In vitro and *in vivo* activity of *Artemisia sieberi* against *Trichomonas gallinae*

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Summary

In Iranian folk medicine *Artemisia sieberi* has been used for treatment of parasite infections in human and animals. The present study was designed to evaluate the *in vitro* and *in vivo* effects of *A. sieberi* essential oil (EO) against *Trichomonas gallinae*. *Trichomonas gallinae* were recovered by wet mount method from infected native pigeons. The *in vitro* assays were accomplished in multi-well plates containing metronidazole (MTZ) as a standard antitrichomonal and EO in final concentrations of 2.5, 5, 10, 20, 50, and 100 µg/ml of culture medium containing 10^4 parasites. The *in vivo* assay was performed on 40 experimentally infected pigeons receiving 25 and 50 mg/kg of MTZ and EO for 7 successive days. Gas chromatographic (GC) analysis was performed to reveal chemical constituents of the EO. At 20 µg/ml, MTZ resulted in no viable trophozoite in culture medium after 24 h incubation period. While the 24 h MIC of EO was 10 µg/ml. Treatment with EO at dose of 50 mg/kg after 4 days led to full recovery of infected pigeons but for MTZ at the same dose 5 days were spent. Major constituents of EO were α-thujone (31.5%) and β-thujone (11.92%). Data of the present study introduced *A. sieberi* as a natural potent antitrichomonal agent effective against *T. gallinae*.

Key words: *Artemisia sieberi*, Essential oil, Metronidazole, *Trichomonas gallinae*

Introduction

Internal parasites of human and animals have been treated with a wide variety of herbal products in traditional medicine. One of these plants used to treat helminthosis in Iranian folk medicine is *Artemisia sieberi Besser* (*Artemisia herba alba* Asso Var. *laxifolia Boiss*) (Mahboubi and Farzin, 2009). *Artemisia sieberi* (Asteraceae) is a classic dry land plant mainly dominated in south west and central Asia (Podlech, 1986). *Artemisia sieberi* is locally named "dermaneh" and is widely distributed in the semi-desert and desert areas of Iran (Mahboubi and Farzin, 2009).

Trichomonas gallinae, the causative agent of avian trichomoniasis is a flagellate belonging to the order of Trichomonadida. The parasite is located in the upper digestive and occasionally in the respiratory tracts of a large variety of birds, mainly in the order Columbiformes and Falconiformes (Boal *et al*., 1998; Rouffaer *et al*., 2014). Avian trichomoniasis is generally manifested as a caseous lesion within the anterior digestive tract of affected birds. The lesions range from mild, often subclinical infections, to severe inflammation which can be acute and fatal and may lead to death by starvation due to the obstruction of the lumen of the esophagus (Gerhold *et al*., 2008). Outbreaks of trichomoniasis have resulted in wide-ranging mortality, mainly in breeding populations (Robinson *et al*., 2010; Lawson *et al*., 2011). *Trichomonas gallinae* has significant health and economic impacts on the poultry industry, especially pigeons and game birds rearing and breeding (Stockdale *et al*., 2015) and is considered as a major factor for regulation and even decline of avian populations (Robinson *et al*., 2010). The drugs of choice for treatment of trichomoniasis are nitroimidazoles. Sub-therapeutic doses and preventive use of these drugs against trichomoniasis, have resulted in emergence of resistant strains of *T. gallinae* (Lumeij and Zwijnenberg, 1990). *Artemisia sieberi* has also been screened for antimicrobial, antifungal, anticoccidial and insecticidal activities (Khosravi *et al*., 2003; Arab *et al*., 2006; Negahban *et al*., 2007; Mahboubi and Farzin, 2009).

Taken together, the present study was designed to evaluate the *in vitro* and *in vivo* effects of *A. sieberi* essential oil (EO) against *T. gallinae*.

Materials and Methods

Essential oil

At full-flowering stage aerial parts of *A. sieberi* were collected from Arak (Markazi Province, Iran) in September 2014. The voucher specimens of the plant were confirmed by Arak Agricultural Sciences University (Arak, Iran). The plant was dried in shadow at room temperature, then by using a Clevenger type apparatus hydrodistilled to extract its EO. Extraction was done according to the method described by (Negahban *et al*., 2007). Gas chromatographic (GC) analysis was performed using a Shimadzu GC-9A with helium as a carrier gas on a DB-5 column (30 m × 0.25 mm i.d, film

thickness 0.25 mm). GC-MS analysis was carried out on a Varian 3400 GC-MS system equipped with a DB-5 column (30 m × 0.25 mm i.d, film thickness 0.25 mm), oven temperature was 40-250°C at a rate of 4°C; transfer line temperature, 260°C; carrier gas, helium with a linear velocity of 31.5 cm/s; split ratio, 1/60; ionization energy, 70 eV; scan time, 1 s: mass range, 40-300 amu.

Parasite

Trichomonas gallinae were recovered by wet mount method from infected native pigeons as follows: forty native pigeons of about 6 to 8 weeks of age were purchased from local breeders in Babol city (Mazandaran province, Iran). Samples were taken from membranous lesions in oropharyngeal area of suspicious birds using microbiology swabs. Wet smears were prepared and examined under a light microscope at ×100 and ×400 magnifications to confirm the existence of *T. gallinae*. Parasite culture was prepared by immersing oral swabs in tryptone/yeast extract/maltose (TYM) medium supplemented with 10% fetal calf serum (Sigma, Germany) and incubated at 37°C (Sansano *et al*., 2009). Cultures were observed over five consecutive days to check the growth of *T. gallinae*. Sub-cultures were done on isolates with 48 h intervals during the logarithmic phase of growth when the parasites showed more than 95% mobility and normal morphology (Seddiek *et al*., 2014).

In vitro assay

The method used for the *in vitro* assay was that described by Munoz *et al*. (1998) with some slight modifications. To examine the susceptibilities of *T. gallinae* to *A. sieberi* EO and MTZ (Alborzdaru, Tehran, Iran), sterile multi well plates were used to incubate the trophozoites with the corresponding EO and drug dilutions. A volume of 100 µL of culture medium containing 1×10^4 parasites pipetted into each well, as well as prediluted MTZ and *A. sieberi* EO to give final concentrations of 2.5, 5, 10, 20, 50, and 100 µg/ml. Tween 20 (0.01% of final concentration) was used as solubilization vehicle for *in vitro* analysis. Control wells received only Tween 20. Subsequently, to generate anaerobic conditions a layer of 50 µL of vaseline was added to wells. All assays were run three times. The wells were examined with an inverted microscope every 24 h for 3 consecutive days. The MIC (the lowest concentration of the drug in the well at which no motile parasite was observed) was also recorded.

The growth inhibition percentage (GI %) was determined as the following equation:

$$\text{Growth inhibition}\,\% = (A - B)/A \times 100$$

where,

A: The mean number of trophozoites in the control group

B: The mean number of trophozoites in the test group (Seddiek *et al*., 2014)

In vivo assay

Protocol of the *in vivo* study (No. 0492/16) was in accordance with laboratory animal welfare guide of Pasteur Institute of Iran and has been accepted by the committee. Forty native pigeons up to 6 weeks of age were pre-examined and confirmed to be free of *T. gallinae* were then experimentally infected by inoculation of 4×10^4 trophozoites in 1 ml of 48 h culture medium. Seven days post-infection, after the birds were examined by wet mount method and by microscopic examination were confirmed to be infected with *T. gallinae*, they were randomly allocated into 5 groups as follows: the first group (CON) infected but not medicated, EO 25 and EO 50 were the groups infected with *T. gallinae* and medicated with the doses of 25 and 50 mg/kg of *A. sieberi* EO, respectively. MTZ 25 and MTZ 50 were infected with *T. gallinae* and medicated with 25 and 50 mg/kg of metronidazole (MTZ). All of the treatments were administered orally (*Per Os*) once a day for 7 successive days. Birds of different groups were located in separate wire cages and fed semisolid mixed grains diet in order to prevent starvation due to difficulty in swallowing because of trichomoniasis infection. The numbers of motile trophozoites recovered from the crop of infected birds were calculated every day for seven consecutive days. Any clinical adverse effects or mortality were recorded during the treatment period.

Statistical analysis

Analysis of variance (ANOVA) was used, followed by Newman Keul's test as the post hoc comparison to determine the source of significant differences (SPSS v.11). The p-value less than 0.05 was considered as statistically significant difference between groups.

Results

Chemical composition of *A. sieberi* EO

Major constituents of *A. sieberi* EO were α-thujone (31.5%), β-thujone (11.92%), camphor (12.3%), and 1,8-cineole (10.09%) as showed in Table 1.

In vitro results

The results for the *in vitro* anti-trichomonalmactivitiy of *A. sieberi* EO and MTZ are shown in Table 2. The results revealed high efficacy of *A. sieberi* EO against *T. gallinae*. At dose of 20 µg/ml, MTZ after 24 h incubation period resulted in no viable trophozoite in culture medium. While the 24 h MIC of *A. sieberi* EO was 10 µg/ml. The 48 h and 72 h MIC of MTZ were 20 and 10 µg/ml but these values for *A. sieberi* were 10 and 5 µg/ml, respectively. Mortality of trophozoites were confirmed by the lack of resumption of growth in the subsequent 48 h cultures.

Results of GI % in MTZ and *A. sieberi* treated groups in 24 h intervals are shown in Figs. 1A and B. It showed that there was significant difference between GI % in MTZ and *A. sieberi* EO treated groups in comparison to control. In dose-GI % graphs, doses of 2.5, 5, 10, and 20 µg/ml MTZ and *A. sieberi* EO resulted in different responses of GI% (Fig. 1).

In vivo results

The *in vivo* assay demonstrated the effectiveness of two doses of MTZ and *A. sieberi* EO against *T. gallinae* (Table 3). Seven days post inoculation of *T. gallinae* (day 0), before initiation of treatments, number of trophozoites recovered from crop of birds was measured and no significant difference was seen among different groups. In the second day of experiment, treatment with all doses of MTZ and *A. sieberi* EO even after administration of only one dose eventuated in significant

Table 1: Chemical composition of *A. sieberi* EO

Compounds	Retention index	% Constituents of *A. sieberi* EO
α-Thujene	922	0.6
α-Pinene	932	1.22
Camphene	945	8.72
Sabinene	969	0.3
β-Pinene	975	0.98
Myrcene	986	0.3
α-Terpinene	1012	0.26
ρ-Cymene	1019	1.03
1,8-Cineole	1028	10.09
γ-Terpinene	1052	0.62
Linalool	1070	0.64
Artemisia alcohol	1080	0.23
α-Thujone	1102	31.5
β-Thujone	1112	11.92
Myrcenol	1123	0.37
Camphor	1140	12.3
cis-Verbenol	1143	0.35
Pinocarvone	1149	1.22
trans-Verbenol	1160	0.89
Borneol	1166	1.2
p-Cymen-8-ol	1176	1.14
Myrtenol	1192	0.3
cis-Piperitol	1196	0.26
trans-Piperitol	1206	0.54
Piperitone	1224	1.8
Thymol	1248	0.3

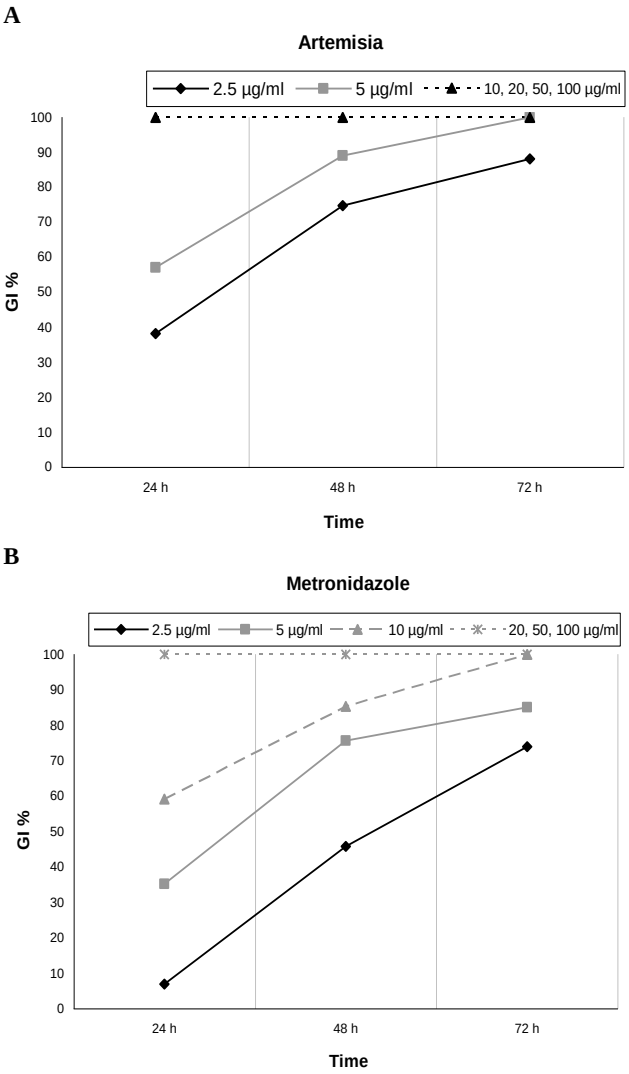

Fig. 1: Growth inhibition percentage (GI %) in *Artemisia sieberi* (A) and metronidazole (B) treated groups in 24 h, 48 h, and 72 h intervals

Table 2: *In vitro* anti-trichomonal activity of metronidazole (MTZ) and *Artemisia sieberi* EO against *T. gallinae*. Data are presented as mean±SD

Time (hour)	Control	Number of trophozoites ×10^4											
		MTZ (µg/ml)						*A. siberi* EO (µg/ml)					
		2.5	5	10	20	50	100	2.5	5	10	20	50	100
24	7.93±0.08^{d}	7.38±0.38	5.14±2.06^{ab}	3.24±0.2^{ac}	0^{a}	0^{a}	0^{a}	4.9±0.72^{ab}	3.4±0.24^{ac}	0^{a}	0^{a}	0^{a}	0^{a}
48	9.41±0.56^{d}	5.1±0.56^{ab}	2.29±0.82^{ac}	1.38±0.1^{ae}	0^{a}	0^{a}	0^{a}	2.38±0.7^{ac}	1.03±0.44^{ad}	0^{a}	0^{a}	0^{a}	0^{a}
72	9.2±0.46^{d}	2.4±0.2^{ab}	1.37±0.72^{ac}	0^{a}	0^{a}	0^{a}	0^{a}	1.09±0.34^{ac}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

$^{a, b, c, d}$ Means with different letters in the same row indicate statistically significant difference (P<0.05)

Table 3: *In vivo* anti-trichomonal activity of metronidazole (MTZ) and *Artemisia sieberi* EO against *T. gallinae*. Data are presented as mean±SD

Days	Number of trophozoites ×10^4				
	MTZ (25 mg/kg)	MTZ (50 mg/kg)	*A. sieberi* EO (25 mg/kg)	*A. sieberi* EO (50 mg/kg)	CON
0	127.28 ± 2.21^{a}	130.56 ± 4.67^{a}	130.8 ± 3.43^{a}	128.56 ± 3.17^{a}	135.44 ± 4.64^{a}
1	84.08 ± 1.50^{a}	41.42 ± 10.28^{b}	36.73 ± 5.04^{b}	20.12 ± 3.35^{b}	140.64 ± 5.55^{c}
2	26.8 ± 1.32^{a}	11.11 ± 0.82^{b}	13.35 ± 1.06^{b}	3.83 ± 1.37^{c}	140 ± 2.33^{d}
3	13.16 ± 0.68^{a}	3.2 ± 0.41^{b}	2.91 ± 0.45^{b}	0^{c}	140.16 ± 4.36^{d}
4	6.41 ± 0.26^{a}	0.36 ± 0.2^{b}	0^{b}	0^{b}	136.16 ± 2.75^{c}
5	3.2 ± 1.56^{a}	0^{a}	0^{a}	0^{a}	126.56 ± 0.68^{b}
6	0^{a}	0^{a}	0^{a}	0^{a}	128.56 ± 0.86^{b}
7	0^{a}	0^{a}	0^{a}	0^{a}	115.68 ± 2.32^{b}

$^{a, b, c, d}$ Means with different letters within the same row indicate statistically significant difference (P<0.05)

reduction of trophozoites in comparison to control group (P<0.05). *Artemisia sieberi* EO at the dose of 50 mg/kg in the 3rd and 4th day of the treatment resulted in significant reduction in number of *T. gallinae* in comparison to all other groups (P<0.05). In the 4th day, no motile trophozoite was recovered from *A. sieberi* EO 50 mg/kg treated birds. One day later (the 5th day), *A. sieberi* EO 25 mg/kg led to full recovery of infected pigeons. For the dose of 25 mg/kg of MTZ treated pigeons, 7 days' time was spent to reach full recovery. No mortality was recorded for treatment groups and no clinical side effects were observed in treated birds.

Discussion

Preventive treatment with nitroimidazoles could result in isolates of *T. gallinae* which can serve as serious threat endangering birds' lives. Nitroimidazole resistant isolates of *T. gallinae* are reported from different parts of the world including Belgium, Spain and the United States (Munoz *et al*., 1998; Rouffaer *et al*., 2014; Gerhold *et al*., 2008). In spite of the fact that nitro-imidazole resistant strains of *T. gallinae* are becoming prevalent, very few researches focused on alternative antitrichomonal resources effective against *T. gallinae*. Antitrichomonal property of the extract of *Clausena lansium* stem bark was studied by Adebajo *et al*. (2009). They concluded that this extract could not fully achieve the efficacy of MTZ against *T. gallinae* (Adebajo *et al*., 2009). Adebajo *et al*. (2006) demonstrated the anti-trichomonal activity of *Murrayakoenigii* (L.) Spreng (Rutaceae), an ancient Indian medicinal herb, and its isolated carbazole alkaloids against *T. gallinae*. Data obtained in their study showed higher efficacy of MTZ in comparison to isolated carbazole alkaloids. Seddik *et al*. (2014) reported that garlic was as effective as MTZ in inhibiting the growth of *T. gallinae* trophozoites in both *in vitro* and *in vivo* assays. They also declared the side effects of cytotoxicity, carcinogenic effects and neurological dysfunction for MTZ and recommended garlic as a safe alternative for prophylactic and therapeutic uses in case of trichomoniasis (Seddiek *et al*., 2014). They found that garlic at the dose of 200 mg/kg after 4 days was effective in treatment of infected pigeons while in the present study *A. sieberi* EO at much lower dose of 25 mg/kg after 4 days resulted in full recovery of infected birds and this period for the dose of 50 mg/kg was as short as just 3 days. Comparison of the results of these two studies reveal higher efficacy of *A. sieberi* against *T. gallinae*.

The other evidence for antiprotozoal activity of *A. sieberi* was in a study done by Arab *et al*. (2006) who reported its effectiveness against *Eimeriatenella* and *E. acervulina*. They reported protective effect of *A. sieberi* in broiler coccidiosis (Arab *et al*., 2006). Further, it was reported that Iranian flora *A. sieberi* was effective against *Plasmodium berghei*. *Artemisia sieberi* showed anti-malarial effects and also was able to reduce parasitemia in infected mice (Nahrevanian *et al*., 2012).

The GC analysis of the *A. sieberi* used in the present study revealed that α-thujone (31.5%) was the major constituent of the EO. It is reported that this monoterpenoid could be found in many plant species, including *Artemesia*, sage, and the Thuja tree (Hold *et al*., 2000). *Artemisia* extracts were used for treatment of gastrointestinal helminthes with records back to ancient Egyptian times. One of the toxic monoterpenoids tested against insects was α-thujone (Lee *et al*., 1997). It was showed that α-thujone acts as a blocker of the $GABA_A$ (γ-Aminobutyric $acid_A$) receptor (Hold *et al*., 2000). On the other hand, paralyzing effect of some antiparasitic agents on helminthes is thought to be associated with $GABA_A$ receptor (Feng *et al*., 2002). So, anthelminthic effect of *A. sieberi* can be attributed to its α-thujone component. Probably α-thujone is one of the most active components of *A. sieberi* with parasiticide action. Further studies on active antitrichomonal component of *A. sieberi* EO and also its activity against *T. gallinae* are recommended.

Data obtained in this study introduced *A. sieberi* as a natural potent antitrichomonal agent effective against *T. gallinae*. Major chemical constituents of *A. sieberi* can be considered as leading compounds in research and development of novel antitrichomonal agents.

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Conflict of interest

The authors declare no conflict of interest.

References

Adebajo, AC; Ayoola, OF; Iwalewa, EO; Akindahunsi, AA; Omisore, NO; Adewunmi, CO and Adenowo, TK (2006). Anti-trichomonal, biochemical and toxicological activities of methanolic extract and some carbazole alkaloids isolated from the leaves of *Murraya koenigii* growing in Nigeria. Phytomedicine. 13: 246-254.

Adebajo, AC; Iwalewa, EO; Obuotor, EM; Ibikunle, GF; Omisore, NO; Adewunmi, CO and Verspohl, EJ (2009). Pharmacological properties of the extract and some isolated compounds of *Clausena lansium* stem bark: anti-trichomonal, antidiabetic, anti-inflammatory, hepato-protective and antioxidant effects. J. Ethnopharmacol., 122: 10-19.

Arab, HA; Rahbari, S; Rassouli, A; Moslemi, MH and Khosravirad, F (2006). Determination of artemisinin in *Artemisia sieberi* and anticoccidial effects of the plant extract in broiler chickens. Trop. Anim. Health Prod., 38: 497-503.

Boal, CW; Mannan, RW and Hudelson, KS (1998). Trichomoniasis in Cooper's hawks from Arizona. J. Wildlife Dis., 34: 590-593.

Feng, XP; Hayashi, J; Beech, RN and Prichard, RK (2002). Study of the nematode putative GABA type-A receptor subunits: evidence for modulation by ivermectin. J. Neurochem., 83: 870-878.

Gerhold, RW; Yabsley, MJ; Smith, AJ; Ostergaard, E; Mannan, W; Cann, JD and Fischer, JR (2008). Molecular characterization of the *Trichomonas gallinae* morphologic complex in the United States. J. Parasitol., 94: 1335-1341.

Hold, KM; Sirisoma, NS; Ikeda, T; Narahashi, T and Casida, JE (2000). Alpha-thujone (the active component of absinthe): gamma-aminobutyric acid type A receptor modulation and metabolic detoxification. Proc. Natl. Acad. Sci. USA., 97: 3826-3831.

Khosravi, A; Shirani, D and Mahmoudi, M (2003). Evaluation of the use of Artemisia sieberi essence on the treatment of cats and dogs with dermatophytosis. J. Vet. Res., 58: 293-295.

Lawson, B; Cunningham, AA; Chantrey, J; Hughes, LA; John, SK; Bunbury, N and Tyler, KM (2011). A clonal strain of *Trichomonas gallinae* is the aetiologic agent of an emerging avian epidemic disease. Infect. Genet. Evol., 11: 1638-1645.

Lee, S; Tsao, R; Peterson, C and Coats, JR (1997). Insecticidal activity of monoterpenoids to western corn rootworm (Coleoptera: Chrysomelidae), twospotted spider mite (Acari: Tetranychidae), and house fly (Diptera: Muscidae). J. Econ. Entomol., 90: 883-892.

Lumeij, JT and Zwijnenberg, RJ (1990). Failure of nitro-imidazole drugs to control trichomoniasis in the racing pigeon (*Columba livia domestica*). Avian. Pathol., 19: 165-166.

Mahboubi, M and Farzin, N (2009). Antimicrobial activity of *Artemisia sieberi* essential oil from central Iran. Iranian J. Microbiol., 1: 43-48.

Munoz, E; Castella, J and Gutierrez, JF (1998). *In vivo* and *in vitro* sensitivity of *Trichomonas gallinae* to some nitroimidazole drugs. Vet. Parasitol., 78: 239-246.

Nahrevanian, H; Sheykhkanlooye, B; Kazemi, M; Hajhosseini, R; Soleymani, M and Nahrevanian, S (2012). Antimalarial effects of Iranian flora *Artemisia sieberi* on *Plasmodium berghei in vivo* in mice and phytochemistry analysis of its herbal extracts. Malaria Res. Treat., doi: 10.1155/2012/727032.

Negahban, M; Moharramipour, S and Sefidkon, F (2007). Fumigant toxicity of essential oil from *Artemisia sieberi* Besser against three stored-product insects. J. Stored Prod. Res., 43: 123-128.

Robinson, RA; Lawson, B; Toms, MP; Peck, KM; Kirkwood, JK; Chantrey, J and Cunningham, AA (2010). Emerging infectious disease leads to rapid population declines of common British birds. PLoS One. 5: e12215. doi: 10.1371/journal.pone.0012215.

Rouffaer, LO; Adriaensen, C; De Boeck, C; Claerebout, E and Martel, A (2014). Racing pigeons: a reservoir for nitro-imidazole-resistant *Trichomonas gallinae*. J. Parasitol., 100: 360-363.

Sansano-Maestre, J; Garijo-Toledo, MM and Gómez-Muñoz, MT (2009). Prevalence and genotyping of *Trichomonas gallinae* in pigeons and birds of prey. Avian Pathol., 38: 201-207.

Seddiek, A; El-Shorbagy, MM; Khater, HF and Ali, AM (2014). The antitrichomonal efficacy of garlic and metronidazole against *Trichomonas gallinae* infecting domestic pigeons. Parasitol. Res., 113: 1319-1329.

Stockdale, JE; Dunn, JC; Goodman, SJ; Morris, AJ; Sheehan, DK; Grice, PV and Hamer, KC (2015). The protozoan parasite *Trichomonas gallinae* causes adult and nestling mortality in a declining population of European Turtle Doves, *Streptopelia turtur*. Parasitology. 142: 490-498.