**Areca catechu L. and Anredera cordifolia (Ten) Steenis supplementation reduces faecal parasites and improves caecal histopathology in laying hens**

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**ABSTRACT**

Some studies have shown that the betel nut *Areca catechu* L. and “binahong” leaves *Anredera cordifolia* (Ten) Steenis have anti-parasite and wound healing properties. This study evaluated the effect of *A. catechu* nut and *A. cordifolia* leaves powder supplementation on faecal parasite number and type, histopathology of the intestine, caecum, associated organs, some serum biochemistry, and egg production of laying hens. Twenty-four 54-week-old ISA-brown laying hens from local layer farmers were assigned randomly into 4-treatment groups: 1) without supplementation (T0), 2) supplemented with 0.25% (T0.25%), 3) 0.5% (T0.5%), 4) 1.0% (T1.0%). We carried out the supplementation for 18 days by administering *A. catechu* nut powder for 3-days, and subsequently, *A. cordifolia* leaves powder for another 3-days for 3-rounds to control the parasite larvae. Faecal parasite count and type were enumerated at the beginning and end of treatment. Egg production was recorded daily during the 18 days experiment. Blood was sampled at the end of the experiment to determine serum albumin, globulin, and transaminases. The intestinal tract, liver, and spleen samples were collected at the end of the study for histopathological examination. Faecal *Ascaridia galli* in control hens increased by 87.5% after 18 days of the experiment, while *A. catechu* nut and *A. cordifolia* leaves powder supplementation prevented such an increase. Supplemented hens have a better reduction of *Railentina cesticillus* compared to control birds. Supplementation improved intestinal and other tissue histopathology, especially in the caecum (free of erosion), improving serum albumin and transaminases without affecting egg production.

**INTRODUCTION**

The challenges of global warming manifested by sudden changes between rainy and hot-humid weather in tropical climates combined with antibiotic resistance are amplifying environmental stress and hardship for local producers. Globally, the nematode infestation in laying hens is widespread [1]. *Ascaridia galli* infection is the most persistent infestation in layers [2–9]. Small-scale-independent farmers in a tropical country such as Indonesia raise commercial layers to produce commercial eggs as the primary income source. Layer farmers bred the most common hybrid chicken the ISA Brown to lay eggs for up to two years or as long as egg production is economically feasible. *Ascaridia galli* is not the only harboured endoparasites in commercial hens but also endoparasites such as coccidian [5–9]. To prevent microbial challenge, farmers use readily available feed.

Herbs and plant metabolites/extracts are gaining wide attention owing to their numerous health benefits, therapeutic characteristic, and nutraceutical properties [10–20]. *Areca catechu* is a species of palm widely found in the tropical Pacific, Asia, and parts of Africa [21,22]. In India and Indonesia, it is a traditional masticatory/chewing herbal medicine. The fruit or nut is locally known as “buah pinang” or betel nut. It comprises polyphenols – mainly flavonoids and tannins (11.1–29.8%), polysaccharides (17.3–25.7%), proteins (6.2–9.4%), fats (8.1–15.1%), fibres (8.2–15.4%), alkaloids (0.11–0.24%) and minerals (1.1–2.5%) [22,23]. The alkaloids include arecoline (7.5 mg/g weight), arecaidine (1.5 mg/g weight), guvacoline (2.0 mg/g weight) and guvacine (2.9 mg/g weight) [22,24] and recently catechu A and catechu B [25]. The arecoline is the primary active substance and the major toxic compound [22,24,26]. It has higher lipophilicity; therefore, it can cross the cell membrane quickly [27]. It induces apoptosis in normal human cells and causes oral mucosal fibrosis and cytotoxicity [28].

The anti-helminthic properties of the Areca nut have been demonstrated in numerous in vitro and in vivo studies. Ethanol extract of Areca nut powder added at 40% showed an effective reduction in the motility of the liver fluke (Fasciola spp.) in the petri dish, resulting in shrinkage and deformation of the
body shape and shrunken edges of the tegument [29]. At 1 g/Kg body weight (approximately 1.5%), Areca nut powder effectively expels round and tapeworm and eggs from indigenous local chicken. However, at a higher dosage of more than 1.5%, the chicken was not in good condition [30]. Areca nut extract possesses anticoxidial activity by reducing faecal oocyte counts, mucosal damage, and caecal lesion in broiler chicken experimentally infected with *Eimeria tenella* [31]. The mechanism appeared to be mediated by nitric oxide production, which was up-regulated in the inflammatory stage (3-days post-infection) and down-regulated 6-days post-infection. Ethanol extract and ethyl acetate fraction of betel nut can efficiently increase the number of goblet cells in the colon and caecum of mice orally infected with parasitic worm *T. muris* infective eggs [32]. Numerous studies showed a protective effect of Areca nut ethanol extract on a wound (excision and burn) healing and were likely to be due to its phenolic antioxidant activity [33–37].

*Anredera cordifolia* (Ten) Steenis (“Binahong”) leaves comprise flavonoids (vitecin, isovitecin, morin, and myricetin) and the sapogenins ursolic acid [38]. It has antibacterial and antioxidant activities [38–41]. Some studies demonstrated that *A. cordifolia* leaves have healing qualities on some wounds (cut, burn, post-partum perineum) [42–50]. A topical application of ethanol extract of *A. cordifolia* leaves twice a day on a 2 cm long excision wound in Guinea pig enhanced the length of excision closure, compared to control with povidone-iodine 10% [45]. A granulation network and re-epithelialization of the open physical wound appeared to be involved in healing [50]. Thus, the wound healing properties could be beneficial to healing the intestine and associated organ lesions due to endoparasite infestation. A study conducted by our group recorded an anti-microbial/endoparasite activity of *A. cordifolia* leaves powder in Saanen goat with mastitis. It exhibited that it could reduce total faecal oocytes [51]. However, there was no detail on oocyte types and their possible mechanism.

As the in-feed drug has been banned or increasingly reduced in animal husbandry practice and layer hens are an important source of quality protein (egg) with long production time, the potential of *A. catechu* and *A. cordifolia* to prevent natural endo-parasitic infestation is warranted to be studied. In a recent work, we found that administration of very low dosage, i.e. 0.025% – 0.1% *A. catechu* nut powder and *A. cordifolia* leaves powder alternately in 42 weeks layer hens reduced liver transaminases that could indicate cell regeneration properties of both additives [44]. However, the low dosage was insufficient to reduce faecal A. galli and other endo-parasite numbers than control without supplementation [5]. Therefore, we further study the supplementation of *A. catechu* nut and *A. cordifolia* leaves powder alternately every 3-days in 54-weeks-old layer hens with a higher dosage, i.e. ten times our first study. We investigated the faecal endo-parasite number, some serum biochemistry, and egg production, but importantly the histopathology of the intestinal tract, liver, and spleen. The histopathological examination of the affected tissues may give a better understanding about the anti-endoparasite properties of both phytogenic additives.

### 2. Material and methods

#### 2.1. Ethical statement

Our study was conducted in ISA Brown laying-hens obtained from the existing small-scale layer farmer, and the hens for supplementation were raised similarly in a wire-battery cage but separately. During the experiment, we applied animal ethics provided the hens with free access to drinking water and feed according to standard hens’ age. Hens were sacrificed at the end of supplementation by cutting the jugular vein according to standard animal welfare. Ethical Committee approved the protocols of this in vivo animal study with approval number: 133/EA/KEPK-FKM/2022.

#### 2.2. Collection of plant material and preparation of powder

Fresh dry *A. catechu* nuts were obtained from a local market, and the pericarp was discarded. Fresh *A. cordifolia* leaves were collected from local farmers and air-dried. The dried plant materials were ground to a powder and filtered through a 25–50 mesh screen to get a homogenous powder. The dried plant powder was kept in a refrigerated container for further use. A detailed description of the preparation process has been provided elsewhere [5,44].

#### 2.3. Supplementation of *A. catechu* nut and *A. cordifolia* leaves powder

We obtained the experimental hens from a small-scale (2500 hens) independent-layer farmer in Semarang-Central Java, Indonesia. The farmer raised Isa Brown hens in a typical V-type three-tiers battery bamboo housing. Each battery cage was 40 cm x 40 cm x 30 cm in size, which housed one hen per cage. The three-tiers battery housing was located in an open space (outdoor) with a roof to protect it from sun and rain. The experimental hens (24) were randomly selected based on age (54-week-old) and body weight (with an average body weight of 1.88 ± 0.02 Kg). We raised the selected hens in standard wire-battery housing separately. The 24-hens were adapted for two weeks, after which they were randomly assigned into four treatment groups: control with no supplementation (T0);
supplemented with 0.25% *A. catechu* nut and 0.25% *A. cordifolia* leaves powder (T2.5%); supplemented with 0.5% *A. catechu* nut and 1.0% *A. cordifolia* leaves powder (T0.5%); supplemented with 1.0% *A. catechu* nut and 1.0% *A. cordifolia* leaves powder (T1.0%). Each group contained 6 hens; hence, there were 24 hens. Each hen was given a 130 g commercial layer diet (mesh) from the farmers (2900 Kkal/Kg ME, 17–19% crude protein, 3–11% crude lipid, 5–6% crude fibre, 3.5% calcium, and 0.45% phosphor, with no antibiotic nor coccidiostat) per day and free access to drinking water. Supplementation was carried out by mixing the powder in the diet so that each supplemented group received the respective dosage. We designed the supplementation of *A. catechu* nut powder for 3-days, subsequently *A. cordifolia* leaves powder for 3-days, and the alternate administration was carried out for a total of 18 days. Figure 1 depicts a summary of the supplementation design.

We designed a 3-days alternate supplementation based on the life cycle of endo-parasites and to control parasite larvae. We assumed that the first round of 3-days of betel nut (with anti-helminth potential) would kill the mature larvae. The next 3-days of *A. cordifolia* could recover the infected tissue with its wound healing properties, while allowing the larval stage of endo-parasites to mature. The second round of 3-days betel nut supplementation would kill the mature larvae (from the larva of the first round), and then 3-days *A. cordifolia* would recover the tissue again. The third or final round of supplementation would kill the residual mature larvae from the previous round (round 2), and the following 3-days *A. cordifolia* supplementation provides the subsequent tissue recovery. To the best of our knowledge, no other publications has utilized a similar approach to ours in supplementing layer hens *A. catechu* and *A. cordifolia* except our previous study [44].

**2.4. Collection of faecal samples, parasites identification, and enumeration**

One day before phytogenic powder administration, we collected fresh faeces from each bird from all groups (total of 24 birds). Fresh faeces from each bird were immediately preserved in 10% formalin.

**Figure 1.** The supplementation designs. (T0): control without supplementation; (T0.25%): supplemented with 0.25% *A. catechu* nut and 0.25% *A. cordifolia* leaves powder; (T0.5%): supplemented with 0.5% *A. catechu* nut and 1.0% *A. cordifolia* leaves powder; (T1.0%): supplemented with 1.0% *A. catechu* nut and 1.0% *A. cordifolia* leaves powder. A total of 18 days supplementation consisted of first round administration of *A. catechu* for 3-days, followed by *A. cordifolia* for 3-days. Subsequently, followed by the second and third round which is the same as the first round i.e. administration of *A. catechu* for 3-days, followed by *A. cordifolia* for 3-days.
for parasite diagnosis [52]. The microscope was equipped with a camera, connected to a computer screen, and moved horizontally and vertically to scan the whole sample to identify and enumerate parasites. Parasites were grouped into types. The enumeration was carried out by experienced staff at the Laboratory of Animal Health Semarang city, Regional Office of Animal Husbandry and Health. Fresh faeces were collected at the end of the experiment from each bird from all groups (23 birds) and preserved in 10% formalin for endoparasite determination.

An increase or decrease in faecal parasite number for each type of parasite was calculated as follows:

Increase faecal parasite number (\%) = 100 - [(faecal parasite number before treatment/faecal parasite number after treatment) x 100]

Reduction of faecal parasite number (\%) = 100% – [(faecal parasite number after treatment/faecal parasite number before treatment) x 100]

2.5. Determination of serum albumin, globulin, AST, and ALT

At the end of the experiment, we collected blood samples from each bird of all groups (23 birds) through the brachial vein. Serum was immediately separated by centrifugation and stored frozen until analyses. The activity of alanine transaminase (ALT) and aspartate transaminase (AST) (previously known as serum glutamic pyruvic transaminase (GPT) and serum glutamic oxaloacetic transaminase (GOT)) were measured by the kinetic method according to the International Federation of Clinical Chemistry [10,11,13].

2.6. Histopathological examination

After blood sampling, birds were sacrificed by cutting the jugular vein according to Standard Animal Welfare. Duodenum, ileum, caecum, liver, kidney, and spleen were removed and preserved in buffered formalin. Preserved samples were embedded in paraffin, cut, and stained with Haematoxylin Eosin (HE) [12]. The samples were analysed by a histopathologist.

2.7. Egg production

We recorded each bird’s egg production and weight during 18 days of supplementation daily.

2.8. Statistical analysis

Excel and SPSS were used to conduct statistical analyses. Parasite numbers for each treatment group were compared to the control group (T0) before and after supplementation using a percentage of reduction, increase, or no change. A descriptive histopathological investigation was performed. Analyses of Variance (Anova) were performed on serum albumin, globulin, AST, and ALT data. Duncan’s multiple tests were carried out when the means among groups were significantly different. Paired T-tests were performed before and after supplementation for serum albumin, globulin, and transaminases. Significance was set at \( p \leq 0.05 \).

3. Results

Table 1 shows the frequency of faecal parasite types and number before and after 18-day supplementation of A. catechu nut and A. cordifolia leaves powder. Before supplementation, helminth A. galli and R.

| Type of helminth | Control (T0) | 0.25% | 0.5% | 1.0% |
|-----------------|-------------|-------|-------|------|
| A. galli        | 1           | 8     | 4     | 0    |
| R. cesticillus  | 7           | 13    | 10    | 11   |
| Tetrameres sp.  | 0           | 0     | 0     | 0    |
| 2 types         | 2 types     | 2 types | 2 types | 2 types |
| A. galli        | 8           | 7     | 4     | 0    |
| R. cesticillus  | 1           | 0     | 0     | 0    |
| Tetrameres sp.  | 0           | 0     | 1     | 0    |
| 2 types         | 1 type      | 2 types | 0 types |

Reduction or increase in endo-parasites number:

- A. galli: 87.5% increase
- R. cesticillus: 85.7% reduction
- T. americana sp.: 100% reduction

Each group consisted of 6 hens, with 5 hens in control as one hen had accident and must be excluded.

(T0): control without supplementation; (T0.25%): supplemented with 0.25% A. catechu nut and 0.25% A. cordifolia leaves powder; (T0.5%): supplemented with 0.5% A. catechu nut and 1.0% A. cordifolia leaves powder; (T1.0%): supplemented with 1.0% A. catechu nut and 1.0% A. cordifolia leaves powder.
cesticillus were observed in all hens with varying numbers in each group. It determines that the hens obtained from local farmers and raised in an outdoor battery housing have a varying endo-parasitic infestation.

After 18-day supplementation, the control birds without supplementation have increased number of A. gulli (87.5% increase). Interestingly, with 0.25% supplementation, the A. gulli number was reduced by 12.5%, whereas with higher supplementation, there was no change in the number of A. gulli. The reduction in the T0.25%, group was slight, leaving 7-parasites (from 8), which was still higher compared to T0.5% and T1.0%. At 1.0% supplementation, the absence of A. gulli was constant. In contrast, in the control group without supplementation showed an increase of 87.5% in A. gulli number.

Table 2 represents a histopathological investigation of the duodenum, ileum, caecum, liver, kidney, and spleen after 18 days of supplementation of A. catechu nut and A. cordifolia leaves powder alternately in 54 weeks laying hens were presented in. In the control group, histopathology of the small intestine (duodenum, jejunum, ileum) and caecum displayed the presence of erosion (Er). In 0.5% supplemented groups, erosion was still present in the ileum and caecum to variable degrees. Strikingly, after 1% supplementation, no erosion can be detected in the caecum, and Figure 2 displays the samples of caecum histopathology of control and T1.0%.

There was inflammation (I) in control duodenum, ileum, and caecum, but none after supplementation at 0.5% and 1%. In the ileum, goblet cells were not found in control birds but found in one of 0.25% supplemented birds, four of 0.5%, and 3- of 1% supplemented birds. There was helminth found in one bird with 0.5% supplementation. One control bird and two 1% supplemented birds had nodular lymphoid (NL).

Liver histopathology exhibited the presence of necrosis (N), infiltration of lymphocytes around blood vessels (Ic), congestion (C), and haemorrhage (H). Necrosis was found in the control liver (4/5 birds) and reduced after supplementation. Infiltration of lymphocytes (Ic) around blood vessels was found in four of 5 control birds. There were only two birds with Ic. after 1% supplementation. Congestion was found in one of 1% supplemented birds. Haemorrhage (Hr) was only found in one control bird and none in the supplemented group.

Several birds from all groups had inflammation in the kidney. There was necrosis in one of 0.5% and 1% of supplemented birds, while congestion (C) was found in one 1% supplemented bird. Spleen’s observation presented that follicle lymphocytes (FL) were found in two of five birds in the control group. No FL was observed after supplementation at all dosages. White pulp (WP) was observed in all supplemented birds but none in the control group. Inflammation was found only in 1 of 0.5% supplemented birds.

Table 3 presents the serum albumin, globulin, and transaminase levels of all groups before and after supplementation. There was a significantly higher level of globulin in groups supplemented with phytogenic additives before supplementation (p < 0.05). However, supplementation did not affect serum albumin, globulin, ALT, and AST (p > 0.05) in all groups.

Paired T-test revealed that serum albumin improved but serum globulin reduced significantly after supplementation (p < 0.05). Serum transaminase were significantly reduced after supplementation (p < 0.05).

Table 4 showed that all groups’ average total egg production and egg weight at the end of phytogenic powder supplementation was not significantly different (p > 0.05). However, the values are higher in all supplemented groups.

4. Discussion

Ascaridia galli is a parasitic roundworm that belongs to the phylum Nematoda. In our case, the birds obtained from local farmers were already infected with the parasite during their rearing. In the tropics, Ascaridia galli is the most prevalent parasite that affects layer husbandry [2–9, 53,54]. Infestation of A. galli in control birds without supplementation increased by 87.5% after 18 days (Table 1), indicating constant infection. On the other hand, a slight reduction occurred (12.5% reduction) at the lowest dosage of supplementation (0.25%) and a higher dosage of supplementation showed no change in faecal A. galli number. Hence, the supplemented groups that did not experience an increase in the number of faecal A. galli suggested that alternate supplementation of A. catechu and A. cordifolia every 3-days can prevent endo-parasite development thereby, preventing an increased parasitic growth that occurred in the control group without supplementation. Based on our methodological hypotheses, alternate supplementation allowed A. catechu nut powder (with anti-helminth potential) to kill the A. galli larvae, and followed by supplementation of A. cordifolia (with anti-wound healing activity) that healed some lesions resulting from the infection without interference from the A. catechu. After healing, another round of 3-days A. catechu nut re-supplementation again killed some more parasites, after which A. cordifolia was re-administered to continue the healing process (Figure 1). Thus, prevention of an increase of faecal A. galli in the supplemented group was likely attributed by the active alkaloid of Areca nut (arecoline, arecaidine, guvacoline, and guvacine), as shown by some studies [29–31]. When both supplements were administered as a mixture, the healing process became disturbed as a higher dosage of A. catechu (1%) could harm the organ’s cell lining and interfere with the healing process. Such harmful effect
| Groups    | T₀ | T₀.25% | T₀.5% | T₀.75% | T₀.9% | T₁.0% |
|-----------|----|--------|-------|--------|-------|-------|
| **Duodenum** |    |        |       |        |       |       |
| Er        | +  | +      | +     | +      | +     | +     |
| Hlm       | -  | -      | -     | -      | -     | -     |
| Jejunum   | +  | +      | +     | +      | +     | +     |
| Hr        | -  | -      | -     | -      | -     | -     |
| I         | +  | +      | +     | +      | +     | +     |
| Gc        | -  | -      | -     | -      | -     | -     |
| ileum     | +  | +      | +     | +      | +     | +     |
| C         | -  | -      | -     | -      | -     | -     |
| Caecum    | +  | +      | +     | +      | +     | +     |
| Hlm       | -  | -      | -     | -      | -     | -     |
| Liver     | +  | +      | +     | +      | +     | +     |
| Gc        | -  | -      | -     | -      | -     | -     |
| C         | -  | -      | -     | -      | -     | -     |
| Kidney    | +  | +      | +     | +      | +     | +     |
| C         | -  | -      | -     | -      | -     | -     |
| Spleen    | +  | +      | +     | +      | +     | +     |
| WP        | -  | -      | -     | -      | -     | -     |
| L         | +  | +      | +     | +      | +     | +     |

**Notes:** Each group consisted of 6 hens, with a control group of 5 hens (one was excluded due to accident).

Er = erosion, Hr = haemorrhage, I = inflammation; ic = inflammatory cells around blood vessel; Hlm = helminth, GC = goblet cell, C = congestion; N = necrosis, FL = follicle lymphocytes, NL = nodular lymphoid, WP = white pulp.

(T₀): control without supplementation; (T₀.25%): supplemented with 0.25% A. catechu nut and 0.25% A. cordifolia leaves powder; (T₀.5%): supplemented with 0.5% A. catechu nut and 1.0% A. cordifolia leaves powder; (T₁.0%): supplemented with 1.0% A. catechu nut and 1.0% A. cordifolia leaves powder.
of *A. catechu* has been demonstrated and could be due to its alkaloid content [22,26]. Therefore, an alternate supplementation for 3-days would give time for each supplement to exert its biological function without interfering with one other.

Another possibility is that when the supplements were administered together, an antagonistic effect was seen, i.e. *A. catechu*, with its cytotoxic activity was capable of killing the larvae but was also cytotoxic to intestinal cells lining [28], that interfered with the healing action of *A. cordifolia*. A study [55] conducted by purposely infecting indigenous chicken with *A. galli* and supplementing a mixture of *A. catechu* nut and *A. cordifolia* leaf powder for only ten days showed that the supplemented group had more *A. galli* eggs per gram in the faeces and the duodenum than the control group. It was unclear why the mixture could not reduce *A. galli* in their study, but we speculated that the administration schedule and combined dosage play an important role. Therefore, our study added significant evidence that 1% supplementation of *A. catechu* and *A. cordifolia* powder alternately every 3-days resulted in a better reduction of faecal *A. galli*.

For *R. cesticillus*, it was naturally reduced by 85.7% in the control group (Table 1). However, all supplemented groups had reduced *R. cesticillus* by 100%, suggesting the ability of alternate supplementation to improve the reduction. *Raillietina* species are found in the jejunum and ileum of the chickens and can reduce growth, causing weakness and digestive tract obstruction, whereas their larval stage (cysticercoid) resides in various invertebrate intermediate hosts, such as ants, beetles, small mini-wasps, or termites [56–58]. *Raillentina cesticillus* is known to spread via flies and cockroaches due to dirty housing [59–62]. With several thousand or more hens, some small-scale farmers have no assistance to do daily sanitation. Furthermore, the outdoor cage-housing with its daily excreta quickly attracted flies and cockroaches, facilitating the spread of the parasites. In control hens, a natural reduction is experienced due to our management, namely daily cleaning of all excreta, feed, and drinking containers. Therefore, the intermediate host was eliminated, and with clean feed and drinking water daily, the hens’ natural immune defence can work better, reducing faecal *R. cesticillus* in the control

![Figure 2](image)

**Figure 2.** Samples of caecum histopathology showing heavy erosion in the control group without supplementation (*T₀* replicate-4, 4x magnification), and free of erosion in 1.0% *A. catechu* nut and 1.0% *A. cordifolia* leaves powder supplemented group (*T₁.₀%* replicate-3, 10x magnification). Histopathology of all groups were presented and summarized in Table 2

| Table 3. Serum albumin, globulin, and transaminase in 54-week-old laying hens before and after 18 days alternate supplementation of *A. catechu* seed and *A. cordifolia* leaves powder. |
|---------------------------------------------------------------|
| **Supplementation** | **T₀** | **T₀.25%** | **T₀.5%** | **T₁.₀%** | **µ** |
| *Albumin (g/dL)* | | | | | |
| Before | 1.64 ± 0.34 | 1.86 ± 0.17 | 1.75 ± 0.20 | 1.73 ± 0.30 | 0.21 |
| After | 2.03 ± 0.08 | 2.22 ± 0.40 | 2.20 ± 0.21 | 2.21 ± 0.31 | 0.22 |
| *P* | 0.08 | 0.04* | 0.00* | 0.03* |
| *Globulin (g/dL)* | | | | | |
| Before | 3.24 ± 1.74* | 5.76 ± 0.57b | 5.37 ± 1.06b | 5.50 ± 1.61b | 0.01* |
| After | 3.43 ± 1.69 | 4.10 ± 0.99 | 3.81 ± 1.13 | 4.60 ± 0.83 | 0.41 |
| *P* | 0.40 | 0.00* | 0.00* | 0.05* |
| *ALT (U/L)* | | | | | |
| Before | 4.75 ± 3.45 | 6.26 ± 3.13 | 4.28 ± 1.04 | 5.55 ± 2.33 | 0.59 |
| After | 4.52 ± 3.07 | 2.93 ± 0.84 | 2.16 ± 0.50 | 3.46 ± 1.53 | 0.17 |
| *P* | 0.65 | 0.07 | 0.00* | 0.05* |
| *AST (U/L)* | | | | | |
| Before | 299.2 ± 84.92 | 274.90 ± 54.58 | 229.53 ± 18.52 | 230.70 ± 38.68 | 0.31 |
| After | 202.06 ± 41.40 | 246.88 ± 45.62 | 205.13 ± 11.09 | 191.23 ± 32.73 | 0.11 |
| *P* | 0.18 | 0.01* | 0.04* | 0.05* |

*AST: aspartate transaminase. ALT: alanine transaminase.*

Each group consisted of 6 hens, with a control group of 5 hens (one was excluded due to accident)

Values represent the average of 6 hens (except control group with 5 hens) ± standard deviation

*P* with no superscript * stands means there is no effect of supplementation (p > 0.05) (Anova). Different superscript within the same row is significant at (p < 0.05) (post-hoc test)

*P* with superscript * stands means significantly different between before and after supplementation (T-test).

(*T₀*: control without supplementation; *T₀.25%*: supplemented with 0.25% *A. catechu* nut and 0.25% *A. cordifolia* leaves powder; *T₀.5%*: supplemented with 0.5% *A. catechu* nut and 1.0% *A. cordifolia* leaves powder; *T₁.₀%*: supplemented with 1.0% *A. catechu* nut and 1.0% *A. cordifolia* leaves powder.)
Table 4. Egg production during 18 days supplementation of A. catechu seed and A. cordifolia Leaves powder in S4-week-old laying hens.

| Egg production | T₀ | T₀.25% | T₀.5% | T₀.1% | p |
|----------------|----|--------|-------|-------|---|
| Total Egg Weight | 44.7 ± 27.8 | 58.83 ± 4.3 | 59.1 ± 5.6 | 47.9 ± 18.2 | 0.96 |
| Average | 7.0 ± 5.1 | 9.2 ± 1.9 | 8.2 ± 6.1 | 8.6 ± 4.1 | 0.20 |

Each group consisted of 6 hens, with a control group of 5 hens (one was excluded due to accident). Values represent the average of 6 hens (except control group with 5 hens) ± standard deviation. Anova was done at p < 0.05. No superscript letter means there is no effect of supplementation on egg production. (T₀): control without supplementation; (T₀.25%): supplemented with 0.25% A. catechu nut and 0.25% A. cordifolia leaves powder; (T₀.5%): supplemented with 0.5% A. catechu nut and 1.0% A. cordifolia leaves powder; (T₀.1%): supplemented with 1.0% A. catechu nut and 1.0% A. cordifolia leaves powder.

Erosion was still present in some hens in the duodenum, jejunum, and ileum, suggesting that infection to the organ from endo-parasite can only be healed partly by supplementation. We speculate that the healing is attributed to both phytoesthetic as both contain polyphenolic compounds with antioxidant properties and re-epithelialization activity [33,34,36,37].

Goblets cells appear at 0.5% and 1% supplementation in the jejunum, ileum, and caecum but not in the duodenum. At 1% supplementation, three hens did not show erosion, and the presence of goblet cells indicated the presence of regeneration as these cells function to produce and maintain mucus layers along the intestinal lining. Two birds in this group had lymph nodules, that indicated an ongoing immune response against endoparasites antigen. The parasites could be hiding under epithelial layers and cause re-infection to occur. Strikingly, for caecum, supplementation of the powder decreased erosion remarkably as compared to control with no supplementation. At 0.25% supplementation, half of the samples were free from erosion, and strikingly at 1%, all samples were free from erosion. It could be due to the inability of sporozoite and merozoite to develop due to A. catechu nut powder (Table 1, after 1% supplementation), while A. cordifolia assists in healing the damaged tissue [33–35,65,66]. As we have previously described, the mechanism could be due to the action of the active alkaloid of Areca nut (arecoline, arecaidine, guvacoline, and guvacine) powder [30]. The lipophilic nature of the alkaloid to penetrate the lipid bilayer of mucosal lining could reach and kill the buried parasite [22,63]. During 3-days of Areca nut administration, the cytotoxicity against parasites was more noticeable than the healing activity. Therefore, the next round of A. cordifolia leaves administration would further support the healing activity mediated by re-epithelialization. Furthermore, the ursolic acid of A. cordifolia can induce epidermal keratinocyte differentiation (via peroxisome proliferator-activated receptor-alpha) that also assists in healing the erosion in the caecum [67]. The caecum is the blind sac of hens before the excretion of undigested feed. The A. cordifolia leaves powder and Areca nut with its fibre could partly reach the caecum and stay longer and hence could act longer to heal all the erosion. It is warranted to study such a possibility further.

Necrosis in the liver showed that after 0.25 and 0.5% supplementation, there is only one tissue sample that showed necrosis. At 1% supplementation, three samples showed necrosis, which could be due to several factors. One is that increasing the dosage of A. catechu nut powder could be toxic to the liver. Arecoline of the beetle nut is cytotoxic and can cause fibrosis [68]. Necrosis appears due to an inflammatory response, after which regeneration occurs and continues with tissue remodelling. However, uncontrolled
tissue remodelling can lead to fibrosis, which could occur at 1% supplementation. Another possibility is that at a lower dosage, Areca nut powder can function as an anthelmintic, antioxidant, antibacterial, and anti-inflammatory [24,28,36,69]. Therefore, in 0.25% and 0.5% of supplemented birds, the number of liver samples with necrosis and lymphocytes infiltration around blood vessels was reduced.

The histopathology of the kidney showed that supplementation did not affect it, as the samples’ initial and final conditions were similar on average. The kidney functions to filter the blood, excreting the end product of metabolism and regulating the concentration of hydrogen ions and minerals in extracellular fluid. To the best of our knowledge, there have been no studies that relate kidneys to endoparasite infestation, and the presence of inflammation in some samples in each group indicated a normal condition of 56-weeks-old layers.

The spleen is a secondary lymphoid organ in chickens. The presence of follicle lymphocytes (FL) in the control group’s spleen indicated an immune response against an antigen. The antigen likely comes from the Marek virus, which usually infects layers worldwide, except in flocks purposely raised under pathogen-free conditions. Significant swelling in the visceral organ during organ sampling supports the possible Marek viral infection. Marek infection occurs in layer chicken from 1 to 3 weeks old with or without clinical signs, and this infection can reduce growth and egg production [12]. No follicle lymphocytes were found in all supplemented birds, but white pulp appeared. White pulp (WP) indicates the presence of coccidial infection, mainly from *Emerica tenella*. Reinfection stimulates lymphocytes to respond more quickly to proliferate, followed by an increase in diameter and weight of the white pulp [70]. Although no *E. tenella* was found in bird faecal samples, the remnants of inflammatory response in the spleen were still observable. White pulp appearance indicates that supplementation improves the immune response against endoparasite infection.

Our present results showed that serum albumin concentration after supplementation improved significantly (p < 0.05) (Table 3). As albumin synthesis occurs in hepatocytes, it indicates an improvement in liver function. The possible improvement is supported by the results that showed supplementation reduced transaminase significantly (p < 0.05). The result is also consistent with our previous study that showed reduced transaminase activity after alternate supplementation using both powders at one-tenth of the present dosage in 42-week-old layers [5,44]. All phytogenic taken via gastro-intestine (GIT) are carried to the liver. A higher dosage used in the present study could put a higher liver workload and cause cell damage, especially from the active alkaloid of Areca nut. However, the polyphenol as the main content of Areca nut (11.1–29.8%) is a well-known antioxidant that could counter-act cell damage by the active alkaloid (0.11–0.24%) [23] and radicals’ generation. Therefore, its use depends on the dosage, administration schedule, and combined use with another herb. Subsequent administration of *A. cordifolia* leaves with epithelialization activity 3-days after Areca nut administration [35] added further support in preventing and healing cells’ damage. Reduction in serum globulin after supplementation indicates a reduction in inflammation due to immune response to endoparasites. Reduction of inflammation is supported by the results of faecal parasites number and histopathology after supplementation, as described previously.

Our results support the anti-endoparasites and wound healing function of Areca nut and *A. cordifolia* leaves powder in vivo layer hens, especially in the caecum, improving serum albumin and transaminase without affecting egg production.

5. Conclusion

Our results demonstrated that supplementation of phytogenic *Areca catechu* nut and *Anredera cordifolia* leave powder alternately every 3-days for 18 days reduced faecal-endoparasites and improved histopathology endoparasites-affected tissues in layer hens, especially in the caecum. The dosage and alternative schedule of supplementation of *A. catechu* and *A. cordifolia* to reduce endoparasites and heal cells’ damage are still open for further studies.

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