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In vitro effect of essential oils from *Cinnamomum aromaticum*, *Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis*

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Summary:

Essential oils may be effective preventive or curative treatments against several flagelated poultry parasites and may become primordial either to organic farms, or as more drugs are bannished. The anti-flagellate activity of essential oils obtained from fresh leaves of *Cinnamomum aromaticum*, *Citrus limon* pericarps and *Allium sativum* bulbs was investigated *in vitro* on *Tetratrichomonas gallinarum* and *Histomonas meleagridis*. On *T. gallinarum*, the minimal lethal concentration (MLC) at 24 hours was 0.25 µl/ml for *C. aromaticum* oil, and 0.125 µl/ml for *C. limon* and *A. sativum* oils. On *H. meleagridis*, MLC was 0.5 µl/ml for *C. aromaticum* oil and 1 µl/ml for *C. limon* and *A. sativum* oils at 24 and 48 hours. Moreover, no synergistic effects were evidenced *in vitro*. The essential oil constituents, based on their GC retention times have been also identified. The major component is trans-cinnamaldehyde (79 %) for *C. aromaticum*; limonene for *C. limon* (71 %) and diallyl tri- and disulfide (79 %) for *A. sativum*. Even if concentration and protocol adaptations are required for successful *in vivo* treatments, it appears that these oils may be useful as chemotherapeutic agents against several poultry parasites.

KEY WORDS : essential oils, *Cinnamomum aromaticum*, *Citrus limon*, *Allium sativum*, *T. gallinarum*, *H. meleagridis*.

MOTS CLÉS : *huiles essentielles*, Cinnamomum aromaticum, Citrus limon, Allium sativum, T. gallinarum, H. meleagridis.

Résumé : Effets *in vitro* d'huiles essentielles de *Cinnamomum aromaticum*, *Citrus limon* et *Allium sativum* sur deux flagellés de volailles, *Tetratrichomonas gallinarum* et *Histomonas meleagridis*

L'utilisation des huiles essentielles en prophylaxie et en thérapeutique anti-flagellés chez les volailles peut être intéressante en élevage "bio" ou en élevage classique privé de nombreux produits allopathiques. Nous avons testé l'activité in vitro *de trois huiles essentielles obtenues à partir de feuilles de* Cinnamomum aromaticum, *du péricarpe de fruits de* Citrus limon *et de bulbes d'*Allium sativum, *contre deux parasites de volailles :* Tetratrichomonas gallinarum *et* Histomonas meleagridis. *Dans le cas de* T. gallinarum, *les concentrations minimales létales (CML) à 24 heures correspondent aux concentrations de 0,25 µl/ml pour l'huile essentielle de* C. aromaticum *et 0,125 µl/ml pour celles de* C. limon *et* A. sativum. *Pour* H. meleagridis, *les CML sont respectivement 0.5 µl/ml pour l'huile essentielle de* C. aromaticum *et 1 µl/ml pour celles de* C. limon *et* A. sativum *à 24 et 48 heures. Lors de ces essais, aucune synergie entre les huiles essentielles n'a pu être mise en évidence. Les composants chimiques de ces trois huiles essentielles ont été identifiés : les composants majeurs sont respectivement le trans-cinnamaldéhyde pour l'huile de* C. aromaticum, *le limonène pour l'huile de* C. limon *et le diallyl tri- et disulfide pour l'huile d'*A. sativum. *À partir des résultats obtenus, il est donc possible d'envisager l'utilisation de ces huiles comme agent thérapeutiques contre ces parasites* in vivo, *même si l'on doit rester prudent du fait que l'extrapolation de résultats* in vitro *à des doses et des protocoles* in vivo *est délicate et parfois décevante.*

INTRODUCTION

Several species of flagellates occur in the digestive tract of poultry. Some are known to be pathogenic, but others apparently are not. *Tetratrichomonas gallinarum* and *Histomonas meleagridis* are two flagellate parasites of the lower digestive tract which have been isolated from chicken, turkey, guineafowl and possibly gallinaceous birds (Kaufmann, 1966). The first is commonly found in the caecum of chickens and other gallinaceous birds (McDougald, 1997). This trichomonad or a closely related species has occasionally been isolated from liver and blood samples. Although lesions have been ascribed to this organism, no confirmation of pathogenicity has come from experimental infection (Kulda *et al.*, 1974). *H. meleagridis* is the cause of enterohepatitis, "Blackhead", mainly in turkeys but also in chickens and other gallinaceous birds. This disease is characterized by diarrhea and typhlo-hepatitis on infected birds with an early clinical sign in turkeys by the presence of a tan-yellow sulfur dropping (Kaufmann, 1966; McDougald, 1997).

Nitroimidazoles, and dimetridazole in particular, were used for many years in food or water for prevention

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Cinnamomum aromaticum oil		*Citrus limon* oil		*Allium sativum* oil	
Compounds	**Composition** (% of total weight)	**Compounds**	**Composition** (% of total weight)	**Compounds**	**Composition** (% of total weight)
Trans-cinnamaldehyde	79.40	Limonene	71.10	Diallyl trisulfide	49.8
Eugenol	1.40	Pinene	9.50	Diallyl disulfide	29.8
Linalol	1.20	Terpinene	8.10	Diallyl sulfide	1.3
Coumarine	0.40	P-cymene	1.40	Limonene	1.2
		Terpineol	low trace	Allyl-methyl disulfide	0.9

Table I. – Chemical composition of the main constituents of the essential oils obtained from fresh leaves of *Cinnamomum aromaticum*, fruits of *Citrus limon* and bulbs of *Allium sativum*.

and treatment of flagellates in poultry (McDougald, 1997). Dimetridazole was identified as potentially carcinogenic for the consumer. It was banned from use in veterinary medicinal products for food-producing animals by the European Council (EEC Council Regulation, 1995) and from feed additive. Moreover, chemical drugs are strictly prohibited in organic productions. Thus, it is imperative to find other drugs for industrial animal production, as well as for organic productions. So, we have developed an *in vitro* drug test to assess the activity of various compounds against *T. gallinarum* and *H. meleagridis* (Callait *et al.*, 2002; Reynaud *et al.*, 1997).

Great interest has been focused on the antimicrobial and antiparasitic properties of natural extracts, and especially volatile oils of natural origin. As an alternative to chemical drugs, they have already demonstrated some efficacy against protozoa such as *Plasmodium falciparum* (Milhau *et al.*, 1999) *Trypanosoma brucei* (Moideen *et al.*, 1999), *Trichomonas vaginalis* (Kaneda *et al.*, 1990; Kaneda *et al.*, 1991; Violon & Chaumont, 1993; Zemek *et al.*, 1987), *Cryptosporidium* sp. (Sreter *et al.*, 1999), *Giardia* sp. (Kaneda *et al.*, 1990; Kaneda *et al.*, 1991) and *Entamoeba histolytica* (Kaneda *et al.*, 1990; Kaneda *et al.*, 1991).

In this study, the anti-flagellate activity of essential oils obtained from fresh leaves of cinnamon *Cinnamomum aromaticum*, lemon pericarps *Citrus limon* and cloves of garlic *Allium sativum*, was investigated using *T. gallinarum* and *H. meleagridis in vitro* susceptibility test. We also tested the synergistic action of these extracts.

MATERIALS AND METHODS

PLANT MATERIAL

Oils were obtained by steam distillation of fresh leaves of *C. aromaticum* (synonym *C. cassia*), pericarps of *C. limon* and bulbs of *A. sativum*. The volatile oils of *C. aromaticum* and *C. limon* were analyzed using a Polyethylene Glycol gas chromatograph column (HP-INNOWax, Hewlett Packard). One ml of each oil was chromatographed in a 60.0 m column, 320 µm of diameter and 0.50 µm nominal film thickness, fitted with a flame ionisation detector. Oven temperature was 100° C to 220° C at 1.5° C/mn with a split ratio of 1:100. The total runtime was 95 mn. *A. sativum* oil was analyzed with a HP-INNOWax instrument (Hewlett Packard) fitted with an Easy 1 bonded phase fused silica capillary column (25 m, 0.32 mm i.d., 0.33 µm film thickness) (Avato, 1998). The identification of the essential oil constituents were based on their GC retention times in comparison with authentic reference compounds (Vernin & Metzger, 1991; Vernin *et al.*, 1986) The compound content of each volatile oil is summarized in Table I.

PARASITE STRAINS

The *T. gallinarum* strain was isolated in Czech Republic from a caecum of duck and then cultivated axenically in Tripticase-Yeast-Maltose media (TYM, pH 7.2) and 10 % heat-inactivated horse serum at 37° C (Kulda *et al.*, 1974).

The *H. meleagridis* HmBR-a strain used in the test was isolated from the dropings of chickens. Parasites and accompanying bacterial flora were cultivated on a non-axenic HmBR-a strain culture maintained in our laboratory since 1998 and cultivated anaerobically at 39° C on Stepkowski media (Stepkowski & Klimont, 1979)

TESTING PROCEDURE

Anti-*T. gallinarum* activity

Aliquots of 2 ml of *T. gallinarum* culture (5.10^4 parasites/ml) were incubated anaerobically for 24 h at 37° C. Twelve two-fold dilutions of a stock solution of each essential oil were prepared in 96-well U bottom plates (Prolabo, Lyon, France) in Tween 80 (Sigma, L'Isle d'Abeau, France). The initial dilution was 4 %; then 100 µl of each was added to culture tube, so that oil dilutions ranged from 2 µl/ml to 0.001 µl/ml. After 24 h of incubation, the lowest dilution of oil in which no

motile and live organisms were observed was defined as the minimal lethal concentration (MLC). Moreover, the number of live parasites in each culture tube was measured on a Malassez cell. All experiment were run three times in duplicate with two standard controls: the negative control contained a dilution of 0.2 % of Tween 80 (2 µl/ml) (Sigma) and the positive control contained dimetridazole at 400 µg/ml. The synergistic activity of these oils was assessed using the same method with an initial dilution of 2 µl/ml of a 50:50 mix of two different oils.

Anti-*H. meleagridis* activity

The *in vitro* activity of these oils was assessed using a similar technique. Aliquots of 1 ml of *H. meleagridis* culture (10^6 parasites/ml) were incubated anaerobicaly for 24 h at 39° C. Four two-fold dilutions of oils were prepared as described above and added to parasite culture in order to obtain final dilutions ranging from 2 µl/ml to 0.25 µl/ml. After incubation for 24 and 48 h, the number of live parasites in each culture was estimated by vital coloration with Trypan blue 0.4 % (Gibco BRL Life Technology, Cergy Pontoise, France). The lowest oil dilution in which no live organisms were observed was defined as the minimal lethal concentration (MLC). Mixtures of two oils (*C. aromaticum* and *C. limon*) using 50:50 proportions and of the three oils (*C. aromaticum*, *C. limom* and *A. sativum*) at 50:30:20 proportions were also tested.

RESULTS

Anti-*T. gallinarum* activity

Table II shows the activity of the three essential oils on *T. gallinarum*. At 24 hours, the MLC correspond to 0.25 µl/ml with *C. aromaticum* oil, and 0.125 µl/ml with *C. limon* and *A. sativum* oils. The parasites grew normally in the negative control but died in the positive control. The synergistic activity of these preparations is summarized in Table II. In the three mixtures, the MLCs were obtained at 0.25 µl/ml with half the quantity of each oil in the tubes, indicating no synergistic effect.

Tetratrichomonas gallinarum	**MLC**
C. aromaticum	0.25 µl/ml
C. limon	0.125 µl/ml
A. sativum	0.125 µl/ml
C. aromaticum : *C. limon* (50:50)	0.25 µl/ml
C. aromaticum : *A. sativum* (50:50)	0.25 µl/ml
C. limon : *A. sativum* (50:50)	0.25 µl/ml

Table II. – Minimal Lethal Concentration (MLC) of *C. aromaticum*, *C. limon* and *A. sativum* oils or mix on the viability of *T. gallinarum*. Serial dilutions used for the tests were: 2 µl/ml, 1 µl/ml, 0.5 µl/ml, 0.25 µl/ml, 0.125 µl/ml, 0.0625 µl/ml, 0.03 µl/ml, 0.016 µl/ml, 0.008 µl/ml, 0.004 µl/ml, 0.002 µl/ml and 0.001 µl/ml.

Histomonas meleagridis	**MLC**
C. aromaticum	0.5 µl/ml
C. limon	1 µl/ml
A. sativum	1 µl/ml
C. aromaticum : *C. limon* (50:50)	0.8 µl/ml
C. aromaticum : *C. limon* : *A. sativum* (50:30:20)	0.8 µl/ml

Table III. – Minimal Lethal Concentration (MLC) of *C. aromaticum*, *C. limon* and *A. sativum* oils or mix on the viability of *H. meleagridis*. Serial dilutions used for the tests were: 2 µl/ml, 1 µl/ml, 0.5 µl/ml and 0.25 µl/ml for essentiel oils and 1.6 µl/ml, 0.8 µl/ml, 0.4 µl/ml and 0.2 µl/ml for the mix.

Anti-*H. meleagridis* activity

For *H. meleagridis*, at 24 and 48 hours, MLCs were 0.5 µl/ml for *C. aromaticum* oil and 1 µl/ml for *C. limon* and *A. sativum* oils. Moreover, the two oil mixtures did not have any synergistic effect (Table III).

DISCUSSION

The three essential oils obtained from fresh leaves of *C. aromaticum*, *C. limon* pericarps and *A. sativum* bulbs have an *in vitro* anti-flagellate activity against *T. gallinarum* and *H. meleagridis*. Their minimal lethal concentrations (MLC) are close for the same parasite, but vary considerably for the two parasites: for *H. meleagridis* it was two times higher with *C. aromaticum* and eight times for *C. limon* and *A. sativum* than *T. gallinarum*. It may explain why, even if many drugs and components have already been shown to be effective against major flagellates like *Tetratrichomonas* sp. or *Giardia* sp., they have been considered less active *in vitro* against *H. meleagridis* (Callait *et al.*, 2002).

Among the other tested oils, garlic and its active components have long been known to exert antiviral, antibacterial and antifungal activity (Reuter & Sendl, 1995). They have proven effective against various protozoan parasites including several species of *Trypanosoma*, *Leishmania* and *Plasmodium*, as well as *Entamoeba histolytica* and *Giardia lamblia* (Aukri *et al.*, 1997; Lun *et al.*, 1994; McClure *et al.*, 1996; Nok *et al.*, 1996). Likewise, Violon & Chaumont (1993) showed that *Cinnamon zeylanicum* essential oil has an *in vitro* effect against *T. vaginalis* with a Minimal Trichomonacid Concentration of 0,05 µl/ml.

The oils were analyzed by GC/MS and their major components were identified. Apart from limonene found at different ratios in *C. limon* and *A. sativum* oils, their composition varied from plant to plant. The major com-

ponents are trans-cinnamaldehyde for *C. aromaticum*, limonene for *C. limon* and diallyl tri- and disulfide for *A. sativum*. Some of these components have already been shown to have antiparasitic effects. Zemek *et al.* (1987) reported that of 24 aromatic compounds of plant origin, eugenol was one of the most potent effector compounds against *T. vaginalis*. In the same report, the activity of trans-cinnamaldehyde was also good. In 1993, Violon explored the activity of various compounds against the same parasite species and found that eugenol, p-cimene and linalol had anti-trichomonas effects (Violon & Chaumont., 1993).

These results prompt us for further experiment *in vivo*. However, even if these *in vitro* tests are useful and indispensable to establish a drug's spectrum, dosages for curative *in vivo* products must be determined with another method. Generally, higher concentrations than those predicted by *in vitro* sensitivity test, and which may be sometimes inadequate or toxic, are required for successful treatment (Niepp, 1964; Revill, 1988). This study suggests that the concentrations of essential oils needed to treat *T. gallinarum* or *H. gallinarum* will be very different.

This *in vitro* study showed that these three essential oils have a definite anti- *T. gallinarum* and *H. meleagridis* effect. This property makes these products or their components tempting candidates for further *in vivo* experimentation. Essential oils could be used non only in organic production to prevent or treat poultry parasites but also in production where chemical drugs are banned. Moreover these products are cheap to produce, and may be used alone or as adjuvant treatments. The analysis of their basic chemical components should be pursued and tested separately on parasites.

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