ethyl acetate fraction. The differences in the compounds were depicted by the peak chromatogram and retention time (min). Representation of the compound chromatogram can be seen in Fig.-3.

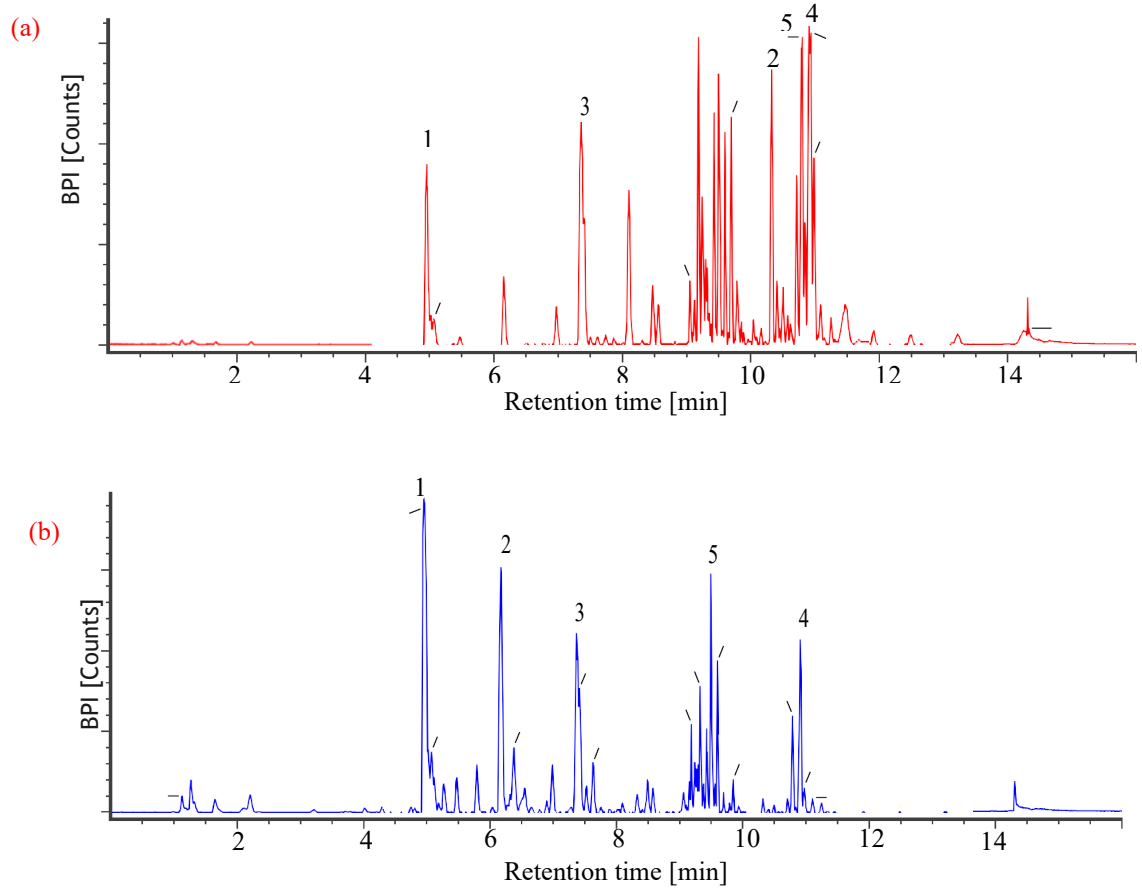


Fig.-3: (a) n-Hexane Fraction (b). Ethyl Acetate Fraction (*Petrocelinum crispum* Mill)

The chemical composition of n-hexane fraction and ethyl acetate fraction from leaves of *Petrocelinum crispum* Mill was analyzed by means of LCMS/MS. The detected constituents are shown in Table-3 and structural compounds can be seen in Fig.-4. Several identified compounds and candidate compounds such as 1,3-Dihydroxy-2-hydroxymethylanthraquinone-3-O-β-D-xylopyranose and Candidate Mass C54H78O9 are contained in the n-hexane fraction was also reported to be present in the ethyl acetate fraction. This explains that the two compounds are soluble in n-hexane and ethyl acetate solvents. Chromatogram the peak compound with the greatest mass is shown by the candidate compound.

Table-3: Compounds in the Fraction of n-Hexane and Ethyl Acetate by LCM/MS Analysis

Sample	No	Senyawa	Observed Retention time (min)	Observed mass (m/z)	Formula
n-Hexane Fraction	1	1,3-Dihydroxy-2-hydroxymethylanthraquinone-3-O-β-D-xylopyranose	4.96	565.1553	C26H28O14
	2	Stigmastan-3,6-dione	10.33	429.3721	C29H48O2
	3	Xanthotoxin	7.37	217.0493	C12H8O4
	4	Candidate Mass C54H78O9	10.92	871.5724	C54H78O9
	5	Candidate Mass C54H78O10	10.80	887.5678	C54H78O10

Ethyl acetate Fraction	1	1,3-Dihydroxy-2-hydroxymethylanthraquinone-3-O-β-D-xylopyranose	4.97	565.1556	C26H28O14
	2	Aviprin	6.18	305.1021	C16H16O6
	3	Xanthotoxin	7.38	217.0494	C12H8O4
	4	Candidate Mass C54H78O9	10.92	871.5738	C54H78O9
	5	Candidate Mass C34H40O9	9.51	593.2760	C34H40O9

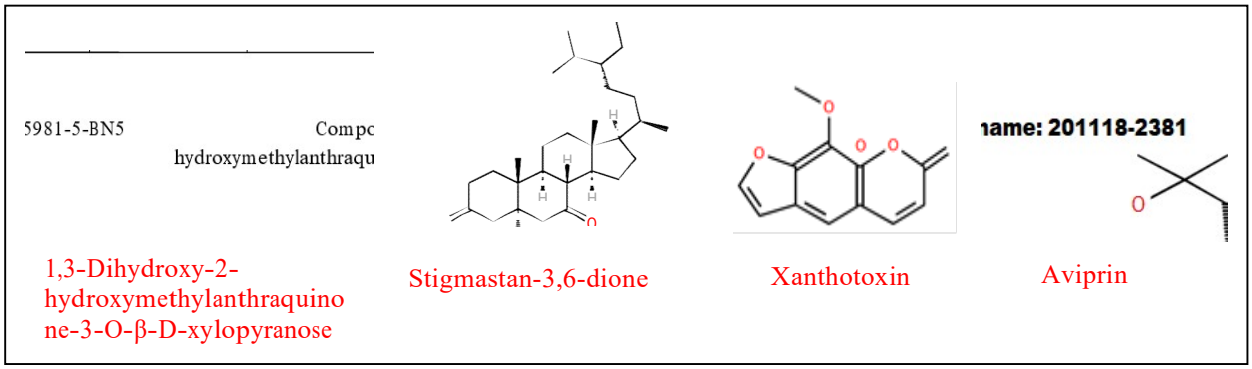


Fig.-4: Structures Compounds from LCMS/MS Analysis

Analysis by LCMS led to the identification, among them were 1,3-Dihydroxy-2-hydroxymethylanthraquinone-3-O-β-D-xylopyranose is an anthraquinone, stigmastan-3,6-dione of the triterpenoid group,[29] aviprin dan xanthotoxin are furanocoumarins.[30,31] The major constituents' previous reports differ in n-hexane fraction from curleysley leaf parsley.[23] Reported that Phenylpropenes (48.7%), myristicin (18.7%), and apiole (27.6%) are the major compounds in the n-hexane fraction of parsley.[23] On the other hand, Tadros *et al.*, 2017 found that flavonoid was the major constituent of ethyl acetate fraction of *Petrocelinum crispum* Mill.[32] Compound diversity could be a result of several parameters including interactions with microorganisms and insects, extraction method, vegetative stage of the plant, geographical origin, cultivar, and storage condition, as well as their.[33]

Prediction of Biological Activity and Skin Toxicity

The predictive probability of active (Pa) and inactive (Pi) for biological activity as an antifungal were studied. The results of the prediction of the biological activity of parsley, compounds showed no Pa value was greater than ketoconazole (Table-4). This seems to be in line with laboratory testing which shows that the zone of inhibition shown by ketoconazole is larger. While the smallest Pa value on skin toxicity was shown by 1,3-Dihydroxy-2-hydroxymethylanthraquinone-3-O-β-D-xylopyranose which was reported to be present in both fraction samples.

Table-4: PASS Prediction for Antifungal Activity and Toxicity Parameters

No	Compounds	Pharmacological Profile				Annotation
		Biological Activity		Skin Toxicity		
		Pa	Pi	Pa	Pi	
1	Ketoconazole	0.640	0.014	0.262	0.258	Dermatitis
2	1,3-Dihydroxy-2-hydroxymethylanthraquinone-3-O-β-D-xylopyranose	0.112	0.035	0.341	0.137	Allergic dermatitis
3	Stigmastane-3,6-dione	0.476	0.035	0.169	0.054	Skin irritative effect
4	Aviprin	0.472	0.035	0.144	0.064	Skin irritative effect
5	Xanthotoxin	0.370	0.057	-	-	

In the prediction of biological activity, the Pa value that is higher than the Pi value means that the compound has the maximum possible activity.[22] From the prediction pass, it was reported that the compounds contained in the n-hexane fraction and the ethyl acetate fraction belonging to the anthraquinone, triterpenoid, and furanocoumarin groups had good inhibitory activity on the fungus

Malassezia furfur. In earlier work on the antifungal properties of anthraquinone derivatives, it was shown to have an growth inhibitory effects against *Sporotrichum schenckii, Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans,* and *Trichophyton mentagrophytes*.[34] Coumarin is a compound with an aromatic heterocyclic structure, containing a benzopane α-pyranone group which can be isolated from completely synthesized in the laboratory, *Umbelliferae, Solanaceae, Rutaceae,* and other plants. Coumarin compounds and their derivatives are reported to have various biological activities, including antiviral, antibacterial, and anticoagulant activities.[35] Meanwhile, silver (I)-coumarin complexes were also reported to exhibit fungicidal activity against *Candida albicans* by interfering with cytochrome synthesis, which in turn caused inhibition of respiration and reduced ergosterol biosynthesis.[36] Although 24-hour coumarin exposure has been reported to alter cell membranes and cell walls, and decrease cytoplasmic volume with structural disorganization in *Candida albicans*, the mechanism of action of coumarin derivatives (aviprin dan xanthotoxin) activity against has *Malassezia furfur* not to be delineated.[37] Based on the results of PASS predictions and inhibitory activity. The inhibitory activity test showed that ethyl acetate fraction containing 1,3-Dihydroxy-2-hydroxymethylanthraquinone-3-O-β-D-xylopyranose compound, aviprin, Xanthotoxin, and two candidate compounds showed the greatest activity although not as big as ketoconazole.

CONCLUSION

The ethyl acetate fraction of parsley (*Petrocelinum crispum* Mill) leaves has moderate inhibitory activity on the growth of Malassezia furfur. As well as the compound xanthotoxin is reported to not have toxic effects. Several bioactive compounds contained in parsley fractions (*Petrocelinum crispum* Mill) were determined by LCMS and the pharmacological activity of each of these compounds was revealed using *in silico* prediction and show promising results.

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