ANTIMICROBIAL EFFECTS OF THE EXTRACTS OF *HYPERICUM THYMIFOLIUM*

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ABSTRACT

Hypercium thymifolium ***(****Ht) a medicinal herb used throughout history, has always been of great interest to people. It is known in Lebanon as "Dazi" and grows wild in vast areas of the country.*

Fresh green leaf samples of Ht were collected from three Lebanese regions, and extraction of essential oil was done using hydro-distillation, followed by Gas Chromatography analysis. The buds of flowering tops of Ht were tested for the level of hypericin with HPLC. The inhibitory effect of the extract on bacteria and yeast were tested using a standard microbiological assay. The odor of its flower is fresh turpentine-like, then fruity, attracting honeybees, especially, in the early morning. The high lipid solubility of the Ht essential oil extract gives a milky liquid with a thin oily layer on the surface. Hydro-distillation yielded 0.5% oil, which is rich in essential oils (limonene16.7%, geranyl acetate 15.54%, terpineol 7.6%, and geraniol 2.5%). Hypericin, which is a bright red pigment dianthrone derivative, is a natural product found in the Ht yellow flower. The total yield of hypericin was around 0.25% (Dry weight). The Ht milky oily extract had outstanding antibacterial properties and was much more potent than the commonly used antibiotics tested. Results of the study indicate that this compound showed strong inhibitory activity against Staphylococcus aureus and was more effective than Sulphamethoxazole. The Ht extract killed 12000 Staphylococcus bacteria, as compared to 137 only by the antibiotic. Its remarkable activity against some gram (+) positive and gram (-) negative bacteria offers enormous potentials for medicinal use.

Keywords: medicinal plant, *Hypericum*, hypericin, essential oil, antimicrobial effects, MIC

INTRODUCTION

Hypericum thymifolium is a plant of the genus *Hypericum*, of which there are 400 species worldwide. Its family is known as Hypericaceae. This plant is commonly called in Lebanon "*Dazi*", in English St.John's wort and in French Millepertuis. Since the time of ancient Greeks, Hyppocrats and Paracelsus used *Hypericum* for treatment and healing of wounds. It is also a mild antidepressant when taken as an infusion (Hayek, 1996).

Hypericum thymifolium is native to Lebanon and grows largely and wildly above 1200m. It is found in dry soils and sunny places. This genus hypericum is widely used in folk medicine to cure warts in humans and livestock. *Hypericum thymifolium* synonym with *H. serpyllifoliu* is described as glabrous perennial and stand erect between 80-100cm usually woody at the base. The leaves are small, green (like thyme) opposite to each other. The flowers are regular, with five persistent-withering yellow petals, which bloom throughout the year (Mouterde, 1931).

Hypericin is a natural product that may be found throughout the plant, but definitely exists in high concentration in its yellow flower. Hypericin, a bright red pigment, "dianthrone derivative", is a polycylic quinone (Petrich *et al*., 1997). It was first mentioned as a main active component, reaching its highest point when the flowers are full but not quite open, responsible for the photosensitivity effect of this plant in grazing animals (Southwell and Campbell, 1991). International interest increased in hypericin and pseudohypericin after researchers from New York University medical center, demonstrated that these compounds strongly inhibit a variety of retrovirus *in vivo* and *in vitro*, including hepatitis C and HIV. No serious toxic side effects were noticed after testing over 800 mice with the compounds (Meruelo *et al*., 1988).

The immediate objectives of this study focus on the extraction and identification of the main constituents of *Ht* and their antimicrobial (bacteriolytic or bacteriostatic) effects on a number of pathogenic bacteria known to be responsible for food-borne infections.

MATERIALS AND METHODS

A Gas chromatograph, Shimadzu 17A, was used for the quantitative and qualitative identification of the essential oils of *Ht*. The methods used were modified according to Hilan *et al*. (1997). The method for identification of hypericin (a main compound of *Ht* extract) was by High Performance Liquid Chromatography Shimadzu CBM (Communications Bus Module), UV-VIS Detector (Enzymatic Therapy Laboratory). All the standards used were from Sigma Ltd.

Collection of samples

Plant material was identified and collected from the east of Sidon, at an altitude between 450-500m, the Chouf Mountain (altitude 950-1000m) and from planted *Hypericum* in the Fanar Laboratory garden (coast), during two successive periods (winter and summer), over 2 years (Herbarium n°. 0012, Agricultural faculty, University of Saint Esprit, Kaslik).

The three samples were subjected to hydro-distillation to extract the essential oil. The oil was stored in the refrigerator at 4° C, after extraction.

Extraction methods of hypericin

Dried flowering tops of *Ht* gathered shortly before or during flowering are weighted, finely powdered and extracted with 25ml methanol.

The method involved for hypericin extraction and analysis was a modified Mathis and Ourisson procedure using Soxhlet extraction with ether to remove chlorophyll and other less polar products, followed by ethanol extraction to obtain the hypericin relatively free of chlorophyll (Mathis and Ourisson, 1964).

The inhibition test of bacteria by *Hypericum thymifolium*

The strains of bacteria were cultured on Mueller Hinton agar. A single colony was isolated and cultured on Kligler Iron agar, incubated at 37°C for 24 hours. Subsequently, a small amount was removed and placed in bijoux bottles containing 3cc of sterile nutrient broth.

One cc of this suspension was added to 9cc of sterile nutrient broth. In order to ensure that bacterial concentrations were adequate for the study of antimicrobial effects, the germs were counted by the dilution technique in series from 10^{-1} to 10^{-6}. One cc of each dilution was cultured on three Petri dishes containing 20ml MHA media, incubated at 37°C for 24 hours. The colonies were then counted and the results recorded, giving the number of bacteria per ml. The *Ht* extract collected through water distillation was sterilized by autoclave. To each 1ml of broth culture, corresponding to a bacterial dilution from 10^{-3} to 10^{-6} bacteria/ml, 5µl, 10µl, and 20µl of *Ht* oil extract were added aseptically respectively. Three mixtures of *Ht* extract concentration in 1ml of bacterial suspension were thus obtained.

Both techniques, bacterial count and bacterial suspension, were carried out at the same time. The 12 bijoux bottles were incubated at 37°C for 10 minutes and cultured on MHA media. All cultures were repeated at intervals of 1hour, 24 hours and 3days and the results recorded. Each test was performed in triplicates (Hilan *et al*., 1997).

RESULTS AND INTERPRETATION

Hypericum thymifolium is green and bloom throughout the year, with yellow flowers. The moisture content of the herb was 60% to 65%.

Leaf and stem collected during the winter months contained the lowest levels of essential oil. The oil yield from the flower was very low. Only fresh green leaves were used. The hydro-distillation yielded 0.5% of the essential oil, for the samples from Sidon; 0.4% from the Chouf mountain, (June/ July 98-99), and 0.5% from the *Ht* planted at the garden of Fanar Laboratory, in July 99.

The taste and smell of the *Ht* extract is characteristically slightly sweet, bitter and astringent. The odor of this oil is fresh, turpentine like, fruity, and rosy like (Weyrestahl *et al*., 1995).

The individual chromatogram peaks of the *Ht* extract components were identified by comparing their retention times, with those of pure essential oil standards. A chromatography profile of the essential oils was thus obtained.

TABLE 1
Relative Retention Indices (RRI) and Major Components of E.O.of *Ht*

Essential oil. Standards	Retention times (min)	Hydro extraction by area %
1- α- pinene	6.713	0.4
2- Myrcene	8. 495	0.05
3- Limonene	9. 964	16.7
4- α- thujone	12.413	0.02
5- β- thujone	12. 413	0.02
6- Camphor	13. 666	0.2
7- Borneol	14.744	0.3
8- Terpineol	15.620	7.6
9- Geraniol	17.926	2.5
10- Linalyl acetate	18.117	0.3
11- Carvacrol	19.406	0.02
12- Geranyl acetate	22.233	15.4

In a previous study, 60% of 280 tested hypericum species were positive for hypericin (Hobbs, 1996). Leaf and stem contained very low levels of hypericin, while flowers contained the highest levels during summer (Southwell and Campbell, 1991) (Meruello *et al*., 1988). The hypericin extract is vivid almost fluorescent red.

The *Ht* extracts from samples collected during the period June/July were shown to contain 0.25% ± 0.01% (dry wt.) of Hypericin ($C_{30}H_{16}O_8$).

The Results of the antimicrobial activity of *Ht* extract are shown in Tables 2 (Candida albicans), 3 (Gram Negative bacteria) and 4 (Gram Positive bacteria).

TABLE 2
No. of Yeast (*Candida albicans*) Remaining After the Inhibition Tests

	Ht ex.	**5 µl**			**10 µl**			**20 µl**		
Yeast	**Time**	**10'**	**1h**	**24h**	**10'**	**1h**	**24h**	**10'**	**1h**	**24h**
Candida albicans 18x 10^6/ml	10^{-3}	50	37	hg	29	24	hg	19	16	hg
	10^{-4}	6	4	hg	5	4	hg	3	1	hg
	10^{-5}	1	1	hg	1	0	hg	0	0	800
	10^{-6}	0	0	1200	0	0	2	0	0	0

hg = high growth
The inhibition was total and definitive after 10 minutes with 20 µl of *Ht* extract.

TABLE 3
No. of Gram Negative Bacteria Remaining After the Inhibition Tests

	Ht ex.	**5 µl**			**10 µl**			**20 µl**		
Bacteria G(-)	**Time**	**10'**	**1h**	**24h**	**10'**	**1h**	**24h**	**10'**	**1h**	**24h**
Escheri. coli 74 x 10^6/ml	10^{-3}	240	300	hg	170	170	hg	104	160	20
	10^{-4}	37	52	hg	22	30	720	10	17	1
	10^{-5}	5	12	720	5	7	33	2	**0**	0
	10^{-6}	1	**0**	0	0	2	0	0	0	0
Pseudo. aerugino. 37 x 10^6/ml	10^{-3}	200	190	hg	175	150	hg	160	0	0
	10^{-4}	36	20	hg	22	20	6	16	0	0
	10^{-5}	5	2	hg	3	2	2	1	0	0
	10^{-6}	1	**0**	hg	**0**	0	0	0	0	0
Salmonel. typhi. 18 x 10^6/ml	10^{-3}	200	hg	hg	180	120	30	125	90	5
	10^{-4}	25	140	hg	21	30	0	19	12	**0**
	10^{-5}	6	8	hg	2	**0**	0	2	0	0
	10^{-6}	1	**0**	0	0	0	0	1	0	0
Proteus mirabilis 24 x10^6/ml	10^{-3}	380	350	700	240	224	33	97	81	**0**
	10^{-4}	40	34	50	27	24	8	18	13	0
	10^{-5}	7	3	4	4	**0**	0	2	1	0
	10^{-6}	0	0	0	3	0	0	0	0	0
Shigella sonnei 15 x 10^6/ml	10^{-3}	350	160	hg	125	100	hg	85	80	100
	10^{-4}	32	20	hg	16	12	hg	10	9	13
	10^{-5}	2	1	hg	2	**0**	hg	1	**0**	0
	10^{-6}	0	0	hg	0	0	8	0	0	0

A bacteriostatic effect was demonstrated in relation to the time of contact and the concentration of *Hypericum thymifolium* extract against *Echerichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa*.

E.coli, 0:157,H7 (resistant bacteria isolated from a hospitalized patient).
74 *E.coli* bacteria were inhibited with 5µl, of *Ht* extract after one hour of contact.
740 *E.coli* were inhibited after 1h of contact with 20µl of *Ht*.
Salmonella typhi, 0:9.12,Vi-H:d
Growth was inhibited after 1h of contact with 5µl of *Ht* extract.
180 bacteria were inhibited after 1h of contact with 10µl, of *Ht* extract.
With 20µl, the inhibition of 1800 bacteria was complete after 24h.
Pseudomonas aeruginosa, (resistant bacteria).
Only 37 bacteria were inhibited after 1h of contact at any *Ht* extract concentration.
With 10µl of *Ht* extract the inhibition was complete after 10 minutes of contact.
Shigella sonnei
150 bacteria were inhibited after 10 minutes of contact with 10µl of *Ht* extract.
But the inhibition was complete after 1h of contact with 20µl, of *Ht* extract.
Proteus mirabilis
240 bacteria were inhibited after one hour of contact with 10µl of *Ht* extract.
Inhibition was complete and definitive after 24h of contact with 20µl of *Ht* extract.

TABLE 4
No. of Gram Positive Bacteria Remaining After Inhibition Tests

Ht extract	**5µl**			**10µl**			**20µl**		
Staphylo.aure 12 x 10^6/ml	**10'**	**1h**	**24h**	**10'**	**1h**	**24h**	**10'**	**1h**	**24h**
10^{-3}	57	120	0	85	6	0	20	5	0
10^{-4}	17	13	0	5	0	0	4	0	0
10^{-5}	1	3	0	0	0	0	0	0	0
10^{-6}	0	0	0	0	0	0	0	0	0
Strepto.fecal. 77 x 10^6/ml									
10^{-3}	100	48	hg	53	5	200	31	24	0
10^{-4}	42	13	9	36	3	9	20	17	0
10^{-5}	8	0	0	3	0	0	2	0	0
10^{-6}	3	0	0	3	0	0	2	0	0

hg = high growth

Staphylococcus aureus
With any concentration of *Ht* extract, the inhibition was complete and definitive after 24 h of contact.
Streptococcus group D
770 bacteria were inhibited after 1h of contact with 5µl of *Ht* extract.
Inhibition was complete and definitive after 24h of contact with 20µl, of *Ht*.

TABLE 5

Efficiency of Essential Oil of *Ht* Against Bacterial Contaminants
Minimum Inhibitory Concentration, (MIC)[1]

Bacteria	Duration of contact	Number of Bacteria	Oil con.µl	Antibio. concentration. µg
E.coli	1h	74	5	Sulphamethoxazole25
Salmonella typhi	1h	18	5	Chloramphenicol 30
Pseudomonas a.	1h	37	5	Resistant
Proteus mira.	10'	24	5	Cephalexin 20
Shigella sonnei	10'	15	5	Chloramphenicol 30
Staphylo. aureus	10'	12	5	Sulphamethoxazole25
Strepto fecalis	10'	770	5	Sulphamethoxazole25

1.The MIC is evaluated like the minimal volume of the *Ht* extract capable of inhibiting the greatest quantity of germs in a minimum of time.

CONCLUSION

Hypericum thymifolium is rich in essential oil: limonene 16.7%; α-pinene 0.4%; borneol 0.3%; terpineol 7.6%. The fact that *Ht* is rich in geranyl acetate 15.4% and geraniol 2.5% (important heavy constituents), endows the oil with organoleptic qualities that are highly appreciated in perfumery. *Ht* contains only a very small quantity of toxic constituents like (α-thujone 0.02%, β- thujone 0.02% and carvacrol 0.02%).

Extracts of flowering tops of *Ht* were shown to contain hypericin between 0.24 % and 0.25 %. Thus, a standardized extract of hypericin content may be the surest way to administer the plant for viral therapy and anti-depressive activity.

This study demonstrated anti-bacterial activity; the extract of *Ht* has a bacteriolytic effect on a number of gram (+) bacteria, and bacteriostatic effect on some gram (-) bacteria. This compound showed significant inhibitory activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and was found to be more effective than Sulphamethoxazole. *Hypericum thymifolium* extract at low concentration showed greater efficiency than commercial antibiotics, especially against antibioresistant bacteria.

Medicinal plants in Lebanon are a squandered resource, badly conserved and badly exploited. Proper exploitation of this agricultural wealth could also be beneficial at the pharmaceutical and economic levels (cosmetics, perfumes). The tendency of this plant to grow on different types of soil raises the possibility of exploiting it on a larger scale as a domesticated alternative, high value, crop.

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