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Modification of pathogenic microbiota and histology of gastrointestinal tract of *Archachatina marginata* (Swainson, 1821) by *Carica papaya* seed meal

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This study investigated the bactericidal efficacy of the ethanolic extract of *Carica papaya* seed on snail gastrointestinal tract (GIT) microbiota and associated histological changes. The bacterial isolates were characterized based on colony morphology, culture characteristics and biochemical tests. Each portion of the gut was further subjected to histological examination to ascertain the effect of this extract on the various regions of the snail gut. Three bacterial species (*Salmonella*, *Klebsiella* and *Escherichia coli*) were isolated from the GIT. *Salmonella* was the major isolate from all the sections of the GIT in the control. *Klebsiella* was the major isolate from all the GIT sections after administering 50 mg/kg body weight (b. wt) of the extract while *Salmonella* was absent. *Klebsiella* was the main isolate after administering 50 mg/kg, 100 mg/kg and 150 mg/kg of extract. *E. coli* and *Salmonella* and *Klebsiella* were isolated after administration of 150 mg/kg b. wt of extract. The histological changes included vacuolation of the crop, and reduction in sub-mucosal fat in the intestinal wall. The extract altered the microbiota of *A. marginata* GIT in a concentration dependent manner.

Key words: *Archachatina marginata, Salmonella, Klebsiella, Escherichia coli,* antibacterial.

INTRODUCTION

Carica papaya (pawpaw) is a commonly consumed fruit in Nigeria. The leaf has been widely used in Nigeria and several parts of the world in traditional medicines (Awais, 2008; Nirosha and Mangalanayaki, 2013; Orhue and Momoh, 2013). In recent times attention has been drawn to ripe and unripe *C. papaya* seed as being medicinal. Recently, reports of their antimicrobial and antifungal properties against common human microbes and plant fungi were made (Dawkins et al., 2003; Akujobi et al., 2010; Chávez-Quintal et al., 2011; Singh and Ali, 2011; Eke et al., 2014; Peters, 2014). In an entirely different contest, deleterious effects of extracts of seed of *C. papaya* from ripe and unripe fruits were made. Azoospermia, degeneration of epididymis and reduced gonad development (reduced fecundity and gonadosomatic index) were among *C. papaya* extact associated negative consequences (Lohiya et al., 2002; Abdelhak et al., 2013; Madan, 2013).

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In another contest not related to fertility, Adeneye et al. (2009) and Naggayi et al. (2015) reported hepato-protective and nephroprotective activities respectively of aqueous *C. papaya* seed extracts. These observations were contradicted by studies made by Dikibo et al. (2012) and Paul and Ligha (2015) where hepatotoxicity was associated with ethanolic and hydromethanolic *C. papaya* seed extracts respectively. Thus, further *in vitro* and *in vivo* studies to harmonize, where possible, these differences in observations are needed.

In Nigeria *C. papaya* trees are common sites. They flourish well adorned with fruits as mark of fertile soil in Nigeria. The full potential of these fruits are not fully utilized as several are allowed to rotten on the tree. Large quantities of the fruit are seen in waste dumps near fruit market because of lack of appropriate storage facilities in Nigeria. This creates the need for innovative strategies to adequately curtail such enormous waste in a nation where food sufficiency seems an intractable challenge, per capita income deplorable and need for diversification of national revenue sources overwhelming. These problems are further complicated by the dwindling oil revenue of the nation (Uzonwanne, 2015). Building storage facilities may not be feasible due to multiple financial commitments of the Federal Government of Nigeria. The use of the seeds of *C. papaya* fruits in traditional medicine and the incorporation of the whole fruit into snail feed may be a useful alternative.

Archachatina marginata (Swainson, 1821), a giant African land snail is widely consumed in Nigeria. Advocacies for snail farming as an alternative source of protein to help alleviate the problems of nutritional deficiencies in Africa has helped enhance snail farming in recent times (NAERLS, 1995; Agbogidi and Okonta, 2011; Afolabi, 2013). Thus, this study had two core objectives which were to (i) investigate the effect of the ethanolic extract of ripe *C. papaya* seed on the gastrointestinal microbiota of *A. marginata*; (ii) and ascertain histological changes of the gastrointestinal tract associated with the extract.

MATERIALS AND METHODS

Collection of samples

The snails used for the study were purchased from Ibagwa and Orba, Nsukka Local Government Area, Enugu State, Nigeria, and transported to the Animal and Breeding Unit, Department of Zoology and Environmental Biology, University of Nigeria, where they were allowed to acclimatize for 2 weeks.The *C. papaya* seeds used for this investigation were collected from ripe pawpaw fruits purchased within the Nsukka metropolis. The seeds collected were dried at room temperature until constant weight was attained.

Preparation of ethanolic extract

After drying, the seeds were ground and weighed. The powdered product was extracted using absolute ethanol in a ratio of 1: 4 (that is, 100 g of crude seed extract in 400 ml of ethanol). Extraction lasted for 24 h and Soxhlet apparatus was used. The extract obtained was filtered off with a muslin cloth; and the filtrate collected and evaporated to dryness. LD_{50} test was carried out, and the sub-lethal ranges that could be used for the experiment determined.

Experimental design

A completely randomized design comprising four treatment groups (A - D), each in triplicates. Each triplicate contained 8 snails placed in one-third sand filled lidded experimental basket. Group A served as control, feed only normal snail feed. The snail in groups B, C and D were, in addition to normal feed, administered 50, 100 and 150 mg/kg body weight respectively of ethanolic *C. papaya* seed extract for 21 days. Extract administration was by oral injection using a syringe aided by an improvised tube used to gently keep the mouth open and extract injected through it. Three snails were randomly selected from each triplicate, sacrificed and the gastrointestinal tract used.

Preparation for and dissection of snail specimen

Prior to examination, the shells were carefully removed and the snail body washed with Elizabeth leaf (*Chromolaena odorata*) collected from Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. This leaf was used to remove slime from the snail before dissection.

The snails were dissected using the methods of Segun (1975). The alimentary canal was sectioned into buccal cavity-oesophageal zone, crop, stomach and intestine. Each region was homogenized separately in normal saline for the bacterial work and formal-saline for the histopathology examination.

Isolation and identification of microorganisms from *Archachatina marginata* gastrointestinal tracts

Each portion of the gut were streaked onto MacConkay's agar plates, using inoculating loop and incubated for 18 - 24 hours at 37°C for bacterial growth. The bacterial isolates were characterized based on colonial morphology, cultural characteristics and biochemical tests as described by Oyeleke et al. (2012). The isolates were identified by comparing their characteristics with those of known taxa using the Bergey's manual of determinative bacteriology (Holt et al., 1994). Four different agar media were used viz: MacConkay's, EMB, Simmon citrate and urease. The MacConkay's agar medium was used to test for lactose and non-lactose fermenters, in each region of the gut. The EMB agar medium was used to test for the presence of *Klebsiella* and as a confirmatory test for *E. coli*. A positive *E. coli* result gives a greenish metallic sheen on the agar plate. Simmon citrate agar medium was used to test for the presence of *Klebsiella* and as a confirmatory test for *Salmonella*. Urease agar medium was used to test for *Proteus enterobacter* and as a confirmatory test for *Klebsiella*. Gram staining was used to further categorize isolates.

Histopathology examination

Each portion of the gut was subjected to histopathological study to ascertain the effect of this extract on the various regions of the snail gut wall. Ten percent (10%) neutral formalin and Carnoy's solution were used as fixatives. Paraplast embedded tissues were sectioned at 5, 8, and 10 microns and stained with Harris' hematoxylin and

Table 1. Bacterial isolates from the gastrointestinal tract of *Acrchachatina marginata.*

GI Sections	MacConkay's	Gram staining	EMB	Simon citrate	Urease	Organism*
Control						
B-Oesophageal	LF	-ve rods	-	+	-	*Salmonella*
	NLF	-ve rods	-	+	-	*Salmonella*
Crop	LF	-ve rods	-	+	-	*Salmonella*
	NLF	-ve rods	-	+	-	*Salmonella*
Stomach	LF	-ve rods	-	+	-	*Salmonella*
	NLF	-ve rods	-	+	-	*Salmonella*
Intestinal	LF	-ve rods	-	+	-	*Salmonella*
50 mg/kg body weight						
B-Oesophageal	LF	-ve rods	-	+	+	*Klebsiella*
	NLF	-ve rods	-	+	+	*Klebsiella*
Crop	LF	-ve rods	-	+	+	*Klebsiella*
	NLF	-ve rods	-	+	+	*Klebsiella*
Stomach	LF	-ve rods	-	+	+	*Klebsiella*
Intestinal	LF	-ve rods	-	+	+	*Klebsiella*
100 mg/kg body weight						
B-Oesophageal	LF	-ve rods	-	+	+	*Klebsiella*
Crop	LF	-ve rods	-	+	+	*Klebsiella*
	NLF	-ve rods	-	+	+	*Klebsiella*
Stomach	LF	-ve rods	-	+	+	*Klebsiella*
Intestinal	LF	-ve rods	-	+	-	*Salmonella*
150 mg/kg body weight						
B-Oesophageal	LF	-ve rods	-	+	+	*Klebsiella*
Crop	LF	-ve rods	+	-	-	*E. coli*
	NLF	-ve rods	-	+	+	*Klebsiella*
Stomach	LF	-ve rods	-	+	-	*Klebsiella*
Intestinal	LF	-ve rods	-	+	-	*Salmonella*
	NLF	-ve rods	+	+	+	*E. coli*

-ve = negative, LF = Lactose fermenter, NLF = Non-lactose fermenter, B-oesophageal = buccal cavity-oesophageal section GI = gastrointestinal. + = present, - = absent. *main prevalent bacteria

eosin or Masson's trichrome. Fresh frozen, calcium-formol post-fixed cryostat sections were also stained with Harris' hematoxylin for histological reference.

RESULTS

Microbial Isolates

Salmonella was the main bacterial isolate from the gastrointestinal tract of the snails fed only normal feed (control group) (Table 1). Lactose and non-lactose fermenters were isolated from the buccal-oesophageal region, crop and stomach of the control, while only lactose fermenters was isolated from the intestine. *Klebsiella* was the major isolated from the gastrointestinal tract of the snails administered 50 mg/kg b.wt of *C. papaya* seed extract in addition to normal snail feed. Non-lactose fermenters were only isolated from the buccal-oesophageal region and crop in the 50 mg/kg b.wt treatment group.

Klebsiella and *Salmonella* were two major bacteria isolated from snails administered 100 mg/kg body weight of *C. papaya* ethanolic seed extract (Table 1). *Klepsiella* was the major isolate from the bucal-esophageal region to the stomach while lactose fermenting *Salmonella* were isolated from the intestine. Non-lactose fermenters were absent in all section in this group except in the crop. The diversity of major bacterial species isolates was highest in the snails administered 150mg/kg body weight of the extracts. *Klebsiella* was the major species isolated from

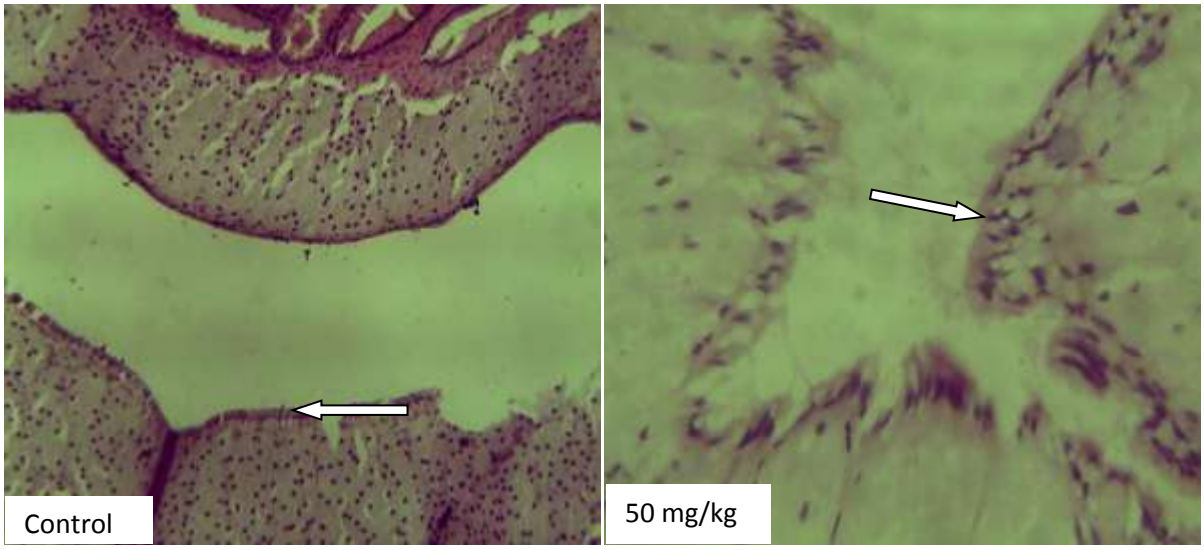

Figure 1. Histological section of the crop of the control and snails administered 50 mg/kg body weight of *C. papaya* seed extract (Magnification x 400). The epithelium of control was normal (white arrow) while that of snails administered 50 mg/kg b.wt of extract showed little vacuolation (white arrow).

the buccal cavity-oesophageal region, crop and stomach; lactose fermenting and non-lactose fermenting *E. coli* were the major in the crop and intestine respectively.

Histological sections

Notable changes in the histology of the gastrointestinal tract were observed in the crop and the intestinal sections (Figures 1 and 2). Slight vacuolations were observed in the cells lining the crop for snails administered 50, 100 and 150 mg/kg body weight of the extract. The layer of sub-mucosal fat thickness decreased in the intestinal wall of the snails administered 150 mg/kg body weight of *C. papaya* seed extract.

DISCUSSION

C. papaya is among the over 10,000 plants known to possess medicinal properties. Extracts of the leaf, seed, stem bark and fruit peels of *C. papaya* have been reported as possessing antimicrobial, antifungal and hepatoprotective potencies. Methanolic, ethanolic and aqueous seed extracts of *C. papaya* exhibited antifungal (Chávez-Quintal et al., 2011; Singh and Ali, 2011) and antimicrobial activities (Dawkins et al., 2003; Akujobi et al., 2010; Eke et al., 2014; Peters, 2014). Chávez-Quintal et al. (2011) reported antifungal activities of ethanolic seed extracts against *Fusarium* sp. and *Colletotrichum gloeosporioides*, even though ethanolic leaf of *C. papaya* was more potent. *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Enterococcus faecalis* and *Salmonella typhi* were among the susceptible microbes. The potency of *C. papaya* seed as antimicrobial as shown from this study in *A. marginata* may be ascribed to its phytochemical constituents. Phytochemicals analysis by Chávez-Quintal et al. (2011) detected high concentrations of saponins and minute concentration of alkaloid and tripertenes in ripe *C. papaya* seed. Similarly, Eke et al. (2010) isolated saponins, flavonoids and alkaloids from unripe *C. papaya* seed. The types and percentage composition of phytochemicals in vegetal determines its efficacies as antimicrobial. Possibly, the low concentrations of alkaloids contributed to the reduced efficacy of ethanolic extract of ripe *C. papaya* seed as antimicrobial against gastrointestinal microbiota of *A. marginata*. This is as alkaloids are known to be effective as antimicrobial. A similarly opinion was shared by Chávez-Quintal et al. (2011), who investigated *in vitro* the efficacy of ethanolic extract of ripe *C. papaya* against fungi.

Susceptibility to antibiotics and antimicrobial extracts is relative to species and strains of microbes. While some strains and species are completely susceptible some are equally resistant. Structural architecture of microbes, biochemical constituents, genetic modifications and reproductive efficiency are among factors that determines an organism's response to unsuitable environments such as that presented by antimicrobial. The susceptibility of *Salmonella* in *A. marginata* administered 50mg/kg b. wt of the extract compared *Klebsiella* was probably due to resistant of *Klebsiella* to the extract. In human, *Klebsiella pneumoniae* is presently one of the microbes of global concern as a result of acquired antibiotic resistant (Barker, 1999; Fair and Tor, 2014; Calballero et al., 2015). The

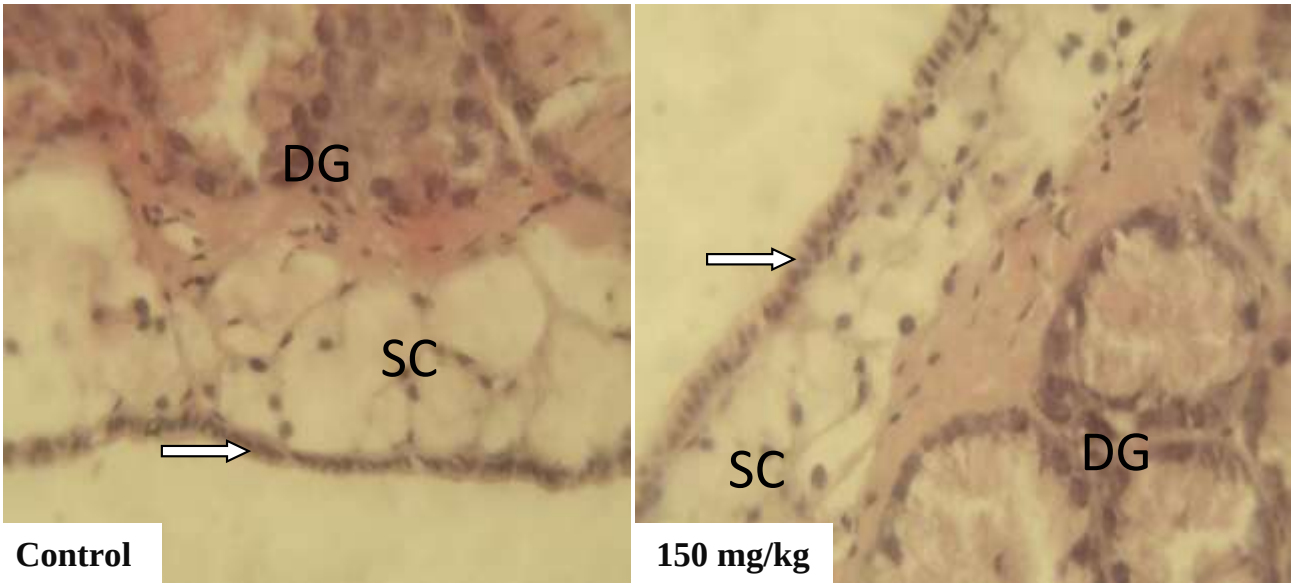

Figure 2. Photomicrograph of a longitudinal section of the intestine of the control and 150mg/kg body weight groups showing the intestinal wall (arrow) lying on typical digestive gland (DG). (Magnification x 400). The layer of sub-mucosal (SC) fat thickness decreased in snail administered 150 mg/kg b. wt of C. papaya compared to control.

inconsistencies in response of *Salmonella* as was observed by its survival at higher concentrations of the extract (100 and 150mg/kg b. wt), however, negates the notion of susceptibility. A susceptible species to a low concentration of an extract ought to remain susceptible at higher concentration even with tendency for increased susceptibility as extract concentration increased (Adetunji and Salawu, 2010; Aruljothi et al., 2014; Peters, 2014). *Salmonella* was susceptible to 100 and 150 mg/kg b. wt concentrations of *C. papaya* seed but not as much as was noticed for 50 mg/kg b. wt. Possibly, the absence or low concentration of antimicrobial phytochemicals such tannins and alkaloid as has been reported by previous studies (Chávez-Quintal et al*.,* 2011; Eke et al., 2014) may have contributed to this, such that response of microbe was irrespective of concentration of extract used. Differences in strains of *Salmonella* may have also contributed to resistance.

Histological modifications associated with *C. papaya* seed extracts are contradictory. Adeneye et al*.* (2009) reported hepatoprotective activities of aqueous extract of *C. papaya* seed in Wistar rats orally fed. Compared to the negative control that received carbon tetrachloride (CCl_4) without *C. papaya* seed extract that had severely congested hepatic central vein, improvement were noticed in terms of moderate to mild decongesting of this vein in those treated with concentrations of *C. papaya* in duration dependent manner (Adeneye et al*.,* 2009). Also, aqueous extract of *C. papaya* was observed to ameliorate the effect of paracetamol induced neprotoxicity in rats (Naggayi et al., 2015). Contrasting observations were made by Dikibo et al. (2012) and Paul and Ligha (2015), who observed hepatotoxic histological changes in Wister rats administered ethanolic and hydromethanolic extracts of *C. papaya* seed respectively; microvascular steatosis, ballooning necrosis of hepato-cytes, pyknosis, parachymal erosion, hemorrhages, mild vacuolation and embolism were some histological pathological changes noted from their study. We also noticed vacuolation and sub-mucosal fat reduction in the intestinal wall of *A. marginata* administered 50, 100 and 150mg/kg b. wt of *C. papaya* ethanolic seed extract. These pathologic histological changes probably resulted from the high saponins content of the seed extract. However, further studies may be needed to conclusively ascertain histological changes associated with *C. papaya* seed extract consumption as antimicrobial. Possible, the extraction solvent which determines the extraction products determine toxic or non-toxic potentials of *C. papaya* seed extracts. More so, anti-fertility activity of the powdered *C. papaya* seed meal in *Oreochromis niloticus* has been reported (Abdelhak et al., 2013). Chloroform and ethanolic extracts of *C. papaya* seed induced reversible azoospermia in langur monkey (Lohiya et al*.*, 2002) and albino rats (Madan, 2013). Toxic potencies of ethanolic extract of *C. papaya* seed was observed when it was used as molluscicide against schistosome intermediate hosts, *Biomphalaria pfeifferi* and *Bulinus globosus* (Adetunji and Salawu, 2010).

C. papaya ethanolic seed extracts possess antimicrobial activities against some gastrointestinal microbes of *A. marginata*. The *in vivo* use of this extract as antimicrobial and their consumption as food supplement require further studies, however, as it may possess some toxic

properties.

Conflict of interests

The authors have not declared any conflict of interests.

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