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Contamination of Intestinal Parasites in Vegetables from Kuching

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ABSTRACT

Background: Contaminated vegetables with intestinal parasites, particularly those eaten raw, represent a proportion of risks for humans acquiring foodborne parasitic diseases worldwide. Unfortunately, the risk is often neglected as limited studies have been reported about the parasitic occurrence from vegetables retailed in Malaysia.

Objective: This study was conducted to determine the occurrence of intestinal parasites in fresh retailed vegetables in Kuching and Kota Samarahan, Sarawak.

Methods: A total of 108 vegetables (comprising leafy and root-type vegetables) were purchased from three supermarkets and three wet markets in Kuching and Kota Samarahan. The samples were processed with 0.95% sodium chloride solution and underlaid with Sheather's sucrose solution. *Cryptosporidium* and *Giardia* were detected using AquaGlo™ G/C antibody reagent and 4',6-diamidino-2-phenylindole stain. Other parasites were detected using Lugol's iodine stain.

Results: A total of 24 out of 108 vegetables samples (22.2%) were contaminated with nematode larvae (range: 0.01 – 0.71 larvae/g), *Cryptosporidium* oocysts (range: 0.01 – 0.03 oocysts/g), hookworm ova (range: 0.01 – 0.02 ova/g) and *Giardia* cyst (0.01 cysts/g). There were no significant differences ($p > 0.05$) for means of parasite concentrations in vegetables from supermarkets and wet markets, as well as parasite concentrations from leafy-type and root-type vegetables.

Conclusion: The findings revealed relatively high numbers of intestinal parasites detected from fresh retailed vegetables, signifying potential foodborne transmission of parasitic infections if the vegetables are not prepared hygienically or cooked well. Besides, a high proportion of such infection risk may be reduced if farmers also take part in proper sanitation practices during vegetable production and transportation.

Key Words: *Cryptosporidium*, *Giardia*, Helminth, Intestinal parasites, Protozoa, Vegetables

INTRODUCTION

Foodborne infections cause worldwide morbidity and mortality, while foodborne infections caused by intestinal parasites are more common in tropical countries. There are many etiological agents of foodborne intestinal parasitic infections (IPIs) in humans and these include parasites associated with contamination of fresh produce such as *Cryptosporidium*, *Giardia*, *Cyclospora*, *Angiostrongylus*, hookworm, *Ascaris lumbricoides*, *Echinococcus*, *Fasciolopsis buski* and *Toxocara* spp.^{1,2} In developing countries, parasitic infections are underreported because of asymptomatic cases and lack of established diagnosis and reporting systems.^{3,4}

Given concerns about heat-labile nutritional contents (e.g. flavonoids, polyphenolic compounds) and natural tastes, many consumers now are more prone to consume vegetables fresh, raw or undercooked.⁵ However, the current trends of healthy diet by eating raw vegetables as well as increasing number of susceptible individuals have exacerbated the risks of vegetable-associated IPIs. Potential of vegetables being a source of parasitic infections should not be underestimated as many parasites are zoonotic and non-endemic in a particular area.⁶

Contaminations involving parasites associated with fresh produces have been documented at many points of cultivation (i.e. pre-harvest and post-harvest contaminations) until

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being in retails. For instance, in water irrigation⁷⁻⁹, the soil of cultivation sites^{10,11}, organic farms¹¹, and directly from vegetables and fruits.^{6,12} In the context of Malaysia, there is still a wide gap of information about parasitological contaminations in vegetables. Thus far, there have been three studies conducted, translating the state of neglect about IPIs.¹³⁻¹⁵ A study by Zeehaida et al.¹⁴ detected *S. stercoralis* rhabdiform larvae in *pegaga* (pennywort), *kesum* (Vietnamese coriander) and water spinach with parasite counts of 152 larvae/kg, 9 larvae/kg and 16 larvae/kg, respectively retailed from wet markets. Whereas, Yusof et al.¹³ found a different variety of intestinal parasites in retailed vegetables from a wet market, namely unidentified fluke, *Strongyloides* larva, *Entamoeba* spp., *Blastocystis* spp. and *Diphyllobothrium* egg from *pegaga* (pennywort), *kesum* (Vietnamese coriander) and *kangkung* (water spinach).

Generally, most human parasites are widespread, nonetheless geographical and basis of location may show different occurrences of such contaminations. Thus, this study was conducted to assess the occurrence of parasitological contamination on fresh vegetables that were retailed in Kuching and Kota Samarahan, Sarawak.

MATERIALS AND METHODS

Sample collection

A total of 108 vegetables were purchased from six markets (three supermarkets [i.e., Supermarket A, B and C] and three wet markets [i.e., Wet markets A, B and C]) in Kuching and Kota Samarahan, Sarawak. Sample collection was conducted with three-time frequency at each market. Three leafy-type vegetables (e.g. cabbage, lettuce, water spinach) and three root-type vegetables (e.g. ginger, potato, carrot) were purchased from each market during each sampling trip as shown in Table 1 and 2. All vegetables were collected fresh from shelves or racks and placed in sterile stomacher bags. Afterwards, they were transported directly to the Molecular Microbiology Laboratory, Faculty of Resource Science and Technology at Universiti Malaysia Sarawak.

Vegetable sample processing

Sample processing was conducted as described by Erdog˘rul and Şener¹⁶. Each vegetable sample was weighed (i.e. whole vegetables including leaves, stem or roots) for 200 g and placed into a sterile stomacher bag and washed with 0.95% sodium chloride solution with shaking at 120 rpm for 20 min. The washed solutions were transferred into conical flasks and left for approximately 12 hr at room temperature. Afterwards, the supernatants were discarded (i.e. leaving about 200 ml supernatants with pellets) and the leftovers were centrifuged at $2,100 \times g$ for 15 min. Later, the supernatants were discarded.

Sucrose flotation method

This method was by the method by Kuczynska and Shelton¹⁷. Briefly, the pellet was mixed with sucrose solution (1.27 specific gravity) until 12 ml in a 15 ml centrifuge tube. The tube was centrifuged at $400 \times g$ for 5 min. Afterwards, extra sucrose solution was added until it formed a negative meniscus. A glass coverslip was placed on top of the meniscus and left for 30 min. Then, the glass coverslip was scrapped, and the washed solution was inserted into a 50 ml centrifuge tube. The sucrose solution was washed by centrifuging at $1000 \times g$ for 10 min. The supernatant was discarded and transferred into a 1.5 ml microcentrifuge tube and spun at 6000 rpm for 10 min. Then, the pellet was brought to 100 µl and divided into 50 µl for *Cryptosporidium* and *Giardia* detection and 50 µl for other parasites detection.

Detection of *Cryptosporidium* and *Giardia*

Immunofluorescence assay for *Cryptosporidium* and *Giardia* complied with the recommended procedure by Waterborne, Inc. (New Orleans, USA). A volume of 50 µl of the sample concentrate after the sucrose flotation was placed on a 4×9 mm well microscope slide (Hendley-Essex, UK). The sample was air-dried and stained with 4',6-diamidino-2-phenylindole (DAPI) (2 µg/ml) for 4 min before being washed with 100 µl of phosphate-buffered saline (PBS). Next, one drop of Aqua-Glo™ G/C antibody reagent (Waterborne, Inc., New Orleans, USA) was added and incubated at 37 °C for 25 min and washed with 100 µl of PBS. Then, a drop of mounting medium containing PBS/glycerol (1:1) was added on the slide and the coverslip was sealed by using nail polish. *Cryptosporidium parvum* and *Giardia lamblia* (oo) cysts (Waterborne, Inc., New Orleans, USA) were used as a positive control, while distilled water was used as a negative control. Lastly, the slide was viewed at $400\times$ and $600\times$ magnifications under a fluorescence microscope (IX51, Olympus, Japan) by using analysis pro software. *Cryptosporidium* oocyst was recognised as spherical-shaped in a range of 4 – 6 µm in size and *Giardia* cyst was identified as spherical to oval-shaped in a range of 5 – 15 × 8 – 18 µm in size. Both appeared as apple green-coloured with distinct bright apple green cell wall.

Detection of other parasites

A volume of 50 µl concentrate was added on a microscope slide and one drop of the Lugol's iodine solution was added on each sample. After the slide was covered with a coverslip, it was observed at $200\times$ to $400\times$ magnifications under a compound microscope (BX15, Olympus, Japan) by using cell^D software (Olympus, Japan) to measure the size of parasite eggs. Identification of the parasites was performed by referring to WHO¹⁸.

Statistical analysis

The concentrations of the detected parasites were expressed as ova/larvae/cysts/oocysts per gram of each sample. Two-sample t-test analysis with equal variance was utilised to find the significant difference of human intestinal parasites occurrence in vegetables sold at supermarkets and wet markets, as well as between human parasites occurrence in leafy and root-type vegetables. The statistical significance was set at $p < 0.05$. This statistical analysis was conducted utilising Microsoft Office Excel 2016.

RESULTS

Overall

A total of 24 out of 108 vegetables samples (22.2%) from six selected markets (i.e. supermarkets and wet markets) examined in this study were contaminated with nematode larvae (range: 0.01 – 0.71 larvae/g) followed with *Cryptosporidium* (range: 0.01 – 0.03 oocysts/g), hookworm (range: 0.01 – 0.02 ova/g) and *Giardia* (0.01 cysts/g) as displayed in Tables 1 and 2, and Figure 1. The parasite concentration in all samples ranged from 0.01 – 0.71 parasites per gram of sample. In general, the types of vegetables found to be contaminated both from the wet markets and supermarkets were mustard greens, lettuce, Chinese kale, spinach, water spinach, sweet potato, ginger and carrot.

Contamination with parasites based on locations and vegetable types

A comparative analysis amongst the three sampled supermarkets found that Supermarket C had the highest number of contaminated sampled vegetables (6/18 vegetables), followed by Supermarket A (5/18) and lastly Supermarket B (1/18) (Table 1 and 2). At Supermarket A, the types of vegetables discovered to be contamination were mustard greens (*Cryptosporidium* = 0.02 oocyst/g), lettuce (*Cryptosporidium* = 0.01 oocyst/g), Chinese kale (*Cryptosporidium* = 0.03 oocyst/g), sweet potato (nematode = 0.02 larvae/g) and ginger (nematode = 0.35 larvae/g). Whilst at Supermarket B, a vegetable detected to be contaminated was ginger (nematode = 0.06 larvae/g). At supermarket C, the contaminated vegetables were Chinese kale (nematode = 0.05 and 0.10 larvae/g; hookworm = 0.02 ova/g), mustard greens (nematode = 0.01 larvae/g), potato (nematode = 0.02 larvae/g), carrot (*Cryptosporidium* = 0.02 oocyst/g) and ginger (nematode = 0.01 larvae/g).

As for wet markets, both Wet Markets A and C had 5 (of 18) sampled vegetables contaminated whilst Wet Market B had 2 (of 18) sampled vegetables contaminated (Table 1 and 2). Types of vegetables that were found to be contaminated in the wet markets include at Wet Market A which were spinach (nematode = 0.01 larvae/g; *Giardia* = 0.01 cyst/g),

water spinach (nematode = 0.03 larvae/g), mustard greens (nematode = 0.03 larvae/g) and ginger (nematode = 0.02 and 0.13 larvae/g). At Wet Market B, the types of contaminated vegetables were Chinese Kale (nematode = 0.01 larvae/g) and potato (0.03 larvae/g). Whilst at Wet Market C, the contaminated vegetables were water spinach (nematode = 0.03 larvae/g), spinach (nematode = 0.03 larvae/g), lettuce (hookworm = 0.01 ova/g), ginger (nematode = 0.04 and 0.71 larvae/g).

The level of parasitological contamination of retailed vegetables at wet markets (i.e. Wet market A, B and C) (total: 12/54 positive samples; 109 intestinal parasites) was higher compared to supermarkets (i.e. Supermarket A, B and C) (total: 12/54 samples; 72 intestinal parasites). However, the two-sample t-test of equal variance analysis showed no significant difference in mean comparison for parasite concentrations in purchased vegetables from supermarkets and wet markets ($p > 0.05$). Comparison of parasitological contamination based on vegetable types revealed that root-type vegetables (total: 11/54 samples; 140 intestinal parasites) was higher contaminated than leafy-type vegetables (total: 13/54 samples; 40 intestinal parasites). Nevertheless, there was no significant difference of parasite concentration from leafy-type and root-type vegetables ($p > 0.05$). The results of the statistical analyses are displayed in Table 3.

DISCUSSION

This study revealed vegetables retailed at supermarkets and wet markets in Kuching and Samarahan were contaminated with intestinal parasites. The highest parasite count detected on vegetables was nematode larvae (range: 0.01–0.71 larvae/g) followed with *Cryptosporidium* oocyst (range: 0.01 – 0.03 oocysts/g), hookworm ova (range: 0.01 – 0.02 ova/g) and *Giardia* cyst (0.01 cysts/g). Nematode larvae were detected from both leafy-type vegetables ($n = 10/54$ samples; i.e. Chinese kale, mustard greens, spinach and water spinach) and root-type vegetables ($n = 10/54$; i.e. ginger, carrot, sweet potato and potato). Whereas, hookworm ova were detected from leafy-type vegetables ($n = 2/54$ samples; i.e. Chinese kale and lettuce). No hookworm ova were detected from root-type vegetable samples. In this study, nematode larvae were detected solely based on morphological identification. Molecular analysis was not performed to identify the species. Besides, hookworm eggs cannot cause infection, but the third-stage of the larvae can penetrate the skin of individuals involved in contacts.^{19,21} *Cryptosporidium* oocysts were detected in leafy-type vegetables ($n = 3/54$ samples; i.e. mustard greens, Chinese kale, lettuce) and root-type vegetables ($n = 1/54$ samples; i.e. carrot). *Giardia* cyst was detected from one leafy-type vegetable ($n = 1/54$ samples; i.e. spinach). However, no *Giardia* cyst was detected from root-type vegetables. Both *Cryptosporidium* and *Giardia* were listed as

the fifth and eleventh top foodborne parasites from the global perspective.² Ingestion of these two protozoan parasites leading to invasion of the intestinal epithelium, thus infected individuals may manifest symptoms of gastroenteritis such as diarrhoea, vomiting, nausea and weight loss.^{22,23} Foodborne IPIs are partly due to the persistency of the parasites on food matrices, viability at normal food storage conditions and low infectious doses. *Cryptosporidium* can adhere strongly on apple surfaces attributed by extracellular matrix of the oocysts²⁴ and internalise into stomata of leafy vegetables.²⁵ *Cryptosporidium* exhibits remarkably high resilience that allows the coccidia survives in harsh external environments. Intestinal parasites (e.g. *Cryptosporidium* and *Giardia*) remain viable at damp and cool storage conditions such as in the household refrigerator. The parasites still manifest infectivity even being stored at 4 °C for 8 days²⁶ and 6 °C for a month.^{24,25} The infectious dose of *Cryptosporidium* is as low as 30 oocysts²⁷, while *Giardia* is as low as 10 to 100 cysts.²⁸

During the initial part of this study, it was postulated that wet markets would be contaminated with higher count of parasites compared to supermarkets. This was assumed due to the lower sanitation practices as the retailed vegetables at wet markets were likely to be traditionally homegrown. Besides, root-type vegetables would be contaminated with a higher number of parasites than leafy-type vegetables as they are exposed to the contaminated soil. However, statistical analysis revealed that the contamination level by the parasites in vegetables retailed at supermarkets versus wet markets, as well as leafy-type versus root-type vegetables, showed no significant differences ($p > 0.05$). Thus, based on the present samples, results indicate that all vegetables (i.e. leafy and root-type vegetables) from both types of the market may possess equivalent risks of sourcing foodborne intestinal parasites to consumers. It is suggested that a high proportion of contaminations might occur in the farms than in retails.

In this recent study, of 108 vegetables examined, high concentrations of intestinal parasite were evident from gingers (0.13, 0.35 and 0.71 larvae/g). This is suggested that it was attributed by its crinkle and irregular shape resulting in large surface area for contact with soil like the finding in Hassan et al.²⁹ with parasitic contamination in root-type vegetables (radish and carrot) were among the highest contaminated. However, the authors enlightened that there were no significant differences in parasite occurrences based on the type of vegetables.²⁹ Nonetheless, leafy-type vegetables are prone to high contamination particularly the ones with an uneven and large area of surfaces. This facilitates stacking of parasites on the leaves especially during washing or watering. The occurrence of IPIs in this study might have sourced during different phases of contaminations. There are three evident routes of parasitological contaminations in food chains; from food and raw materials on the farm, contaminated water used

in the processing, and transmission via food preparers during production, service or domestic settings.³¹ Tackling real risks is crucial to be established before any actual incidents take place. At the farm level, such contamination can be sourced from the contaminated fertiliser, soil and water.³² Whereas, at the processing and preparation stages, contamination can take place from materials used in the processing and by food preparers. Washing vegetables with contaminated water possesses a risk of contamination. Inversely, inadequate washing may contribute to the persistency of the sticking foodborne parasites on the vegetable surfaces. Several studies have demonstrated that inadequate washing may cause persistency of parasites in vegetables.³³⁻³⁶ Furthermore, personal hygienic practices among vegetable preparers or hawkers (e.g. washing hands, cutting nails), where the areas under the fingers are difficult to clean and may contain the highest number of microorganisms among all parts of human body.³⁷

Based on many studies that assessed risk factors of intestinal parasitisms in Malaysia, it was found that there were significant associations between demeanour of consuming raw vegetables with the high rate of IPIs.³⁸⁻⁴² Moreover, the risks of IPIs are also high in immunocompromised individuals. For instance, a study by Li et al.⁴³ presented eating raw fruits and vegetables, that were never or occasionally washed, represented a risk factor to IPIs among the patients with a chronic disease. Vegetables are normally fried, boiled or steamed but in some instances, they are consumed raw such as in burger, salad, coleslaw, vegetable juices and chutney to retain natural tastes and heat-sensitive nutrients.⁴⁴ As to prevent and control IPIs, disinfections are the crucial stage for reducing the risks through vegetable consumption.⁴⁵

CONCLUSIONS

The findings provided evidence that retailed vegetables were contaminated with intestinal parasites. This highlighted the potential of transmitting parasitic infections via vegetables especially if the vegetables are not prepared hygienically (e.g. washed, cooked, steamed, boiled) to render the parasites non-infectious for safe human consumption. Nevertheless, steps can be taken to reduce the risk if farmers adopt proper sanitation practices during vegetable production and transportation.

ABBREVIATIONS

DAPI: 4',6-diamidino-2-phenylindole; IPIs: intestinal parasitic infections; PBS: phosphate-buffered saline

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Availability of Data and Materials: All data generated or analysed during this study are included in the published article.

Authors' Contributions: LMB was involved in the study designation; CSKG was involved in the sample collection; AST and CSKG were involved in the laboratory experiments; AST, LMB, YAL and RLR were involved in the manuscript drafting. All the authors read approved the final version of the manuscript.

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Table 1: Intestinal parasites detected from leafy-type vegetables purchased from supermarkets and wet markets in this study

Market	First sampling visit	Second sampling visit	Third sampling visit
Supermarket A	Cabbage	Cabbage	Spinach
	-	-	-
	Mustard greens <i>Cryptosporidium</i> = 0.02 oocyst/g	Mustard greens	Mustard greens
	-	-	-
	Lettuce <i>Cryptosporidium</i> = 0.01 oocyst/g	Chinese kale <i>Cryptosporidium</i> = 0.03 oocyst/g	Pak choy
	-	-	-
Supermarket B	Cabbage	Cabbage	Pak choy
	-	-	-
	Mustard greens	Mustard greens	Mustard greens
	-	-	-
	Lettuce	Choy sum	Choy sum
	-	-	-
Supermarket C	Cabbage	Chinese kale Hookworm = 0.02 ova/g Nematode = 0.05 larvae/g	Cabbage
	-	-	-
	Mustard greens	Mustard greens Nematode = 0.01 larvae/g	Mustard greens
	-	-	-
	Lettuce	Lettuce	Chinese kale Nematode = 0.10 larvae/g
	-	-	-

Table 1: (Continued)

Market	First sampling visit	Second sampling visit	Third sampling visit
Wet market A	Spinach	Spinach Nematode = 0.01 larvae/g <i>Giardia</i> = 0.01 cyst/g	Water spinach
	-	-	-
	Water spinach	Water spinach Nematode = 0.03 larvae/g	Cabbage
Wet market B	-	-	-
	Lettuce	Lettuce	Mustard greens Nematode = 0.03 larvae/g
	-	-	-
Wet market C	Gain lan	Spinach	Water spinach
	-	-	-
	Mustard greens	Chinese kale Nematode = 0.01 larvae/g	Cabbage
Wet market C	-	-	-
	Lettuce	Lettuce	Mustard greens
	-	-	-
Wet market C	Water spinach Nematode = 0.03 larvae/g	Chinese kale	Spinach Nematode = 0.03 larvae/g
	-	-	-
	Mustard greens	Mustard greens	Lettuce Hookworm = 0.01 ova/g
Wet market C	-	-	-
	Lettuce	Water spinach	Cabbage
	-	-	-
Total = 13/54 positive samples (24.1%)			

Bold denotes positive sample with intestinal parasite(s), '-' denotes negative sample with intestinal parasite(s)

Table 2: Intestinal parasites detected from root-type vegetables purchased from supermarkets and wet markets in this study

Market	First sampling visit	Second sampling visit	Third sampling visit
Supermarket A	Potato	Potato	Potato
	-	-	-
	Carrot	Carrot	Sweet potato Nematode = 0.02 larvae/g
Supermarket B	-	-	-
	Garlic	Ginger	Ginger Nematode = 0.35 larvae/g
	-	-	-
Supermarket B	Potato	Potato	Potato
	-	-	-
	Carrot	Carrot	Carrot
Supermarket C	-	-	-
	Garlic	Ginger	Ginger Nematode = 0.06 larvae/g
	-	-	-
Supermarket C	Potato	Potato Nematode = 0.02 larvae/g	Potato
	-	-	-
	Carrot	Carrot <i>Cryptosporidium</i> = 0.02 oocyst/g	Carrot
Wet market A	-	-	-
	Ginger	Ginger Nematode = 0.01 larvae/g	Ginger
	-	-	-
Wet market A	Potato	Potato	Potato
	-	-	-
	Turmeric	Sweet potato	Carrot
Wet market A	-	-	-
	Ginger	Ginger Nematode = 0.02 larvae/g	Ginger Nematode = 0.13 larvae/g
	-	-	-

Table 2: (Continued)

Market	First sampling visit	Second sampling visit	Third sampling visit
Wet market B	Potato	Potato	Potato
	-	-	Nematode = 0.03 larvae/g
	Carrot	Carrot	Sweet potato
	-	-	-
Wet market C	Sweet Potato	Ginger	Ginger
	-	-	-
	Potato	Potato	Potato
	-	-	-
Wet market C	Carrot	Carrot	Sweet potato
	-	-	-
	Ginger	Sweet potato	Ginger
	Nematode = 0.71 larvae/g	-	Nematode = 0.04 larvae/g
Total = 11/54 positive samples (20.4%)			

Bold denotes positive sample with intestinal parasite(s), '-' denotes negative sample with intestinal parasite(s)

Table 3: Two-sample t-test analysis of mean comparison of parasite concentrations in purchased vegetables from wet markets and supermarkets, and comparison parasite concentration in leafy-type and root-type vegetables

	Two-tailed ($\alpha = 0.05$)
Comparison parasite concentration in vegetables purchased from supermarkets and wet markets	0.324
Comparison parasite concentration in leafy-type and root-type vegetables	0.211

Figure 1: Photomicrographs of detected human intestinal parasites from the purchased vegetable samples in this study. **A:** *Cryptosporidium* oocyst ($5.89 \times 4.69 \mu\text{m}$) positively stained with AquaGlo™ G/C antigen reagent (Waterborne, Inc., USA), **B:** *Cryptosporidium* oocyst as in 'A' that was negative with DAPI, **C:** *Giardia* cyst ($11.04 \times 6.15 \mu\text{m}$) positively stained with AquaGlo™ G/C antigen reagent (Waterborne, Inc., USA), **D:** *Giardia* cyst as in 'C' that was negative with DAPI, **E:** Hookworm ova ($76.03 \times 48.59 \mu\text{m}$), **F:** Hookworm ova ($90.16 \times 49.82 \mu\text{m}$), **G:** Nematode larvae ($388.77 \times 19.68 \mu\text{m}$), **H:** Nematode larvae ($370 \times 50 \mu\text{m}$) and **I:** A group of nematode larvae ($331.16 - 362.25 \times 16.80 - 20.08 \mu\text{m}$).