



Antifungal activity of *Melilotus officinalis* of Iraq

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ABSTRACT

The aim is to evaluate the antifungal activity of *Melilotus officinalis* widely grown in Iraq and to identify the phytochemical responsible for this activity. The antifungal activity of the water soluble fraction was investigated using agar diffusion method. The fraction was analysed to detect the active principle using TLC, HPTLC, UV and IR spectroscopy. *Melilotus officinalis* of Iraq exhibited a strong antifungal activity against five diagnostic fungi in concentration of 1mg % and above. TLC of the acid hydrolysed glycoside indicates the presence of kaempferol flavonoid by comparing with standard. HPTLC, UV and IR spectroscopy confirmed the presence of this flavonoid. *Melilotus officinalis* widely grown in Iraq exhibited a strong antifungal activity against pathogenic fungi. Kaempferol glycoside is responsible of this potent antifungal activity as it is the major phytochemical present in the polar portion of the extract.

Keywords: melilot, kaempferol, glycoside, kaempferol aglycone, antifungal activity

INTRODUCTION

Melilotus officinalis, known as yellow sweet clover, yellow melilot and common melilot, a plant belong to the family Fabaceae[1]. *Melilotus officinalis* was used in traditional medicine in treatment of problems related to varicose veins such as painful and heavy legs, cramps in the legs and itching [2].

In Iran the dry extract of *Melilotus officinalis* is marketed under the name Semelil (ANGIPARS™) which is used as herbal formulation for treatment of chronic wounds, particularly diabetic foot ulcers [3]. The healing of diabetic foot ulcer was attributed to the strong antifungal activity exhibited by this plant together with coumarin which acts by reduction of oedema and inflammation by increasing venous and lymphatic flow [4, 5, 6].

This glycoside has been used for many years in traditional medicine to treat infectious disease for this reason kaempferol and its glycoside isolated from *Melilotus officinalis* used for antiviral, antibacterial and antifungal agent [7].

EXPERIMENTAL SECTION

Plant material

The aerial parts of flowering *Melilotus officinalis* were collected from Abu-Graib in Baghdad at the end of March (2013). The plant was authenticated by the National Herbarium of Iraq and were dried in shade for several days at room temperature and then grinded as powder.

Extraction of kaempferol glycoside

Powdered plant aerial part (50 g) was extracted by Soxhlet apparatus with ethanol (80%, 250 mL) till exhaustion. The extract was concentrated by evaporation under vacuum. Water (100 g) was added and the suspension was partitioned with petroleum ether (2x 100ml). The pet ether layer was discarded and the aqueous layer was extracted with ether (3x100ml) and chloroform (100 mL). The remaining water layer was extracted with butanol (3x 100 mL). Evaporation of butanol was performed under high pressure to obtain the glycoside.

The antifungal activity of the total ethanolic extract together with butanol dried layer was investigated by agar dilution method.

Acid hydrolysis of phenolic glycosides

Powdered plant (100 g) was heated with water (100 mL) at 60 C° for 30 mins. The mixture was filtered and the filtrate was centrifuge until a clear solution obtained.

HCl (1N, 5mL) was added to the filtrate (80 mL). The mixture was refluxed for 1 hour. Ethyl acetate (100 mL) was added after cooling to extract the aglycone. The organic layer was evaporated and the kaempferol was purified by TLC [8].

Thin layer chromatography

The R_f value of isolated kaempferol after acid hydrolysis was compared with standard kempferol in three solvents system. Preparative TLC was performed using 0.5 mm thickness silica gel. Elution of the isolated band was conducted with A.R methanol.

Antifungal activity

Five diagnostic pathogenic fungi used in this work were supplied from the Department of Microbiology, College of Sciences. These fungal species included *Microsporium canis*, *Trichophyton mentagrophytes* var. *interdigitale*, *Trichophyton mentagrophytes* var. *mentagrophytes*, *Trichophyton rubrum* and *Tricophyton violaceum*. The subculture for fungi was prepare by suspend the media (6.5g) in purified water (100ml), heat with frequent agitation and boil for one minute to completely dissolve. The media was autoclaved at 121°C for 15 minute, cooled for several minute and poured in Petri dishes until solidify. Each one of these fungi inoculated on the media and incubated for 5-7 days at 25 °C to obtain young, actively growing cultures consisting of mycelia and conidia.

RESULTS AND DISCUSSION

TLC of isolated kaempferol after acid hydrolysis was compared with standard in three solvent systems (table 1).

Table 1: R_f of standard and isolated kaempferol in three solvent systems

Solvent system	R _f of standard kaempferol	R _f of isolated kaempferol
Toluene: Acetone: Chloroform 40: 35:25	0.65	0.65
Formic acid: Acetone: Chloroform 0.85: 1.65: 7.5	0.75	0.73
Chloroform: Methanol 90:10	0.48	0.44

Isolated kaempferol (preparative TLC) was identified by m.p. 277 which was identical with that reported in the literature [9].

Identification of kaempferol (IR and HPTLC)

The plant was reported to contain kaempferol 7-O-glycosides (figure 1) [7].

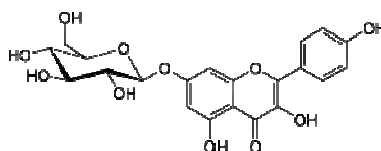


Figure 1: Kaempferol glycoside

The isolated kaempferol after acid hydrolysis was identifying by IR. , spectrum of kaempferol showed absorption at 3420 cm^{-1} , due to hydroxyl group; 2830 cm^{-1} , due to C-H group; 1720 cm^{-1} , due to C=O group; 1600, 1610, 1560, 1510, 1450, 1400 corresponding to aromatic ring (Figure 2)

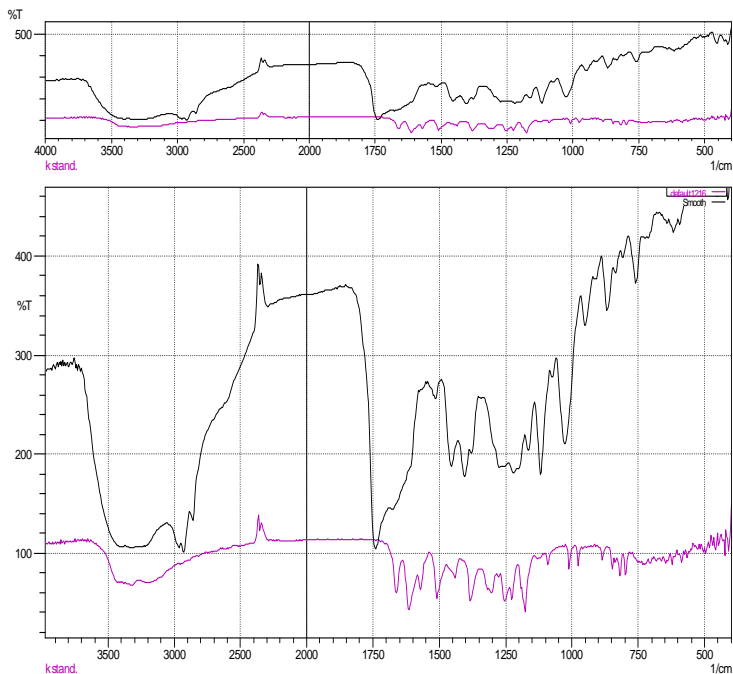


Fig 2: IR spectroscopy for isolated kaempferol and kaempferol standard

For qualitative analysis, HPTLC was carried out for identification of isolated kaempferol, the HPTLC were made for isolated kaempferol and kaempferol standard (Figure 3 and 4).

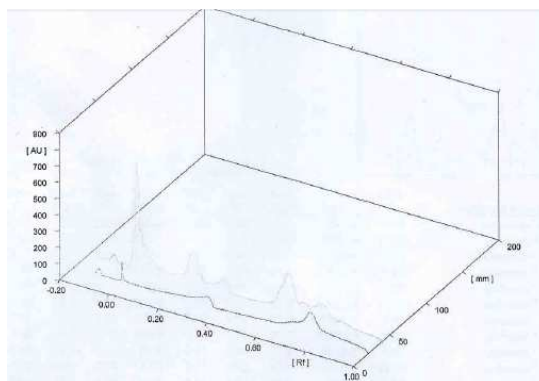


Figure 3: HPTLC for kaempferol

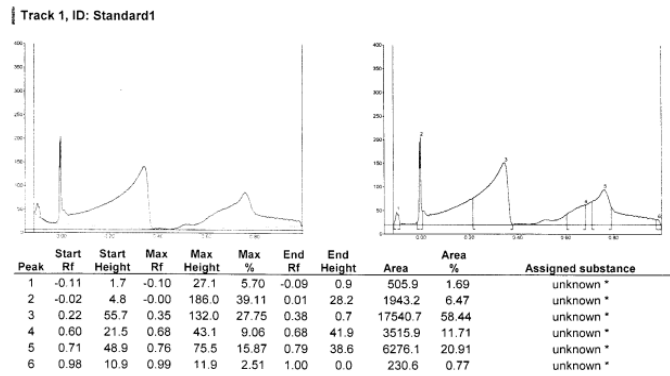


Fig 4: HPTLC of all tracks (kaempferisolated and standard), 236nm

Antifungal activity

The five diagnostic pathogenic fungal species were tested previously checked for purity and for phenotypes. These fungal species were incubated under suitable growth conditions (Figure 5)



Figure 5: Five diagnostic pathogenic fungi growth

The five different fungal grew in two concentrations (0.5% and 0.25%, triplet), It is worth mentioning that 0.25% concentration of extract gave more growth than 0.5% concentration, i.e. when the concentrations of extract decreases the growth of fungal increases. In these growth plates there were a partial growth inhibition when pathogenic fungi treated with 0.5% and 0.25% concentration of ethanol extract. One species only from five species of pathogenic fungi was *Trichophyton mentogrophytes* (fungal 1) was more sensitive and marked reduction in growth culture, while both *Trichophyton rubrum* (fungal 2) and *Microsporum canis*(fungal 5) produced resistance to this low concentration (Figure 6).



Figure 6: Partial growth for fungi under 0.5% concentration

The concentration of ethanolic extract was increased (1%, 2% and 3%) to find a concentration that obtains a full inhibition zones. The spots present in the plates media was a trace of small origin media inoculums but not a fungal growth complete inhibition resulted with 1-3% concentration (Figure7).

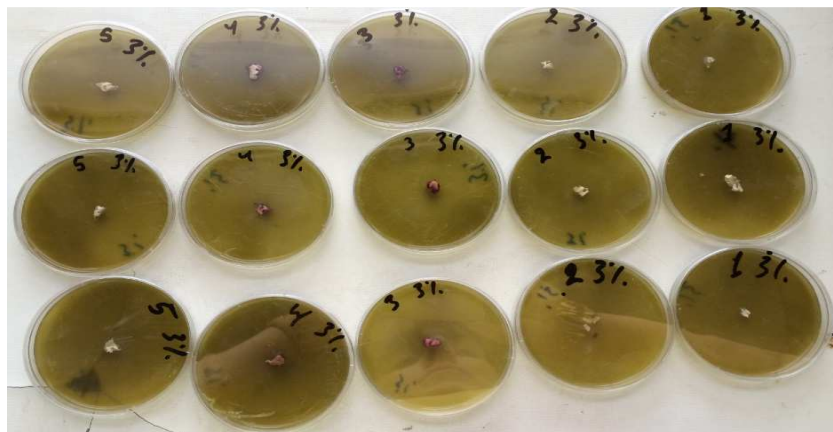


Fig 7: Complete inhibition of the growth by concentrated ethanolic extract (3%)

Complete inhibition zones were also obtained from butanol extracts (1, 2 and 3%) concentration which indicates that the glycoside of the plant is responsible for this strong antifungal activity (Figure 8).

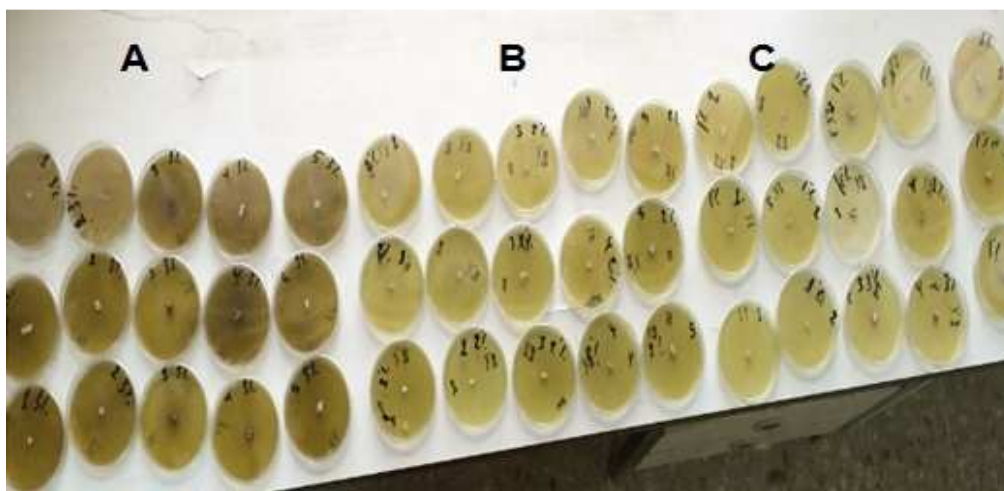


Figure 8: Complete inhibition zones for butanol layer (concentration 1, 2 and 3%)

SAS analysis (2012) is used as a statistic evaluation for the tow low concentration which resulted in partial inhibition. DMSO was used as control (Table 2) [10].

Table 2: SAS statistic evaluation

Conc. %	Control	Fungal 1/cm			Fungal 2/cm			Fungal 3/cm			Fungal 4/cm			Fungal 5/cm		
		1st	2nd	3 rd	1 st	2nd	3 rd	1st	2nd	3rd	1st	2nd	3 rd	1st	2nd	3 rd
0.5	7.5	1	1.5	1.1	1.6	1.6	1.3	3	2.9	3	4.6	5	4.8	4.8	4.6	4.6
0.25	7.5	2	2	2	2.7	1.9	1.9	4.4	4.1	3.8	6.6	6.6	6.6	5.5	5.8	6

The variation in the inhibition of two different conc. (0.5% & 0.25%) against five dignostic fungal and the mean value and LSD values were shown in Table 3

Table 3: Mean and LSD values

Conc. (%)	Mean ± SE of G.I (%)					LSD value
	Fungal 1	Fungal 2	Fungal 3	Fungal 4	Fungal 5	
0.5	83.6 ± 4.39	79.8 ± 3.47	60.2 ± 3.61	36 ± 2.35	37.6 ± 2.64	12.305 *
0.25	73.3 ± 3.04	62.6 ± 2.91	45.6 ± 1.82	11.8 ± 0.79	27.3 ± 1.08	10.684 *
LSD value	7.924 *	9.177 *	9.536 *	8.326 *	7.365 *	---

* ($P \leq 0.05$).

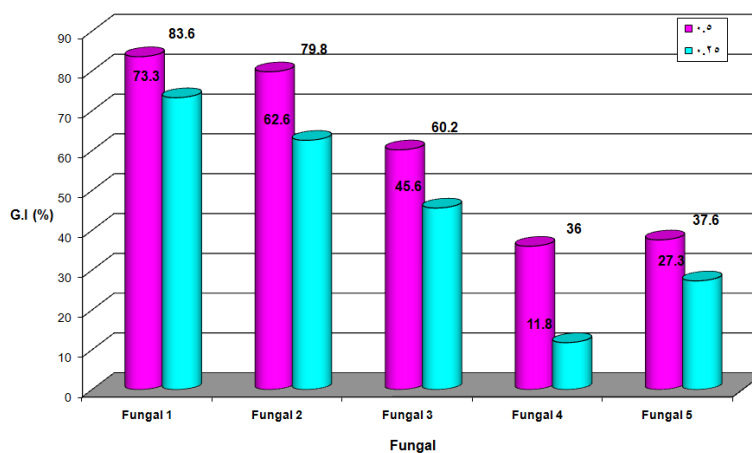


Figure 9: Plates for the Statistical evaluation for this comparability

Comparison study was presented for these results which indicate that a partial growth inhibition was obtained when pathogenic fungi were treated with 0.5% concentration of ethanolic extract. *Tricophyton mentogrophytes* was more sensitive and produced a marked reduction in growth culture (Figure 9)

This result confirmed the literature reports in review article which stated that kaempferol glycosides were found to be responsible for strong antifungal activity of plant extract [11].

CONCLUSION

The ethanolic total extract together with butanol dried layer were exhibited a partial inhibition zones when used in low concentration 0.25-0.5%. Complete inhibition zones were obtained from 1-3% concentration of the extract. Kaempferol glycoside is responsible of this potent antifungal activity as it is the major phytochemical present in the polar portion of the extract. Kaempferol aglycon was identified by melting point, TLC, IR and HPTLC after acid hydrolysis of the glycoside.

Acknowledgment

I would like to express my thanks to the college of Pharmacy al-Mustansiriyah University for grant and support of this work.

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