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Chemical characterization of two Egyptian *Melilotus* species to reveal their anti-inflammatory properties

Nermin A. Ragab[a], Salma A. El Sawi[a], Elsayed A. Aboutabl[b], Ali M. El Halawany[b], Amany A. Sleem[c], Mona M. Marzouk[d]

[a] *Pharmacognosy Department, National Research Centre, 33El Bohouth St., Dokki, Giza 12622, Egypt*
[b] *Faculty of Pharmacy, Cairo University, Kasr-el-Aini Street, 11562, Cairo, Egypt*
[c] *Pharmacology Department, National Research Centre, 33El Bohouth St., Dokki, Giza 12622, Egypt.*
[d] *Phytochemistry and Plant Systematics Department, National Research Centre, 33 El Bohouth St., Dokki, Giza 12622, Egypt*

Abstract
Melilotus species (Fabaceae) have provided extensive anti-inflammatory traditional uses, and thus, they will continue to be among the major plant sources of possibly new anti-inflammatory drugs. In continuation of our previous chemical and biological studies on *Melilotus indicus* (L.) and *Melilotus messanensis* (L.)., the present work constructs a comparative anti-inflammatory evaluation of both plants and highlights their bioactive potential concerning their chemical components. The phenolic and flavonoid contents of the aqueous methanol extract (AME) of both plants were estimated spectrophotometrically and calculated to be (24.522 and 29.696 mg Gallic/g extract) and (7.429 and 11.344 mg Catechin/g extract) in (*M. indicus* and *M. messanensis*), respectively. Chronic toxicity tests of AME for each species were investigated after long-term daily oral administration (100 mg/kg B.wt.) to the experimental animals, where no significant change in their body and visceral organ weights. Also, the level of some biochemical markers like creatinine, urea, glucose, triglycerides, cholesterol, AST, and ALT was measured in the animal blood sera and found that there are no fluctuations occurred to their typical levels, therefore, both plants were mostly safe. Furthermore, AME, petroleum ether extract (PEE), and defatted AME (DAME) of *M. indicus* and *M. messanensis* were subjected to anti-inflammatory assay using carrageenan-induce paw edema models and exhibited significant potentials through inhibition of the rat paw edema diameter (13 and 12%), (11 and 10%), and (14 and 15%) for *M. indicus* and *M. messanensis*, respectively, in comparison with indomethacin (6%).

Keywords: *Melilotus messanensis, Melilotus indicus,* Chronic toxicity, Anti-inflammatory

1. Introduction

Melilotus Mill. is a genus in the family Fabaceae (subfamily: Trifolieae) known as grassland and weeds of cultivation. *Melilotus* genus comprises about 22 species widely distributed all over the world [1]. Geographically, their presence is not limited to a certain country or continent but it is widely spread all over the world, *viz*.: Pakistan, India, Europe, and Africa [2,3].

The most dominant traditional use that distinguishes this genus is its anti-inflammatory effect which is in turn confirmed by appreciable some pharmacological studies, where, Melilots herbs are consumed in the treatment of ulcer, bronchitis, asthma, rheumatism, allergy, arthritis, as poultice for inflammations and swollen joints [4–6]. On the other side, *in vitro* studies of some *Melilotus* species confirmed its inhibitory effect on pro-inflammatory mediators and leukocytes migration [7], whereas a significant anti-inflammatory activity of *Melilotus* species were illustrated in rodents' models, *viz*, carrageenan-induced edema and rat cecal ligation and puncture induced model [8] [9]. Furthermore, advanced clinical trials in patients with chronic lymphedema and postoperative edema definite the anti-inflammatory potentials of *Melilotus officinalis* drug [10].

In previous findings, the extracts of Some *Melilotus* species extracts and various isolated compounds were tested as *in vitro* and *in vivo* anti-

*Corresponding author e-mail: nerminmaherragab@gmail.com.;(Nermin A. Ragab)
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inflammatory agents. Most of the tested extracts showed moderate to significant activities [8,11–13]. Also, some isolated compounds of *M. indicus* exert inhibition one to ten times higher than that of control [14]. *M. indicus* extract induced considerable downregulation of TNF-α in serum samples equivalent to the anti-inflammatory effects of standard drug-treated groups [8].

Despite the wide geographic propagation, rich ancient traditional values as well as scientific information on phytochemical and biological properties of genus *Melilotus*, the supreme anti-inflammatory importance *Melilotus* species *viz; M. messanensis,* remains somewhat repressed. Therefore, one of the objectives of the current study is finding a possible anti-inflammatory drug of this genus which prosperously grows in Egypt.

Although herbal drugs are considered safe, recent studies proved the correlation between herbal drugs misuse and hepatic and renal toxicity [15]. On completion of our previous studies proving the safety of plants subject of study in terms of acute toxicity [16–18], the scope of the present study is implied to validate the safety of the plants under study on long term administration of *M. messanensis* and *M. indicus*.

Our previous studies presented the chemical identity of *M. messanensis* and *M. indicus*. The investigation of their DAMEs by chromatographic techniques led to the isolation of fifteen phenolics from *M. messanensis* and sixteen phenolics from *M. indicus.* On the other hand, PEEs were analyzed by GC/MS assay, which led to the detection of 44 and 35 nonpolar compounds (fatty acids methyl esters and unsaponifiable compounds) in *M. messanensis* and *M. indicus*, respectively. Furthermore, chemical profiling for *M. messanensis* and *M. indicus* using LC-ESI-MS analysis which led to the characterization of 39 metabolites of both plants [16,17]. Thereby, the present study correlates the possible phytoconstituents underlying the anti-inflammatory activity.

2. Material and Method

2.1. Plant material

M. messanensis and *M. indicus* were collected as weeds of cultivation on 25 February 2022, from 6th October City, Giza, Egypt. The samples were verified by Prof. Dr. Mona M. Marzouk. The labelled specimens N1_3216 and N2_14216 were hold in the herbarium of National Research Centre for *M. messanensis* and *M. indicus,* respectively.

2.2. Preparation of plant extracts

Five hundred gm of the air-dried plant samples of each species were ground individually then extracted by maceration in 70% MeOH/H_2O, followed by 50% MeOH/H_2O, repeatedly, till exhaustion, at room temperature. The combined extracts were distilled off under reduced pressure and low temperature to obtain a residue of AME, 71g and 37g for *M. messanensis* and *M. indicus*, respectively. Each AME residue was suspended in 500 ml H_2O and exhaustively partitioned with light petroleum ether (40–60 °C). The PEE, 6g and 4g for *M. messanensis* and *M. indicus,* respectively, was dehydrated over anhydrous Na_2SO_4, evaporated, and preserved for analysis. The remaining DAME, 39g and 27g for *M. messanensis* and *M. indicus*, respectively, was also evaporated and kept for investigation. All compounds are >95% pure by HPLC analysis.

2.3. Phytochemical studies

2.3.1. Determination of total phenolic content

The Folin Ciocalteu method was adopted to evaluate the total phenolic content of both plants which is according to [19]. In a test tube, 100 µL plant extract was diluted to 3.5 mL with distilled water and 250 µL oxidizing agent is added namely, Folin-Ciocalteau reagent. After 5 min, the neutralization of the mixture is done with 1.25 mL of 20% aqueous sodium carbonate (Na_2CO_3) solution. After 40 min, the measurement of the absorbance was at 725 nm using the solvent as blank. Determining the total phenolic content was resolved by means of a calibration curve prepared with gallic acid and conveyed as µg of gallic acid equivalent (mg GAE) per g of sample.

2.3.2. Determination of total flavonoid content

Aluminium Tri-chloride ($AlCl_3$) colorimetric assay [19] was adopted to verify the total flavonoid content of both plant extracts. In brief, 100 µL of plant extract was mixed with 300 µL of 5% sodium nitrite ($NaNO_2$). After 6 min, we added 300 µL of a 10% $AlCl_3$ solution, and this prepared mixture was diluted to 2.5 mL using distilled water. After 7 min, 1.5 mL of 1 M NaOH was added, at 5000g for 10 min, the mixture was centrifuged. The supernatant absorbance was measured at 510 nm against the solvent as blank. A calibration curve established with catechin and expressed as milligrams of catechin equivalent (mg CE) per g of sample used to establish the total flavonoid content.

2.4. Biological studies

2.4.1. Chronic toxicity

Thirty albino rats were divided into three groups, each of 10 rats. The first group was retained as a control and received 1 mL saline as a daily oral dose. The second and third group received daily oral dose of 100 mg/ kg b.wt. /day of the AME extract of *M. indicus* and *M. messanensis*, respectively. Administrations were continual for sixteen weeks. From the retro orbital venous plexus of each rat, Blood samples were obtained at zero-time, after 8 and 16 weeks. Isolation of serum was proceeded by centrifugation and divided into different portion to be further analyzed to evaluate triglycerides, total

cholesterol, creatinine, glucose urea, aspartate amino transferase (AST/GOT) and alanine transferase (ALT/GPT). Furthermore, the body weight of each rat was noted. Finally, the animals were sacrificed and some of their internal organs were dissected off, then any morphological changes were investigated for and weighed accurately [20].

2.4.2. Anti-inflammatory activity

Thirty adult male albino rats (for each plant) were divided into five groups (six animals/ group). The first group; rats that received 1 ml saline serving as a control. The second, third and fourth group of animals received AME, PEE and DAME (100 mg/kg b.wt.), respectively. The fifth group; rats that received standard drug; indomethacin (20 mg/kg b.wt.). One hour after oral administration, 0.1 ml of 1% carrageenan was given to all animals as a sub-plantar injection of solution in saline in the right hind paw. After drug administration, every hour (four times) , paw diameter is determined [21]. Edema is determined by the following equation: % of change (edema) = [(diameter of rat paw at zero–diameter of rat paw after one hour (or 2, 3 and 4) /diameter of rat paw at zero] x 100. Potency = (% of change standard/ % of change plant group) x100. All the mentioned procedures were approved by the Medical Ethics Committee, National Research Centre (no.17158).

2.4.3. Statistical analysis

For anti-inflammatory and chronic toxicity analysis, data are presented as standard error of the means (± SEM) for six rats in each group. Statistical significance was assessed using the ANOVA test followed by the post hoc, Duncan's multiple range test. A probability value below 0.05 ($P < 0.05$) was statistically significant.

3. Results

3.1. Phytochemical investigation

The phenolic and flavonoid contents of the AME of both plants were estimated spectrophotometrically and calculated to be (29.696 and 24.522 mg $_{Gallic}$/g $_{extract}$) and (11.344 and 7.429 mg $_{Catechin}$/g $_{extract}$) in (*M. messanensis* and *M. indicus*), respectively. Calibration curves were established using standards.

3.2. Biological activity

Chronic toxicity

According to previous studies done by our pharmacological team, LD_{50} of the AME of *M. messanensis* and *M. indicus* were found to be 5.8g extract/kg b.wt. and 4.7g extract/kg b.wt., respectively. This findings shows the safety of *M. messanensis* and *M. indicus*. [16,17], corresponding to these results. Chronic toxicity tests of the AME of each of the two species were investigated.

Effect of oral administration of M. messanensis and M. indicus extracts on body and organ weights

The body and visceral organ weights of experimental animals after short- and long-term administration of AME of *M. messanensis and M. indicus* did not show any significant change. Their increase in bodies' weights is due to the normal daily food and water uptake, then animals were sacrificed, followed by, dissection of their internal organs and weighed accurately. The results showed that there was no significant change when compared to the control group and no organ showed a decrease or increase in its weight Tables (1 & 2).

Table1: Body weight (g) after long term administration of AME of *Melilotus messanensis* and *Melilotus indicus*

Group	Body weight (g)		
	Zero time	**8 weeks**	**16 weeks**
Control	128.6 ± 3.9	153.9 ± 5.9*	182.3±5.6*
M. messanensis	125.9 ± 3.8	158.7 ± 6.8*	185.7 ± 6.8*
M. indicus	131.7 ± 4.1	163.4 ± 4.6*	189.1 ± 4.3*

*Statistically significant difference from zero at P<0.01

Table2: Relative organ weight (mg/100kg b.wt) after long term administration of AME of *Melilotus messanensis* and *Melilotus indicus*

Organs	Relative organ weight (g/100 g b.wt)		
	Control	***M. messanensis***	***M. indicus***
Kidney	0.85 ± 0.03	0.92 ± 0.01	0.95 ± 0.01
Heart	0.51 ± 0.06	0.65 ± 0.05	0.64 ± 0.02
Lungs	0.81 ± 0.01	0.92 ± 0.01	0.95 ± 0.01
Spleen	0.43 ± 0.01	0.44 ± 0.02	0.48 ± 0.03
Liver	3.80 ± 0.02	3.81 ± 0.04	3.87 ± 0.4
Testis	1.50 ± 0.01	1.51 ± 0.02	1.59 ± 0.01
Seminal vesicles	0.41 ± 0.01	0.42 ± 0.03	0.43 ± 0.02

No significant difference from control

Table3: Biochemical changes after long-term administration of AME of *Melilotus messanensis* and *Melilotus indicus*

Group	Time (weeks)	Biochemical changes						
		Cholesterol mg/dL	**Triglyceride mg/dL**	**Glucose mg/dL**	**Creatinine mg/dL**	**Urea mg/dL**	**AST U/L**	**ALT U/L**
Control	**Zero**	82.1±6.5	76.9±3.9	82.3±2.1	1.1±0.01	22.3±0.3	35.1±1.5	32.8±1.4
	8	80.2±8.6	74.2±2.6	83.6±2.2	1.2±0.01	21.1±0.4	33.2±1.3	32.4±1.3
	16	81.5±3.3	73.9±2.8	81.1±2.7	1.1±0.02	21.8±0.3	34.3±1.1	34.8±1.6
Mm	**Zero**	83.6±2.4	71.1±1.2	86.2±2.1	1.3±0.04	21.2±0.6	32.8±1.7	29.8±0.7
	8	81.5±2.9	73.5±2.9	83.1±1.6	1.2±0.03	22.1±0.7	31.9±1.6	31.1±1.3
	16	82.3±2.5	72.1±2.8	81.6±1.3	1.1±0.02	21.8±0.8	30.5±1.3	30.3±1.2
Mi	**Zero**	81.2±2.8	73.8±1.2	85.3±2.4	1.3±0.03	21.6±0.8	30.9±1.4	33.1±1.1
	8	83.1±2.3	72.1±1.3	83.1±2.3	1.2±0.01	22.3±0.7	31.6±1.2	32.4±1.4
	16	81.3±2.1	71.3±2.3	79.2±1.5	1.1±0.03	23.3±0.5	32.1±1.6	31.8±1.3

Mm; *Melilotus messanensis,* Mi; *Melilotus indicus*. No significant difference from zero

Effect of oral administration of M. messanensis and M. indicus extracts on biochemical markers

Table (3) revealed that when the level of some biochemical markers like creatinine, urea, glucose, triglycerides, cholesterol, AST and ALT were measured in blood serum after long administration of *M. messanensis and M. indicus*, the animals' biomarkers levels showed no significant changes compared to normal levels. No changes were observed in the levels of the biomarkers measured or the total body weights and organ weights of the rats. This verifies the safety of both plants to a great extent at the dose of 100 mg/kg b.wt.

Typically, assessment of test materials for acute anti-inflammatory activity is done through their ability to reduce or prevent paw edema induced by carrageenan. Additionally, non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, is used as standard drug.

Results of the acute anti-inflammatory test were done by oral administration of doses of different extracts. As the control group developed a continuous increase in paw edema. Oedema inhibitory values were based on 4 hr post carrageenan treatment and calculated by subtracting the diameter of the control rat paw from that of the treated rat paw [11,22]

Evaluation of Acute anti-inflammatory Activity

Paw swelling, or footpad edema is a convenient approach used to evaluate the acute anti-inflammatory effect [21]. Carrageenan is the irritant used in this model which stimulate the paw swelling.

Results showed notable Oedema inhibitory values of plant treated rats, where, the highest activity exhibited by PEE (10%), AME (12%) and DAME (15%), of *M. messanensis,* in comparison with indomethacin (9% of change) (Figure 1). In *M. indicus* group**,** the highest activity exhibited by PEE (11%), AME (13%) and DAME (14%), in comparison with indomethacin (9%of change) (Figure 1). Results of the potency (based on comparison to standard drug) showed the highest potency was gained by PEE of *M. messanensis* (90), followed by, PEE of *M. indicus* (81) (Figure 2).

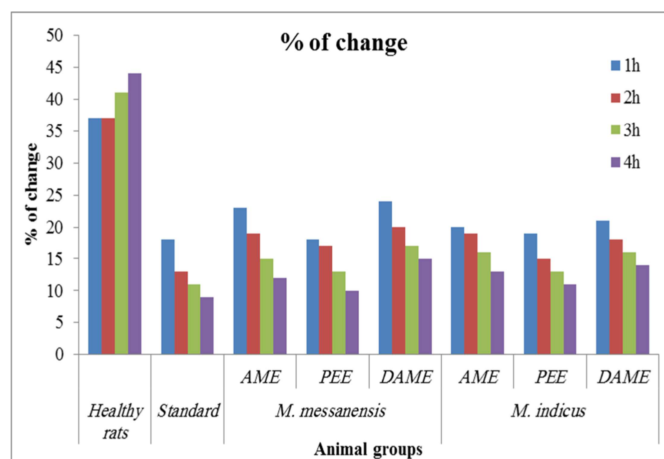

Figure 1: Acute anti-inflammatory activities of *Melilotus messanensis* and *Melilotus indicus* extracts compared to standard and healthy animals.

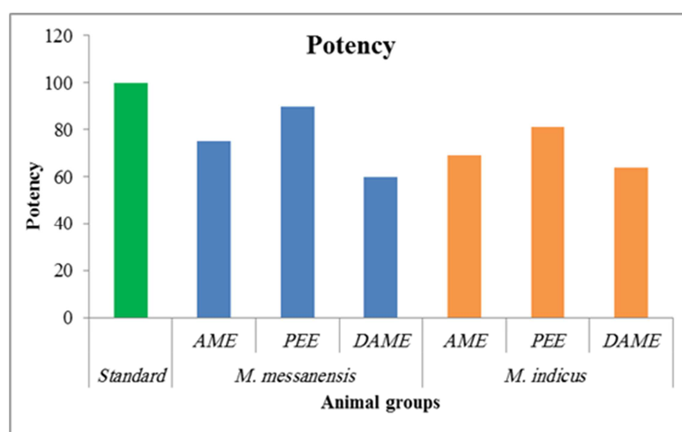

Figure 2: Potency of *Melilotus messanensis* and *Melilotus indicus* extracts on carrageenan induced rat paw edema compared to indomethacin

4. Discussion

The total phenol and total flavonoid content of *M. indicus* were determined as 45µg (0.045 mg) (gallic acid equivalents *pe*r mL of plant extract) and 550 µg (0.55mg) (rutin equivalents *per* mL of plant extract), respectively [2]. These Natural constituents represent the first line defense mechanism in human body system and prevent the harmful effects of inflammation [23].

Accessing the toxic effect of herbal substance is productively accomplished by examining the haematological parameters in rodents to give a high analytical assessment of toxicity in humans [24]. Several parameters were measured after long-term oral administration of the AME of *M. messanensis and M. indicus* to reveal the effect of each extract on body weights, cholesterol, triglycerides, glucose, creatinine, blood urea and liver enzymes (AST and ALT) serum levels and relative organs' weights of albino rats. *M. messanensis and M. indicus* extracts showed no toxic effect through retaining the normal body weight and relative organ weight. The monitored biomarkers in the present study confirmed the safety of *M. messanensis* and *M. indicus* extracts on long term administration.

Collected literature reported a significant anti-inflammatory activity of *M. indicus* extracts [8], in contrast, *M. messanensis* extracts lack any anti-inflammatory evaluation in previous studies. This directed our attention to construct a comparative anti-inflammatory evaluation of both plants under the same laboratory measures.

Previous finding confirmed the richness of PEE of both plants with bioactive fatty acid methyl ester, Saturated , mono-, di- and tri-unsaturated fatty acids and sterols (Table S1 & S2) [16,17]. This explain the high potency of PEE [25,26]. Previous studies deduced that β-sitosterol decreases the percentage of paw volume in carrageen induced paw edema at 10 mg/kg with respect to control. Also, lupeol has ability to restore the serum levels of cytokines, lipid peroxide and superoxide dismutase [27].

The effectiveness of the polar extract might stem from its flavonoid constituents. Flavonoids are recognized for having multi diversified pharmacological responses, for example, anti-inflammatory, anti-allergic, virucidal ,bactericidal and anti-cancer agents [28]**.** The identified compounds of plants under study through the chromatographic techniques and the LC-ESI-MS assay in our previous studies (Table S3, Figure S1) [16,17] are well known as anti-inflammatory agents, *viz*, rutin [29], quercetin [30,31], luteolin 8-C-glucoside, robinin, cloven [32] and kaempferol [33]. Additionally, kaempferol glycosides and quercetin glycosides exhibited great effectiveness in inhibiting arachidonic acid-induced edema. Moreover, some of flavonoid C-glycosides displayed heightened inhibitory ability of LPS-induced NO production. Prior *in vivo* studies demonstrated that oral administration of flavonoid glucosides possesses a comparable or potentially greater anti- inflammatory activity than flavonoid aglycones [32].

Also, flavonoids are interacting in many signaling pathways and the appearance of inflammatory genes: they modulate the activities of enzymes that metabolize arachidonic acid and inhibit the production of proinflammatory cytokines [23].

5. Conclusion

The safety of the aqueous methanol extracts of the plants under study is validated through median lethal dose and chronic toxicity test. Current evidence suggests that the pronounced anti-inflammatory effect of *M. messanensis* and *M. indicus* may be, due to the presence of quercetin, rutin, kaempferol and fatty acid constituents as the principal elements, with the underlying anti-inflammatory effect of the active fractions of both species. These results extend our previous findings and demonstrate that *M. messanensis* could be itemized as a typical example

of a natural anti-inflammatory drug, thus avoiding the undesirable effects of synthetic drugs. Forthcoming *in vivo* research of dose-biological response, metabolic transformations, and mechanisms involved are required in present bioactivity.

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