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TESTING OF PLANT EXTRACTS AS ANTIPARASITIC AGAINST GASTROINTESTINAL HELMINTHS WITH TRADITIONAL AND NEW TECHNOLOGIES

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Abstract: Plant extracts represent interesting alternatives to synthetic antiparasitic drugs against gastrointestinal helminths ("worms"). These have been used intensively over the last decades and antiparasitic resistance is increasing worldwide. In the present study three plant extracts (*Malva sylvestris, Chamomilla recutita* and *Althaea officinalis*) were assessed for their antiparasitic properties against gastrointestinal worms. A traditional microscopy method (larval development assay, LDA) and a molecular analysis (Droplet digital PCR, ddPCR) were performed using a laboratory strain of *Haemonchus contortus* (target organism). All three extracts had a certain antiparasitic effect up to 20 mg/ml concentration, but a stronger effect of *M. sylvestris* and *A. officinalis* than *C. recutita* was observed, even at lower concentrations. Correlation between both methods (LDA and ddPCR) was low (R^2=0.39), but it will be investigated with further studies. The availability of molecular methods for the screening of alternative antiparasitic substances is relevant to the organic husbandry since it would provide a robust, standardized and objective tool to get access to effective alternatives to traditional antiparasitic drugs against gastrointestinal worms.

Introduction: Gastrointestinal parasites are some of the most important hurdles in health management of extensive and intensive organic livestock production, causing significant economic losses. In the last few years, due to the increasing incidence of antiparasitic resistance and the development of organic livestock farming, plant products have become important alternatives for the prevention and treatment of the gastrointestinal worms' infestation (Pisseri et al., 2013). Chemical composition and biological activities of these plants are the major criteria used to find potential interesting candidates worth to be further evaluated as antiparasitic against gastrointestinal worms. Traditionally, antiparasitic properties against gastrointestinal worms are evaluated with techniques like egg/larval count after parasite exposure to the herbal product, but all these methods rely on subjective interpretation by the operator. Therefore, a molecular technique is potentially a more accurate test that would reduce the subjectivity of traditional methods. Aims of the present

study were: to evaluate some herbal extracts for their antiparasitic properties against gastrointestinal worms and to compare a traditional microscopic detection method (larval development assay, LDA) with a new innovative technique (molecular analysis with Droplet digital PCR, ddPCR) for plant products screening.

Material and methods: Three plants extracts from *Malva sylvestris, Chamomilla recutita* and *Althaea officinalis* were tested. First, a LDA was performed by exposing *Haemonchus contortus* larvae to 12 serial concentrations (40, 20, 10, 5, 2.5, 1.25, 0.62, 0.31, 0.15, 0.078, 0.039 and 0.019 mg/ml) of the aqueous extracts of the abovementioned plants in a 96-well microtiter plate. One row (reference) was used to confirm the viability of the substrate for the larval development (Váradyová et al, 2018). The same plate was then sent to the Swedish University of Agricultural Sciences (SLU) to evaluate the correspondence between the quantity of larvae observed in LDA by microscopy and the quantity of DNA copies of Internal transcribed spacer-2 (ITS-2) amplicon from *H. contortus*. This assessment was done for each one of the 12 concentrations of plant extract using the ddPCR (BioRad) as detection method. Briefly, DNA was extracted from frozen larval cultures and ddPCR was run using extracted DNA samples as template and UnivHC primers (Elmahalawy et al., 2018). Samples were placed in 96-well plates according to manufacturer's instructions. Droplets were generated and dispensed into a new 96-well plate using an automated droplet generator (QX200, BioRad). The new plate was sealed and transferred into a thermal cycler (MyCyclerTM Thermal Cycler) where PCR reaction was performed. After the amplification step, the plate containing the droplets was loaded into the droplet reader (QX200, BioRad) and analyzed using QuantaSoft software (Baltrušis et al., 2019).

Results: Results of the effects of each plant extract concentration tested recorded through LDA are reported in Table 1. No differences among the plants extracts were observed at the lower concentrations (i.e. dilutions from 0.019 mg/ml to 10 mg/ml). However, the average number of eggs and larvae appeared lower than reference in all the three tested extracts at 10 mg/ml. From the concentration of 1.25 mg/ml of plant extract, it has been possible to observe an interesting trend where greater numbers of *H. contortus* (eggs and larvae) in *C. recutita* than in *M. sylvestris* and *A. officinalis* were recorded, witnessing a lower antiparasitic effect of the former plant extract. At 20 mg/ml of plant extract, the difference previously described became statistically significant. The LDA and ddPCR techniques were compared through the regression method (Figure 1). The best fitting curve was the logarithmic function whose equation ($y = 4.7201 \ln(x) + 3.7068$) showed the higher coefficient of determination ($R^2=0.385$).

Table 1 – Number of parasitic forms (eggs and larvae) resistant to the treatment using the microscopy method of larval development assay (LDA)

	Plant extract concentrations (mg/ml)											
Treatment	**40**	**20**	**10**	**5**	**2.5**	**1.25**	**0.62**	**0.31**	**0.15**	**0.078**	**0.039**	**0.019**
Malva sylvestris	0.0	1.5a	4.0	31.0	31.5	25.5	34.5	42.5	40.0	37.0	41.3	44.5
Chamomilla recutita	0.0	22.0b	16.0	46.5	43.0	46.5	36.0	44.5	41.0	37.0	43.5	49.5
Althaea officinalis	0.0	4.0a	12.5	34.5	36.5	41.5	29.0	37.5	46.5	50.0	38.5	52.0
Significance ($P < 0.05$)	-	0.014	0.340	0.214	0.428	0.170	0.906	0.725	0.864	0.170	0.656	0.391
Reference	34	43	44	40	32	37	47	42	38	45	46	39

[a,b] $P < 0.05$

Discussion: The literature is rich in studies on the use of plant and plant-derived substances as antiparasitic against gastrointestinal worms (Váradyová et al., 2018). Their use is often documented in traditional medicine. Despite the increasing amount of published studies, standardised procedures to test their activity and effectiveness are lacking, as well as an agreement in the scientific community that could lead to a recognised status of antiparasitic products for at

least some of the plants studied. *Malva sylvestris* has been indicated to possess considerable bioactivities as antiseptic and antibiotic agent. However, no antiparasitic effect has been documented. In the present study, we observed a good antiparasitic effect by the reduction of eggs and larvae at the dilution of 20 mg/ml of *M. sylvestris* plant extract. This effect was similar to the one observed for *A. officinalis* and overcame the one of *C. recutita*.

Conclusions: The two methods tested to evaluate the antiparasitic activity of the extracts appeared not to be strongly related, and more studies are needed to improve the ways to detect and assess the antiparasitic properties of new substances. Practitioners within the organic sector can see this as a first trial to overcome one of the major problems related to the evaluation and identification of new plant products: the lack of standardised methods. Even if many factors can influence the outcome of ddPCR analysis, the possibility of getting absolute amount of DNA copies has only recently become available even in this research field.

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Image:

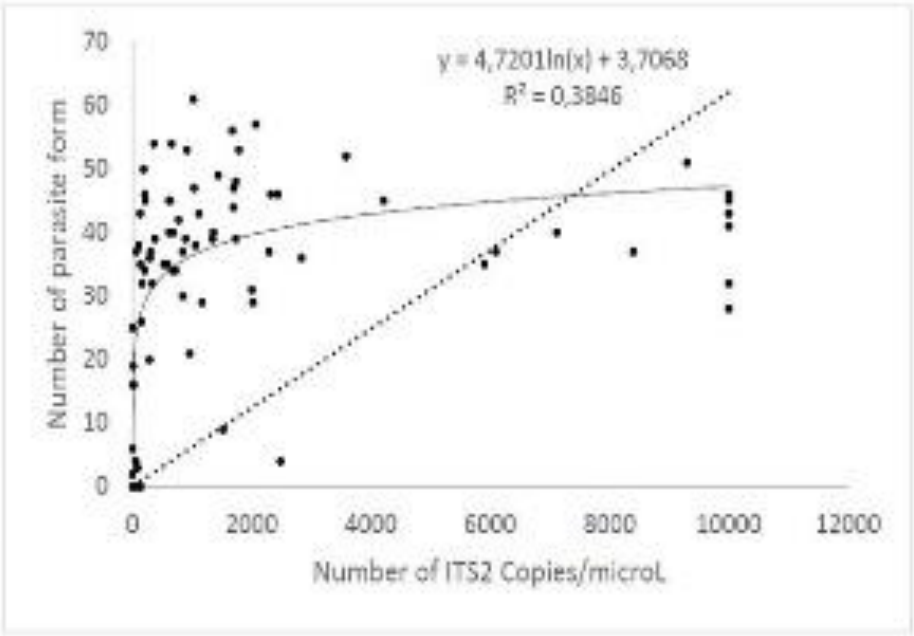

Figure 1: Relationship between parasite count and number of copies determined through ddPCR

Disclosure of Interest: None Declared

Keywords: Anthelmintic, assessment, ddPCR, plant extracts, technology