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Toxic properties and allelopathic activity of *Melilotus officinalis* (L.) Pall.

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Abstract

Melilotus officinalis (L.) Pall., known as yellow sweetclover (Fabaceae), is widely used in medicine and agriculture. At the same time, yellow sweetclover is a weed and invasive plant in Siberia. In Russia, *M. officinalis* is cultivated as a valuable medicinal, fodder and honey plant. Its widespread use is due to its high ecological plasticity. In recent years, an interest in cultivation of *M. officinalis* as a low maintenance multipurpose crop has increased in biological agriculture. The herb *M. officinalis* contains a rich complex of biologically active compounds. However, along with positive properties, this species, though with a rich chemical composition and high physiological activity, is toxic towards different groups of living organisms. The toxic effect of *M. officinalis* extracts is primarily due to the presence of coumarin. A high allelopathic activity of *M. officinalis* was revealed. The phytotoxic effect of herb extracts on germination of crop and weed seeds was studied in detail. Data on the fungicidal and insecticidal activity of *M. officinalis* were obtained. Laboratory and in situ studies showed that the aboveground part of *M. officinalis* is a potential source of biopesticides with a broad-spectrum effect (bioherbicidal, insecticidal and fungicidal).

Keywords

Melilotus officinalis, coumarin, biological toxicity, allelopathy, biopesticides, repellent activity, biological agriculture

Introduction

Melilotus officinalis, known as yellow sweetclover, is widespread in Russia. In most regions of Siberia (Omsk, Novosibirsk, and Kemerovo regions, Altai Territory and the Republics of Altai and Khakassia), it is a native species. The plant grows in steppe meadows, sometimes saline, along roads, ditches, in fallow lands and in crops as a weed, on the plain in the forest, forest-steppe and steppe zones, and in the mountains – up to the middle belt of mountains. The species actively disperses within its natural and secondary ranges, including dispersion with crop seeds (Mikhailova et al. 2019). *M. officinalis* is often found as a weed plant (ruderal and segetal), and in some regions (Tomsk and Irkutsk regions, the Republics of Tyva and Buryatia, Krasnoyarsk Territory) of Siberia it is considered an invasive species (Khrustaleva 2016).

Melilotus officinalis is cultivated as a valuable medicinal, fodder and honey plant. The widespread use of sweetclover is due to its high ecological plasticity. *M. officinalis* is drought-resistant, winter-hardy, and undemanding to soil fertility; it can grow in a wide range of edaphic conditions (Luo et al. 2016). The plant exhibits high phytomeliorative properties and is recommended for improving soil fertility and phytoremediation of soils contaminated with heavy metals and hydrocarbons (Steliga and Kluk 2021).

In Russia, *M. officinalis* is typically grown as a medicinal raw material (*Meliloti herba*). In conventional medicine, the herb *M. officinalis* is included in the composition of sedative drugs (Vidal, electronic link). In traditional medicine, the aboveground part of *M. officinalis* is used to treat bronchitis and bronchial asthma, inflammatory processes in the ovaries, menstrual disorders, flatulence, furunculosis, etc. (Dubrovskikh, electronic link, Al-Snafi 2020). The experimentally proven pharmacological effects of the herb *M. officinalis* are as follows: antimicrobial, antioxidant, anti-inflammatory, hepato- and neuroprotective, antitumor, anxiolytic, rheological, immunostimulatory, etc. (Al-Snafi 2020, Abyshev et al. 2014, Venugopala, Patent 2006, Podkolzin et al. 1996). In Europe and China, the plant extract is used to treat inflammation, arthritis, rheumatism, phlebitis, venous insufficiency, hemorrhoids, brachygalgia, and bronchitis (Khare 2007).

The medicinal and poisonous properties of *M. officinalis* and its widespread cultivation and distribution in some countries as an invasive species triggered an intensive study of its allelopathic activity and biotoxicity.

The aim of the study was to analyze sources of information concerning the toxic properties of *M. officinalis* towards different groups of living organisms.

Material and methods

A literary search and a review of scientific articals available in the search databases was carried out using the keywords «*Melilotus officinalis*» and «biopesticides», «*Melilotus officinalis*» and «biological toxicity», «biological agriculture». The paper used the resources of PubMed search engines, eLibrary, Scopus, Web of Science, for the review we used articles containing evidence-based experimental data on the toxic properties of *Melilotus officinalis* depending on their impact on different groups of organisms.

Results

Chemical composition

The herb *M. officinalis* contains a rich complex of biologically active compounds: phenolic compounds (coumarins, flavonoids, tannins), alkaloids, purines, essential oil (up to 0.02%), fatty oil (up to 4.3%), proteins (up to 17 .6%), and mineral salts (Al-Snafi 2020). Phenolic compounds are represented mainly by coumarins, flavonoids, and derivatives of phenolcarboxylic acids. The predominant compounds include unsubstituted coumarin among coumarin compounds, rutin among flavonoid compounds, and ferulic acid among derivatives of phenolcarboxylic acids. The aboveground part of the plant contains flavonoids (luteolin, vitexin, hyperoside, herniarnin, hesperidin, robinin, quercetin, kaempferol, rutin, and others), coumarins (umbelliferon, scopoletin, 4-hydroxycoumarin, melilotin, melitoside), and derivatives of phenolcarboxylic acids (chlorogenic, coffee, cinnamon, and others) (Bubenchikova 2004).

About 60 components were found in the essential oil produced from the aboveground part of the plant by exhaustive hydrodistillation, the main of which was chamazulene, 4-epi-akoronene, benzyl alcohol, and some others (Efremov et al. 2012).

The content of polysaccharides and amino acids (glutamine, arginine, valine, threonine, etc.) (Fedoseeva, Kharlampovich 2013), fatty acids, and triterpenes was determined (Anwer et al. 2008).

The study of flowers assessed the content of the essential oil, coumarins (up to 0.87%), mucus, choline, resins, tannin, and flavone glycosides (kaempferol, quercetin, myricetin) (Nikolaev et al. 2019, Khare 2007).

The seeds contain the amino acid canavanine and alkaloid trigonelline (Khare 2007).

Toxic properties of sweetclover towards animals

Coumarins are considered to be the main group of pharmacologically active compounds of *M. officinalis*, which determine both therapeutic and toxic effects in humans and animals (Abyshev et al. 2014).

Basic data on the toxicity of *M. officinalis* refer to veterinary toxicology. In forage production (green feed, hay and haylage), it is necessary to consider the content of coumarin in the grass, which can indirectly cause severe poisoning and death in different animal species. Sweetclover poisoning ("sweetclover disease") was described by F.W. Schofield in 1921 (Schofield 1923) He unambiguously showed that spoiled sweetclover forage can cause fatal bleeding when fed to cattle or experimental animals. He also revealed the relationship between the presence of mold (typically located inside the stem) and the disease onset and concluded that the disease was caused either by the mold itself or by a decomposition product, yet he did not prove his conclusion.

The history of the discovery of 3,3'-methylenebis-(4-hydroxycoumarin), later named dicoumarol, its isolation from spoiled sweetclover hay (*Melilotus alba* and *M. officinalis*), crystallization, identification and synthesis is well known (Campbell and Link 1941; Campbell et al. 1940; Stahmann et al. 1941; Zobnin et al. 2013).

Cases of animal intoxication with dicumarol have been reported regularly in the US and Canada (Alstad et al. 1985; Puschner et al. 1998; cited from Berny et al. 2005).

Dicoumarol is produced by the growth of fungi of the genera *Aspergillus* or *Penicillium* in *Melilotus* spp. The fungi secrete melilotic acid, mycotoxin responsible for dimerization of 4-O-coumarin in dicoumarol. The latter acts as an anticoagulant rodenticide: it reduces the level of prothrombin in the blood and affects blood coagulation. When an animal consumes toxic hay or silage for several weeks, dicoumarol changes the proenzymes required for prothrombin synthesis, which causes the hemorrhagic syndrome of hypoprothrombinemia (preventing active enzyme formation). It can also interfere with the synthesis of factor VII and other vitamin K-dependent coagulation factors. Dicumarol concentrations of 20–30 mg/kg of hay ingested over several weeks are sufficient to cause poisoning in cattle. The signs of poisoning are most pronounced in cattle and to a limited extent in pigs and horses, while sheep are sufficiently resistant. The time between consumption of toxic sweetclover and the onset of clinical disease varies and depends on the dicumarol content in the particular *M. officinalis* cultivar, the age of the animal, and the amount of feed consumed. If dietary dicumarol is low or variable, animals may consume it for months before signs of disease appear. Hay containing a dicoumarol doze equal to 10–20 mg/kg can be fed for 100 days before poisoning develops. A 60–70 mg/kg doze of dicumarol can cause poisoning after 21 days of its consumption. A 50 μg/g doze of dicumarol causes severe clinical signs within 15 days (Puschner et al. 1998).

The first indication of dicumarol poisoning in cattle is often death of one or more animals. Poisoned animals may be stiff and lame due to bleeding into muscles and joints. Cardiovascular signs (severe hemorrhage causes anemia, mucosal pallor, heart palpitations and death), respiratory signs (epistaxis), renal signs (hematuria), reproductive signs (excessive hemorrhage during calving), and ocular signs (bleeding into the anterior chamber of the eye). Death is generally caused by massive hemorrhage or bleeding. In horses, poisoning can lead to excessive bleeding, pallor of the mucous membranes, and weakness. A prothrombin time exceeding 40 seconds indicates faulty blood coagulation. Blood prothrombin, activated partial thromboplastin time, and blood coagulation time become markedly increased (Baumann et al. 1942; Radostits et al. 1980; Puschner et al. 1998).

Low-coumarin varieties of *M. officinalis* are safe and do not cause a disease (Goplen 1980).

Dicoumarol became a new rodenticide and the first anticoagulant patented in 1941 as a pharmaceutical (Link 1959; Zobnin et al. 2013).

In the economic use of *M. officinalis* in feed formulations, its predisposition to the accumulation of fungal metabolites toxic to animals should be considered. A comparative study of mycotoxin contamination of legumes on natural fodder lands in the European part of Russia showed that more than 80% of *M. officinalis* samples were contaminated with the following mycotoxins: ergoalkaloids, alternariol, cyclopiazonic acid, and emodin; ochratoxin A and PR toxin were found in smaller amounts (Burkin et al. 2017).

At present, active breeding aimed at reducing the content of coumarin in sweetclover grass is underway (Dzyubenko et al. 2018), and forage conservation technologies are being improved (Sagalbekov et al. 2016).

Toxicity of sweetclover towards humans

The herb *M. officinalis* is commonly used in herbal medicine as an angioprotective and antiplatelet agent to treat angina pectoris, chronic heart failure, and varicose veins. High dosages of *M. officinali* can cause side effects such as dyspepsia, drowsiness, and headache (Lesiovskaya 2014). Moreover, improper harvesting of medicinal plant materials leads to dicumarol formation. Hemorrhagic syndrome can develop in patients treated with preparations made on the basis of such raw materials (Zobnin et al. 2013).

Traditionally, coumarins are used as a flavoring agent for food and alcoholic beverages. Coumarin was synthesized in 1868, and it was first put on the market as a flavoring substance (Abraham et al. 2010). However, coumarin was found to cause liver toxicity in human, and carcinogenicity and mutagenicity in animals, and it was withdrawn from usage in the USA in 1954. In 1964, coumarin was banned in the UK based on the experimental data on the ability of coumarin to cause benign and malignant tumors in the liver, kidneys and lungs of animals. In 1988, the European Union set a tolerable daily intake for coumarin from natural spices and herbs in food of 2 mg/kg (Lake 1999, cited in Shikh et al. 2015; Abraham et al. 2010), which was then reduced to 0.5 mg/kg, since hepatotoxicity in human could not be ruled out. In relation to safety, obvious differences in metabolism of coumarins present in foods and used as fragrances in cosmetics were revealed between susceptible species and other species, including humans. Conversion of this compound in the human body proceeds predominantly through the 7-hydroxylation pathway, which is safe for the body (Lake, 1999; Abraham et al. 2010).

Melilotus officinalis is included in the list of plants prohibited for use in food supplements (Nikolaev et al. 2019).

Allelopathic activity *in situ* and *in vitro*

There is a renewed interest, particularly among organic growers, in using yellow sweetclover (*M. officinalis*) as cover crop. Experiments were initiated in 1999–2002 in Canada to compare the effect of high- and low-coumarin cultivars of *M. officinalis* on weed suppression. Sweetclover termination at 70% bloom was often more effective in suppressing weeds than termination at the bud stage. In the summer and fall after termination, surface residues of Yukon, a high-coumarin and drought-tolerant cultivar, reduced *Chenopodium album* L. density by > 80% compared with the no sweetclover check and essentially eliminated *Descurainia Sophia* (L). Webb. In the following spring, Yukon reduced *Kochia scoparia* (L.) Schrad. density by > 80% and *Avena fatua* L. biomass by > 30% compared with the no sweetclover check. It may be possible for organic growers to manage weeds with sweetclover in a reduced tillage system that leaves most of the plant residues on the soil surface (Moyer et al. 2007). It was suggested that *M. officinalis* suppresses weeds due to the allelopathic activity of the compounds released during its decomposition (Blackshaw et al. 2001).

Melilotus officinalis has the complex phytosanitary activity of spring wheat predecessor to agrocenoses of the southern forest steppe in Western Siberia (Novosibirsk Region). Yellow sweetclover improved the phytosanitary condition of soil by decreasing population densities of phytopathogens – agents of common root rots, weeds and phytophages (Toropova et al. 2014; Toropova et al. 2021).

Russian and Japanese scientists (Mardani et al. 2016) screened 178 species of Caucasian flora for the presence and degree of allelopathic activity. Laboratory experiments performed using the sandwich method showed that *M. officinalis* belongs to the group of highly toxic allelopathic plants. Aqueous extracts of the herb remarkably inhibited germination of lettuce (*Lactuca sativa*) seeds.

Aqueous extracts of *M. officinalis* with different concentrations (1, 3, and 5%) inhibited germination and growth of wheat and peas (Umer et al. 2010). Similar results were obtained for other crop test plants (Shinwari et al. 2013).

The allelopathic activity of aqueous extracts from leaves (15, 20, 25 and 30%) of *M. officinalis* was studied in relation to germination of seeds and growth of wheat seedlings. High concentrations of the extract (25–30%) significantly reduced seed germination, root length, and aerial part of wheat seedlings. Yet *M. officinalis* extracts were inferior in phytoinhibitory activity to leaf extracts of *Centaurea maculosa* (Siyar et al. 2017).

According to C.X. Wu et al. (2014), coumarin plays a predominant role in the inhibitory activity of *M. officinalis* extracts. The effect of coumarin, which inhibits plant seed germination and seedling growth, is consistent with the effect of aqueous extracts of *M. officinalis* on other plants (Wu et al. 2015).

Of particular interest is the study of alleloherbicidal activity of *M. officinalis* in China (Wu et al., 2010; 2014; 2015; 2016; Wang and Qi 2014). C.X. Wu et al. (2016) reported the results of chemical studies of the herb *M. officinalis* and biotesting on different groups of plants. This study aimed to isolate, identify and quantify the predominant allelochemical composition of *M. officinalis* using organic solvent extraction, chromatography, thin layer chromatography (TLC), gas chromatography-mass (GC-MS) and nuclear magnetic resonance (NMR), as well as to assess its inhibitory activity on weeds using bioassays. The most active allelochemical components were extracted using petroleum ether. Coumarin, 2H-1-benzopyran-2-one, was isolated and recognized as the most predominant and active allelochemical. The coumarin content in the original extract of *M. officinalis* was 46.78 μg/ml or 1.152% of dry matter content. At a dose of 40 μg/ml, coumarin significantly inhibited seed germination and seedling growth in *Lolium multiflorum* Lam., *Polygonum aviculare* L., *Trifolium pretense* L., *Veronica persica*, *Poa pratensis* L., *Chenopodium album*, and *Plantago asiatica* ($P<0.05$). Coumarin exhibited significant inhibitory activity on both seed germination and seedling growth of all plants studied ($P<0.05$). It completely suppressed seed germination and seedling growth in *Lolium multiflorum*, *Polygonum aviculare*, and *Trifolium pratense*. Coumarin showed a strong inhibitory effect on seed germination and seedling growth in numerous weeds, which suggests that it can be used as a natural herbicide. According to A. Kaur and R. Kaur (2017), flowering leaves and flowers of *M. officinalis* can be used as a raw material for production of biopesticides.

There are few data on the effect of *M. officinalis* on insects, in particular on its reppelent activity. Insecticidal activity of methanol extracts from *M. officinalis* was tested on 3rd instar larvae of the Egyptian cottonworm (*Spodoptera littoralis*). *M. officinalis* extracts were moderately toxic (LC50 – 5.6 μg/ml). The relative growth rate, the rate of food intake and efficiency of conversion of the digested food were calculated. Clear correlations were found between weight increase, quantity of the ingested food, and quantity of excrements produced during the whole assay period. These results indicate an antifeedant activity of the tested extract of *M. officinalis* (Pavela and Chermenskaya 2004).

The repellent and insecticidal activity of *M. officinalis* on *Tenebrio molitor* L. were studied experimentally. The repellent activity of the dry crushed aboveground part of *M. officinalis*, added to oatmeal and spring wheat, was investigated against *T. molitor* larvae. The repellent efficacy of *M. officinalis* was observed in relation to *T. molitor* larvae when added to oatmeal. The insecticidal activity of an aqueous extract from the aboveground part of *M. officinalis* was studied on larvae and imago of *T. molitor*. *M. offcicinalis* was found to have a pronounced insecticidal effect on *T. molitor* imago, yet none of the effect was found for larvae (Gulik et al. 2021).

Conclusion

An increased interest in the cultivation of *M. officinalis* as a multi-purpose agricultural crop (medicinal, fodder, melliferous), particularly in biological agriculture, requires a comprehensive analysis of its chemical composition and physiological activity. A high coumarin content in *M. officinalis*, especially in wild plants, can have a toxic effect on the biocenosis. Laboratory and in situ studies have shown that the aboveground part of *M. officinalis* is a potential source of broad-spectrum biopesticides (bioherbicidal, insecticidal and fungicidal). Genetic variation in germplasm of *Melilotus officinalis* is the foundation of successful plant breeding, and wild germplasm is a valuable source of new alleles associated with desirable characteristics, such as higher or lower coumarin content (Zhang et al. 2018).

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