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Anthelmintic activity of *Chrysophyllum cainito* and *Psidium guajava* ethanolic bark extracts against *Ascaridia galli* of chicken

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Abstract

Limited substantiations are available supporting the pharmacological properties of herbal plants utilized in ethno-veterinary medication which remained sustainable in local communities in spite of advancements in animal health today. This study evaluated through *in vitro* and *in vivo* anthelmintic assays the folkloric use of *Chrysophyllum cainito* and *Psidium guajava*, which are among the selection of documented florae in the Philippines being used in ethno-veterinary medicine. *In vitro* anthelmintic evaluation showed time-dependent and concentration-dependent efficacies. The ovicidal action of *Chrysophyllum cainito* bark ethanolic extract at 60mg/ml has recorded 94.65% inhibition capacity, whereas *Psidium guajava* bark ethanolic extract generated 92.64% and 96.28% efficacies at 30mg/ml and 60mg/ml dilutions, respectively. The wormicidal activity of the former elicited 88.88% at 60mg/kg, while the latter yielded 88.88% and 94.44% mortalities to worms at 30mg/ml and 60mg/ml dilutions, correspondingly. Probit analysis on the lethal concentration (LC50) against eggs and worms was logged at different magnitudes for both plants. *In vivo* assessment by means of fecal egg count reduction (FECR) rate has signified biologically, that the tested plants undoubtedly possess anthelmintic property.

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Introduction

The scientific dwellings in modern animal health is averted with issues on drug resistance and residues attributed to injudicious use of veterinary drugs; a menace which may possibly upsurge as global problem in the future. The undisciplined custom of employing anthelmintics both for therapeutic and non-therapeutic purposes raises distress to authorities. Indeed, most farmers are reliant on the use of synthetic anti-parasitic drugs, and less application of organic approach (Waller, 2006).

The control and prevention of these parasites have depended largely on the application of several synthetic preparations of anthelmintics (Kumarasingha *et al*., 2016). In view of this concern, experts are assertive in seeking alternative approaches of controlling worm infection in animals, such as the elimination of *Ascaridia galli*, which is the most known parasite of birds that causes severe illness, pathological deformities and financial losses even in modern fowl production systems (Garedaghi, 2011; Soulsby, 1982).

The documented resistance of gastrointestinal nematodes to commercial dewormers has intensified the essential need to evaluate natural products, which can supplant the current approaches of controlling these parasites (Macedo *et al.,* 2012). Innovative dealings to control helminth infections are essential at this hour in order to halt this very concern on anthelmintic resistance (Giri *et al.,* 2015). The identification of anthelmintic plant extract with promising pharmacologic properties may contribute to the development of phytotherapeutic products with lower risk of resistance in contrast to conventional medication currently employed (Ferreira *et al*., 2013). Yigezu *et al.* (2014) highlighted the need for further methodical evaluations of plant materials used in ethno-veterinary medicine. As such, limited investigation finding is known on the pharmacological properties of *Psidium guajava* and *Chrysophyllum cainito* specifically on the anthelmintic activity. This therefore, dictates the need to search for substantial scientific evidences on the beneficial use of the aforementioned herbal plants that would offer alternatives to animal raisers in managing worm infection using vegetation found in the surroundings which are safe, efficient and inexpensive.

Materials and methods

Ethical Consideration

All methods engaged in the study were in accordance to the protocol of the Bureau of Animal Industry (BAI)-Manila, through the approval of the Institutional Animal Care and Use Committee (IACUC) of Isabela State University. *Chrysophyllum cainito* and *Psidium guajava* plants were authenticated by the Bureau of Plant Industry (BPI), National Plant Quarantine Services Division 02, Tuguegarao City, Philippines.

Plant Preparation

Collected barks were air dried, conservatively pulverized and immersed in 95% ethanol for 16 hours. After a series of filtration, pure extracts were obtained through a concentrator machine (Genevac EZ-2 Series). Stock solution was set at 100mg/ml concentration (Zuharah *et al.,* 2015; Nagappan, 2012) and then different dilutions were prepared encompassing 7.5mg/ml, 15mg/ml, 30mg/ml, and 60mg/ml. A few samples of the processed extracts were submitted for qualitative phytochemical analyses.

In Vitro Anthelmintic Assay

Adult *Ascaridia galli* female worms were retrieved from native chickens and incubated overnight at 37 °C to give-off fertile eggs. Ova were cultured with 0.1% H_2SO_4 (sulfuric acid) for six weeks to embryonate into vermiform stage (Ferdushy *et al*., 2012). In the ovicidal evaluation, every treatment was triplicated containing 0.5 microliter egg suspension. Replicates of each treatment group were added with 1000 microliter of the prepared working solutions. These were incubated at 27 °C and changes in eggs morphology were assessed after 3 days. The ovicidal action was expressed based on the percentage of ova with impaired segment or with unusual intra capsular mass (Ramadan and Znada, 1992). In the wormicidal assessment, *Ascaridia galli* worms were checked initially for liveability. After the period of incubation, the viability and motility of the worms were closely

observed. The adult worms of both sexes were randomly distributed to treatment groups. Worms were dispersed in petri dishes and then added with 10mL of the working solutions and incubated at 37°C. Worm death was observed at 1, 3, 6 hours intervals. After 6 hours, all worms were lifted from the working solutions and re-suspended in lukewarm 0.9% NaCl for 5 minutes to spot the revival of the worms. Dead and immobile worms were characterized based on motility upon poking. The lethal activity was expressed on the percentage of immobile worms after exposure (Cabardo *et al.*, 2017).

In Vivo Anthelmintic Assay

A total of eighty (80) Sasso chicks (*Gallus gallus domesticus*), 3-week of age and immunised against NCD and IBD were used. Birds were individually restrained and inoculated with the embryonated *Ascaridia galli* eggs at virulent dose of at least 250 embryonated eggs each animal (Bazh and El-Bahy, 2013). The progress of infection was based on the onset of egg shedding through the faeces which were taken at 6 weeks post-inoculation. The minimum number of ova per gram used to selected infected birds was at least 100 EPG (Yazwinski *et al.*, 2003). Confirmed infected birds comprised of mixed sex were randomly assigned to treatment and control groups having four (4) replicates with five (5) animals each. The groups were identified as follows: T1- Infected and treated with single dose of *Chrysophyllum cainito* bark ethanolic extract at 2000mg/kg BW, T2- Infected and treated with single dose of *Psidium guajava* bark ethanolic extract at 2000mg/kg BW, T3- infected and treated with single dose of Levamisole HCl (4g/bird) and T4- infected and given with 5mL distilled water (Wilkins®) per bird. Faecal egg count reduction (FECR) rate was examined in the respective treatment groups starting from day 0 pre-treatment and then at 7th and 14th day post-treatments. Following modifiedmc Master testing method, mc Master slide with analytical sensitivity of 25 eggs per gram was used to determine FECR rate. Faecal egg count reduction percentage (FECR%) was calculated using the formula described by Lone *et al.* (2013).

$$\text{FECR\%} = \frac{\text{Pre-treatment egg count per gram} - \text{Post-treatment egg count per gram}}{\text{Pre-treatment egg count per gram}} \times 100$$

Ovicidal and wormicidal activities were examined through multivariate-analysis of variance (MANOVA), while fecal egg count reduction (FECR) rate was analysed using one-way analysis of variance (ANOVA). Tukey-kramer test was employed to assess the multiple comparisons of means between treatments. All results were considered statistically significant at 5% level. The estimation of lethal concentration (LC_{50}) of plant extracts in the ovicidal and wormicidal activities was calculated using a probit analysis. Statistical program used was NCSS Version 12.

Results and discussion

Qualitative phytochemical screening of *Chrysophyllum cainito* and *Psidium guajava* ethanolic bark extracts has elucidated the presence of primary secondary metabolites primarily tannins, saponins and terpenoids.

Ovicidal Activity

As shown in Table 1, *Chrysophyllum cainito* at 60mg/ml (T6) has yielded 94.65% efficacy, while *Psidium guajava* at 30mg/ml (T9) and 60mg/ml (T10) elicited 92.64% and 96.28% inhibitory activities, respectively. All these recorded Fig.s were statistically identical with the 100% ovicidal action of Levamisole HCl ($p > 0.05$). The recorded ovicidal efficacy was explained by Fernandez *et al.* (2014) associating the numerous bioactive substances present in plant extracts, averting the growth of the eggs. Alemu *et al.* (2014) ascribed the efficacy of plant source tannins on egg hatching inhibition capacity, while Doligalska *et al.* (2011) implicated saponins in the interruption of egg hatching and moulting of larvae. Convincingly, there was a dose-dependent destructive effect on the morphology of *Ascaridia galli* eggs in the set of concentrations for both extracts denoting anthelmintic activity at the early stage of the parasite. Fig. 1 presents the pronounced damages in the physique of egg cells caused by the prepared extracts, characterized by presence of uncommon intra capsular masses typified by deformed and dissolved zygotes and injured egg

capsules. Vargas-Magaña *et al.* (2014) defined the apparent mechanisms of how plant extract inhibits egg transformation such as the blocking action on eggshell permeability, interference of some enzymes linked to egg hatching and obstruction of hatching receptors found in eggshells.

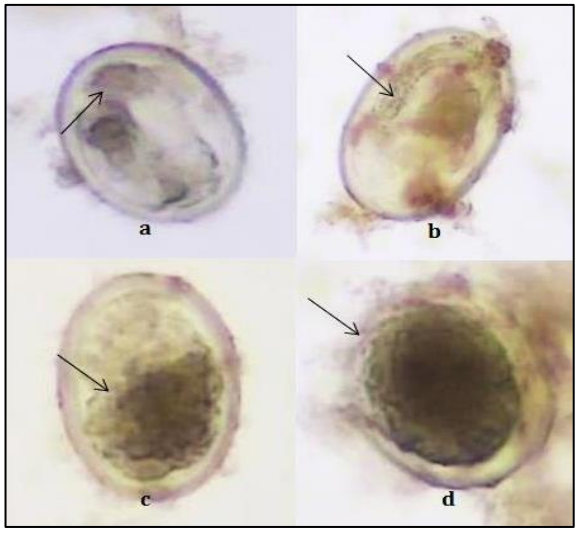

Fig. 1. *Ascaridia galli* eggs displaying diverse aspects of impairment caused by the plant extracts; where (a, b, c) exhibit dissolved zygotes or egg cell masses and (d) showing injured egg capsule.

Table 1. Impaired *Ascaridia galli* eggs at varying plant extract concentrations.

Treatments	Mean % of impairment
T1 (Levamisole HCl)	100.00 ± 0.00 [e]
T2 (Tap Water)	0.00 ± 0.00 [a]
T3 (*C. cainito* at 7.5mg/ml)	22.17 ± 5.91[b]
T4 (*C. cainito* at 15mg/ml)	57.76 ± 3.66 [c]
T5 (*C. cainito* at 30mg/ml)	78.21 ± 5.31[d]
T6 (*C. cainito* at 60mg/ml)	94.65 ± 4.63 [e]
T7 (*P. gujava* at 7.5mg/ml)	35.13 ± 1.59 [b]
T8 (*P. gujava* at 15*mg*/ml)	70.75 ± 4.16 [d]
T9 (*P. gujava* at 30*mg*/ml)	92.64 ± 7.71[e]
T10 (*P. gujava* at 60mg/ml)	96.28 ± 3.23 [e]

[a] Different superscript letters indicate significant differences at $p < 0.05$.

[b] Different superscript letters indicate significant differences at $p < 0.05$.

[c] Different superscript letters indicate significant differences at $p < 0.05$.

[d] Different superscript letters indicate significant differences at $p < 0.05$.

[e] Different superscript letters indicate significant differences at $p < 0.05$.

Wormicidal Activity

After 3-hour exposure to extracts (Table 2), only *Psidium guajava* at 60*mg*/ml (T10) elicited 83.33% lethality which was statistically comparable to the 100% activity of Levamisole HCl. Consecutively at 6-hour, *Chrysophyllum cainito* at 60mg/ml (T6) registered 88.88% efficacy, equating the mortality recorded by the standard drug ($p > 0.05$). *Psidium guajava* bark ethanolic extract has sustained its action successively, wherein dilutions at 30mg/ml (T9) and 60mg/ml (T10) have elicited 88.88% and 94.44% worm mortalities, respectively. This is analogous to the recorded activity of Levamisole HCl ($p > 0.05$); though the result was a little lower than the standard drug at all-time points. Notably, all set of concentrations for both plants displayed anthelmintic activity in time-dependent and dose-dependent modes.

The elicited deaths among worms may be initiated by tannins which adversely disturb the integrity of the cuticle (Fernandez *et al.,* 2013). According to Argentieri *et al.* (2008) adult worms have a critical structure (protective cuticle), an extracellular matrix forming their exoskeleton which is made-up of collagen proteins. This elucidation correlates the exposition of Eguale *et al.* (2007) ascribing the anthelmintic activity of plant ethanolic extract to its lipid soluble nature, that augments quick transcuticular absorption of bioactive components into the physique of the parasite, that later result to paralysis and death. Additionally, the synergistic action of the identified components may have caused the lethal effect to worms. Tannins influence directly the worm's activity by complicating the sheath protein make up of nematodes, thereby inhibiting ensheathment and impediment of DNA synthesis (Hoste *et al.*, 2011). Equally, saponins have intercalating action on the parasite's cell membranes which result in disruption and subsequent upsurge in cell uptake (Botura *et al.,* 2013; Argentieri *et al.*, 2008). Whereas, the synergy of numerous terpenoids which are complex mixture of compounds may act on several multiple molecular targets inherent to the parasite (Katiki *et al.*, 2011).

Table 2. Mean percentage of lethality to Ascaridia *galli* worm at varying plant extract concentrations and observation periods.

	Observation period		
Treatments	1 hour	3 hour	6 hour
T1 (Levamisole HCl)	94.44 ± 9.62 [c]	100.00 ± 0.00 [c]	100.00 ± 0.00 [c]
T2 (Tap water)	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
T3 (*C. cainito* at 7.5*mg*/ml)	0.00 ± 0.00 [a]	5.55 ± 9.62 [a]	22.22 ± 9.62 [ab]
T4 (*C. cainito* at 15*mg*/m*l)*	5.55 ± 9.62 [a]	22.22 ± 9.62 [ab]	38.88 ± 9.62 [abc]
T5 (*C. cainito* at 30mg/ml)	22.22 ± 9.62 [ab]	38.88 ± 9.62 [ab]	50.00 ± 16.66 [abc]
T6 (*C. cainito* at 60*mg*/ml)	33.33 ± 0.00 [b]	66.66 ± 16.66 [bc]	88.88 ± 9.62 [c]
T7 (*P. gujava* at 7.5*mg*/ml)	11.11 ± 9.62 [ab]	22.22 ± 9.62 [ab]	38.88 ± 9.62 [abc]
T8 (*P. gujava* at 15*mg*/ml)	27.77 ± 9.62 [ab]	50.00 ± 16.66 [b]	72.22 ± 25.45 [bc]
T9 (*P. gujava* at 30*mg*/ml)	50.00 ± 6.66 [b]	66.66 ± 0.00 [bc]	88.88 ± 9.62 [c]
T10 (*P. gujava* at 60*mg*/ml)	55.55 ± 9.62)[b]	83.33 ± 16.66 [c]	94.44 ± 9.62 [c]

[a] Different superscript letters indicate significant differences at $p < 0.05$.
[b] Different superscript letters indicate significant differences at $p < 0.05$.
[c] Different superscript letters indicate significant differences at $p < 0.05$.

The induced 50% lethality on eggs was logged at 14.01mg/ml and 9.77mg/ml for *Chrysophyllum cainito* and *Psidium guajava* bark ethanolic extracts, respectively. Whereas against worms was observed at 21.01mg/ml for *Chrysophyllum cainito* and 9.17mg/ml for *Psidium guajava*. The probit analysis report articulates that at a lower concentration, *Psidium guajava* has a better gradation in impairing ova and in inhibiting the motility of worms than *Chrysophyllum cainito* (Fig. 2).

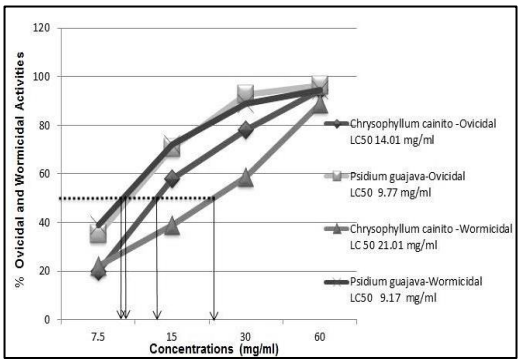

Fig. 2. Dose-response relationship and lethal concentration (LC50) of *Chrysophyllum cainito* and *Psidium guajava* ethanolic bark extracts against *Ascaridia galli* eggs and worms.

In Vivo Anthelmintic Assay

As for the *in vivo* finding involving Sasso chickens dosed with 2000mg/kg of the prepared extracts (Abdel Azis *et al.,* 2018), result revealed (Table 3) that at day 7 post-treatment, *Chrysophyllum cainito* (T3) and *Psidium guajava* (T4) ethanolic bark extracts induced fecal egg count reduction (FECR) of 38.16% and 42.97%, correspondingly, in contrast to the 93.3% efficacy of Levamisole (T1). Remarkably, FECRs improved at day 14 post-treatment, where T3 yielded 55.46%, T4 generated 62.26% and Levamisole HCl (T1) excelled to 100%.The generated FECRs for the two plant extracts for the given observations were statistically similar, but did not equate the effectiveness recorded by the standard drug. The accounted fecal egg count reduction rate (FECR) correlates the elucidation that compounds that are effective *in vitro* do not necessarily work similarly *in vivo*. Better bioavailability and anthelmintic action are entirely dependent on the compound's solubility and stability inside the animal's gastro-intestinal tract (Katiki *et al.*, 2013), influenced by animal's gut florae and *ph* and other factors that may alter the worm's response to treatment (Abdel Aziz *et al.,* 2018).

Table 3. Mean percentage of faecal egg count reduction (FECR) at 2000mg/kg dosage.

	Faecal egg count reduction (FECR)		
Treatments	Pre-Treatment (epg)	Post-Treatment Reduction%	
		Day 7	Day 14
T1 (Levamisole HCl)	298.43	93.30 ± 6.84 [c]	1100.00 ± 0.00 [c]
T2 (Tap water)	292.18	- -10.82 ±15.49 [a]	-42.86 ± 28.68 [a]
T3 (*Chrysophyllum cainito*)	278.12	38.16 ± 5.84 [b]	55.46 ± 56.69 [b]
T4 (*Psidium guajava*)	292.18	42.97 ± 4.42 [b]	62.26 ± 5.50 [b]

[a] Different superscript letters indicate significant differences at $p < 0.05$.
[b] Different superscript letters indicate significant differences at $p < 0.05$.
[c] Different superscript letters indicate significant differences at $p < 0.05$.

Conclusion

The primary secondary plant metabolites demonstrated in the extracts are conceivably the ones responsible for the documented anthelmintic activities both *for in vitro* and *in vivo* assays.

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Abbreviations: Fecal egg count reduction (FECR).

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