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Subchronic toxicity studies of cocoa pod husk pectin intended as a pharmaceutical excipient in Sprague Dawley rats

[Estudios de toxicidad subcrónica de la pectina de la cáscara de la vaina de cacao destinada a ser un excipiente farmacéutico en ratas Sprague Dawley]

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Abstract

Context: Excipients play a key role in the quality of medicines and contribute to viable delivery systems**.** This has intensified the search for new natural polymer pharmaceutical excipients. Cocoa pod husks (CPHs) are a rich source of pectin. A study of CPH pectin showed that it possesses the requisite physicochemical properties to be employed as a multi-functional pharmaceutical excipient. However, the safety of this natural polymer has not been evaluated.

Aims: To conduct sub-chronic toxic effects of CPH pectin in Sprague Dawley rats to assess its safety as a pharmaceutical grade excipient.

Methods: CPH pectin at doses of 0.714, 7.14, and 71.4 mg/kg were administered to male and female Sprague-Dawley rats by oral gavage over a 90-day period. Parameters assessed were food and water intake, urinalysis, serum biochemistry, wet organ weights, histopathology and pentobarbital-induced sleeping time.

Results: CPH pectin at the orally administered doses had no significant effects on feed and water intake nor on biochemical parameters, except elevations in alkaline phosphatase at the medium and high dose in the female rat. There were also reductions in creatine kinase in both male and female rats at the medium dose after 60 days, suggesting a potential cardioprotective effect of CPH pectin.

Conclusions: There were no adverse effects of CPH pectin on the kidneys, wet organ weights and histopathology of the rat tissues. Subchronic administration of cocoa pod husk pectin therefore, has no significant toxic effects.

Keywords: histopathology; oral gavage; pectin; pharmaceutical excipient; *Theobroma cacao*.

Resumen

Contexto: Los excipientes desempeñan un papel clave en la calidad de los medicamentos y contribuyen a sistemas de administración viables. Esto ha intensificado la búsqueda de nuevos excipientes farmacéuticos de polímeros naturales. Las cáscaras de vainas de cacao (CPH) son una rica fuente de pectina. Un estudio de CPH pectina mostró que posee las propiedades fisicoquímicas requeridas para emplearse como un excipiente farmacéutico multifuncional. Sin embargo, la seguridad de este polímero natural no ha sido evaluada.

Objetivos: Evaluar los efectos tóxicos subcrónicos de la pectina CPH en ratas Sprague Dawley para valorar su seguridad como un excipiente de grado farmacéutico.

Métodos: Se administró pectina CPH a dosis de 0,714; 7,14 y 71,4 mg/kg a ratas Sprague-Dawley machos y hembras por sonda oral durante un período de 90 días. Los parámetros evaluados fueron la ingesta de agua y alimentos, el análisis de orina, la bioquímica sérica, el peso de los órganos húmedos, la histopatología y el tiempo de sueño inducido por pentobarbital.

Resultados: La pectina CPH, en las dosis administradas por vía oral, no tuvo efectos significativos en la ingesta de alimento y agua ni en los parámetros bioquímicos, excepto las elevaciones de la fosfatasa alcalina a la dosis media y alta en las ratas hembras. También hubo reducciones en la creatina cinasa en ratas machos y hembras en la dosis media después de 60 días, lo que sugiere un posible efecto cardioprotector de la pectina CPH.

Conclusiones: No hubo efectos adversos de la pectina CPH en los riñones, el peso de los órganos húmedos y la histopatología de los tejidos de la rata. Por lo tanto, la administración subcrónica de la pectina de la cáscara de la vaina de cacao no tiene efectos tóxicos significativos.

Palabras Clave: excipiente farmacéutico; histopatología; pectina; sonda nasogástrica; *Theobroma cacao*.

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INTRODUCTION

Effective treatment of disease can be realized from suitable pharmaceutical formulations derived from a combination of the active drug substance and essential additives/ excipients (Heer and Swami, 2013). The performance of these excipients is crucial as they optimize the therapeutic effect of the active drug ingredients. Consequently, excipients play a key role in the quality of medicines and contribute to viable delivery systems. Excipients need to be evaluated for their pharmaceutical quality and safety before use (Abdellah et al., 2015). The traditional concept of the excipient as any component other than the active principles, has changed from an 'inert' and cheap vehicle to an essential constituent with multi-functional roles in formulation and drug delivery (Karolewicz, 2016).

Developments such as advances in drug delivery technologies, the growing unmet needs in pharmaceutical formulation, the need for compatible excipients with innovative active principles being developed, as well as patients' needs have intensified the search for novel pharmaceutical grade natural polymers (Hamman and Steenekamp, 2012). Some of the challenges in excipient research and development are the regulatory restrictions in the approval of new synthetic excipients. Research in natural polymers, suitable for use as excipients and their pharmaceutical applications has generated growing interest and attention in recent times. These natural polymers are economical, readily available, and amenable to modifications, biocompatible, biodegradable, renewable nature and have an eco-friendly profile (Ogaji, 2012).

Pectins are polysaccharides or polymers of natural origin. They contain linear chains of (1, 4)-linked α-d-galacturonic acid residues, with methyl esters of uronic acid. They are useful as emulsifiers, stabilizers, thickeners, and gelling agents in the food industry (Chan and Choo 2013). Pectin has been extensively studied as a functional excipient in pharmaceutical formulation for oral and controlled drug delivery (Sriamornsak, 2011). There are some pharmaceutical excipients originating from the food industry, which are generally regarded as safe (GRAS). However, there are concerns about excipients, regarded as food additives with preservative action, which are associated with hepatotoxicity (Helal et al., 2017), nephrotoxicity (Al-Gayyar et al., 2016) and pulmonary toxicity (Pilcer and Amighi, 2010). Previous reports of excipients associated with cardiotoxicity and damage to major organs have been observed (Souza et al., 2014). Hence, the structure and function of an organ could be a toxicological target following exposure to certain excipients. For example, the cardiovascular, respiratory, renal and hepatic systems are most susceptible to toxic injury. Factors influencing the response of an organ to a toxicant include the disposition of the toxicant in the body e.g. physicochemical properties, metabolic fate and metabolites of the substance and the ability of the organ involved to repair itself after the induced damage (Hock, 2013). Pharmaceutical excipients are to be subjected to the same toxicological evaluations as required for active drug substances. This in principle should be applicable to excipients including food additives, even though they are incorporated into formulations at low concentrations (Abdellah et al., 2015). It is therefore necessary to evaluate the safety profile of promising excipients that are under development (Baldrick, 2010).

Cocoa pod husk pectin is extracted from the waste generated after the recovery of cocoa beans from the cocoa (*Theobroma cacao* L., family *Sterculiaceae*) fruit in the form of cocoa pod shells or cocoa pod husks (Chan and Choo, 2013). The cocoa pod husks, which constitute about 52-76% of the cocoa fruit are considered as waste, an environmental menace and are largely unexploited, but is a rich source of pectin (Vriesmann et al., 2011).

Previous studies indicate that CPH pectin has an ash value of 1.0%, a moisture content of 0.19 ± 0.06%, swelling index of 357.3 ± 4.6, 274.7 ± 4.6 and 360.0 ± 0.0 in 0.1 M HCl, phosphate buffer pH 6.8 and distilled water, respectively. The degree of esterification of 26.8 ± 2.5% was indicative of a low methoxyl pectin. Morphological characteristics on examination showed irregular shapes, non-uniform sizes and rough surfaces. This was indicative of the ability of CPH pectin to entrap drug particles in the pores resulting in modified, retarded or sustained drug release (Adi-Dako et al. 2016). A study by Yapo and Koffi (2013) indicated that CPH pectin has an intrinsic viscosity of 162-304 mL/g and an average

molecular weight of 43-82 kDa under the specified experimental conditions.

CPH pectin has been shown to have the requisite physicochemical properties for use as a pharmaceutical excipient, nutraceutical agent and antibacterial preservative in the pharmaceutical, food and allied industries (Adi-Dako et al., 2016). However less is known about its safety in long term administration. This study seeks to evaluate the toxicity profile of CPH pectin, intended to be a pharmaceutical excipient, in pharmaceutical formulations in the treatment of diseases requiring long term continuous administration, in Sprague Dawley rats.

MATERIAL AND METHODS

Materials

Pentobarbital (Sigma Aldrich, U.S.A), Elitech Clinical Systems Test kits (France): albumin, total protein, aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), creatine kinase (CKMB), creatinine and urea. CPH pectin (moisture content: 0.19%; ash value: 1%; degree of esterification: 26.8%) extracted from minced fresh cocoa pod husks by hot aqueous extraction and freeze dried at 0-120 mBar and -41°C (Labconco, USA) as previously reported (Vriesmann et al., 2011; Adi-Dako et al., 2016) was used for the study.

Animal husbandry and maintenance

Healthy male and female Sprague Dawley rats (SDRs), approximately six weeks old were obtained from the Animal House of the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem in the Eastern Region of Ghana. The animals were housed in stainless steel wire mesh cages with soft wood shavings and with feed from Agricare Co. Ltd., Accra, Ghana. The animals were supplied with water *ad libitum*. The proposal was granted approval (CPM/A95/SF145) by the CPMR Committee for Animal Care and Use. The study was conducted in accordance with CPMR guidelines as well as the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (CNR, 2010).

Animal grouping and treatment

The study protocol was carried out according to the OECD guidelines, as described by Afiriyie et al. (2013), and Amoateng et al. (2016). Twenty-four male SD rats were grouped into four experimental groups of six animals each. This was repeated for twenty-four female SD rats, with four experimental groups of six animals each. It was ensured that the female rats were nulliparous and non-pregnant. The control (Group A) was administered distilled water orally at 2 mL/200 g/ daily. Moreover, the study was conducted based on an earlier report, by Adi-Dako et al. (2017), indicative of the optimal quantity of CPH pectin capable of eliciting the desired modified release profile for treatment of disease.

The second set of test animals (Group B) was given 0.714 mg/kg, the third set (Group C) was given 7.14 mg/kg, and the last set (Group D) was given 71.4 mg/kg of CPH pectin, at a dose volume of 2 mL/200 g, by oral gavage, respectively. All the animals received treatment for the 90-day period. The animals were observed daily for any clinical signs of toxicity, or abnormality (Afriyie et al., 2013; Amoateng et al., 2016; Adi-Dako et al., 2017).

Food and water intake

Measurement of food and water intake were performed by placing animals in each experimental group in metabolic cages at the start of treatment. The amount of food and water was calculated and supplied. The remaining was measured the next day to determine the differences, which indicated their daily intake.

Urinalysis

Urinalysis was performed on fresh urine sample collected from each animal on days 30, 60 and 90, respectively and urine volume determined.

Urinalysis for glucose, bilirubin, ketone bodies, specific gravity, pH, proteins, uribilinogen, nitrite and leucocytes in the urine were determined using standard procedures with a commercial kit, URIT 10V reagent strips (Urit Medical Ltd, China) (Afriyie et al., 2013).

Serum biochemistry

Blood samples were collected from the tail vein into gel separator tubes and were centrifuged at 3000 rpm for ten minutes after which the resulting serum was then stored at -20^{0}C and used for analy-

sis the next day. The analysis involved the AST, ALT, ALP, CKMB, total protein, albumin, creatinine, and urea determination with a Microtech 3000 analyzer, (Vital Scientific, Netherlands). This was done for day 0, and every 30 days over the 90-day period. The analysis was conducted in accordance with an earlier study with slight modification in the scheduled of timing for analysis (Liu et al., 2017).

Gross pathology

At the end of the study period, the rats were euthanized and immediately autopsied. All visible tissues and organs were macroscopically examined, harvested and stored in 10% formalin. Gross necropsy and post mortem examinations were conducted.

Wet organ weights

The wet organ weights relative to the body weights were determined for the heart, liver, lungs, spleen and kidneys at the end of the experimental period.

Histopathology

Tissues from the heart, liver, lung, and kidney were obtained earlier and preserved in 10% formalin. They were routinely processed, embedded in paraffin and sectioned with a Leica rotary microtome at 3-5 um. After this the sections were stained with haematoxylin-eosin for the microscopic examination (Poole and Leslie, 2009).

Pentobarbital-induced sleeping time

Intraperitoneal injections of pentobarbital were administered at 40 mg/kg body weight, in the lower half of the abdomen of the rats. The onset and duration of sleep was timed by the disappearance and return of the "righting reflex". This is the ability of the animal to turn spontaneously to the "on quarters" position when placed on its back. The sleep time in minutes was determined and analyzed (Quaye et al., 2017).

Statistical analysis

Statistical analysis of the data was done using GraphPad prism version 5.03. The results obtained were expressed as mean ± standard error of mean (SEM), n=6. Significant differences between dose groups and controls were evaluated by performing a two-way ANOVA and a one-way ANOVA where necessary. If the test showed significant differences, the Bonferroni or Dunnett's *post hoc* analysis was performed, respectively. P values less than 0.05 were considered as significant values.

RESULTS

There was no mortality associated with the study. Table 1 shows the feed intake pattern of Sprague Dawley rats throughout the 90 Day study period. Feed intake was unaffected by administration of CPH pectin ($p>0.05$). Table 2 shows the water intake of male and female SDRs, which were unaffected by administration of CPH pectin with no remarkable changes ($p>0.05$).

Table 3 shows the results of the effect of CPH pectin administration on biochemical parameters at termination (day 90). Biochemical parameters were unaltered ($p>0.05$), except for the ALP values in the female rats on day 30, at the medium and high doses. Values obtained indicated an isolated significant increment in ALP activity after day 30 for the female rats (Table 4). All other ALP values, for both male and female rats, at all dose levels were unaffected (Table 3). Urea levels showed marginal changes except for the female rats that received the high dose CPH pectin, which showed an isolated increase in urea ($p<0.05$). All other parameters evaluated showed similar results. Table 5 shows the results of the effect of CPH pectin on the clinical chemistry on days 30-90 of serum CKMB activity. An evaluation of CKMB activity showed a general reduction of CKMB levels ($p<0.05$) as compared to the control male and female rats (Table 5). In addition, there were remarkable reductions associated with the medium and high dose groups for both male and female rats after day 60.

Table 6 shows the results of urinalysis conducted at termination of treatment (day 90). Urinalysis parameters remained unaltered throughout the 90-day study ($p>0.05$). There were no traces of leucocytes, urobilinogen, bilirubin, blood, nitrite, glucose and ketones in the urine of both male and female rats. Table 7 shows the percentage organ weight to

body weight ratio for rats at termination of treatment (day 90). The wet organ weight relative to body weight showed no remarkable changes during the 90-day administration of CPH pectin ($p>0.05$) except for the female rats that received high dose CPH pectin (71.4 mg/kg) ($p<0.05$).

Table 1. Feed intake pattern of Sprague Dawley rats throughout the 90-day study period.

Week	Control	CPH pectin (mg/kg)		
		0.714	7.14	71.4
0				
M	11.00 ± 1.46	12.83 ± 1.08	13.00 ± 1.93	14.00 ± 1.00
F	8.33 ± 0.62	7.83 ± 0.54	6.83 ± 0.48	6.83 ± 0.91
4				
M	13.33 ± 0.62	13.33 ± 0.67	14.67 ± 0.62	14.00 ± 0.26
F	13.00 ± 0.23	12.50 ± 0.99	10.33 ± 0.67	12.00 ± 0.52
8				
M	13.50 ± 0.50	12.17 ± 0.79	11.67 ± 0.94	13.00 ± 0.93
F	12.83 ± 0.40	11.5 ± 0.67	10.00 ± 0.37	11.67 ± 0.62
13				
M	12.33 ± 0.33	13.50 ± 0.92	12.67 ± 0.88	14.83 ± 0.79
F	13.33 ± 0.88	10.67 ± 0.33	11.50 ± 0.34	11.17 ± 0.60

Data are presented as mean ± SEM. No significant differences were observed between groups, Bonferroni test, $p > 0.05$ (n=6).M: Male; F: Female; CPH: Cocoa pod husks.

Table 2. Water intake of Sprague Dawley rats throughout the 90-day study period.

Week	Control	CPH pectin (mg/kg)		
		0.714	7.14	71.4
0				
M	14.17 ± 1.38	14.50 ± 1.34	15.17 ± 1.68	17.00 ± 1.37
F	18.67 ± 1.67	14.83 ± 1.80	17.33 ± 1.76	17.33 ± 2.04
4				
M	17.00 ± 0.63	15.83 ± 0.48	17.83 ± 3.46	14.50 ± 0.34
F	21.17 ± 3.67	18.17 ± 3.27	14.33 ± 1.33	17.33 ± 1.15
8				
M	19.17 ± 0.91	17.83 ± 1.56	22.17 ± 5.11	17.83 ± 2.14
F	18.20 ± 2.01	16.00 ± 1.13	12.50 ± 0.92	16.33 ± 2.32
13				
M	19.50 ± 1.65	20.17 ± 2.81	27.83 ± 7.60	21.17 ± 2.02
F	25.00 ± 4.73	17.00 ± 2.11	14.67 ± 0.56	16.50 ± 2.53

Data are presented as mean ± SEM. No significant differences were observed between groups, Bonferroni test, $p > 0.05$ (n=6). M: Male; F: Female; CPH: Cocoa pod husks.

Table 3. Results showing the effect of CPH pectin on biochemical parameters at termination of treatment (day 90).

Biochemical parameter	Control	CPH pectin (mg/kg)		
		0.714	7.14	71.4
Albumin (g/L)				
M	28.60 ± 0.84	29.28 ± 0.67	28.37 ± 1.11	27.59 ± 1.14
F	36.24 ± 3.60	38.66 ± 3.86	37.23 ± 3.39	37.90 ± 3.90
ALP (U/L)				
M	522.83 ± 54.61	448.17 ± 44.57	412.00 ± 37.40	402.33 ± 30.27
F	312.00 ± 21.84	286.00 ± 24.55	262.83 ± 59.18	264.33 ± 29.46
ALT (U/L)				
M	71.50 ± 4.14	65.5 ± 2.79	69.33 ± 6.02	65.17 ± 4.57
F	64.00 ± 2.45	51.50 ± 2.62	49.00 ± 1.71	50.67 ± 3.55
AST (U/L)				
M	170.87 ± 14.51	165.45 ± 9.61	167.38 ± 13.24	181.2 ± 24.40
F	150.52 ± 6.21	134.48 ± 10.61	149.13 ± 9.75	127.22 ± 5.09
Creatinine (µmol/L)				
F	45.87 ± 2.75	41.1 ± 2.28	44.33 ± 3.92	43.00 ± 2.52
M	54.52 ± 4.85	45.42 ± 3.88	53.91 ± 3.83	53.96 ± 0.47
Urea (mmol/L)				
M	4.83 ± 0.25	4.83 ± 0.18	5.07 ± 0.38	5.67 ± 0.27
F	8.37 ± 0.52	7.08 ± 0.40	7.25 ± 0.32	9.92 ± 0.30*
Total protein (g/L)				
M	55.32 ± 4.63	59.65 ± 3.47	62.45 ± 5.72	62.12 ± 1.93
F	92.80 ± 7.05	103.93 ± 6.79	113.52 ± 4.73	118.20 ± 12.60
CKMB (U/L)				
M	1086.70 ± 183.70	1031.20 ± 103.40	974.20 ± 169.30	712.00 ± 120.90
F	576.00 ± 64.03	528.70 ± 73.20	487.30 ± 43.30	471.00 ± 25.20

Data are presented as mean ± SEM. *p < 0.05 significant differences were observed between groups respect to the control group, Bonferroni test, (n=6). M: Male; F: Female; CPH: Cocoa pod husks; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; CKMB: creatine kinase myocardial B fraction.

Table 4. Clinical chemistry evaluation on days 30-90 of serum alkaline phosphatase (ALP) activity for female rats.

Day	Control	CPH pectin (mg/kg)		
		0.714	7.14	71.4
30	378.17 ± 19.92	275.50 ± 41.37	620.17 ± 40.32*	620.83 ± 63.00*
60	460.83 ± 54.24	427.33 ± 68.32	430.67 ± 31.69	453.33 ± 64.06
90	312.00 ± 21.84	286.00 ± 24.55	262.83 ± 59.18	264.33 ± 29.45

Data are presented as mean ± SEM. *p < 0.05 significant differences were observed between groups respect to the control group, Bonferroni test, (n=6). M: Male; F: Female; CPH: Cocoa pod husks.

Table 5. Clinical chemistry results on days 30-90 serum creatine kinase myocardial B fraction (CKMB) activity.

Day	Control	CPH pectin (mg/kg)		
		0.714	7.14	71.4
30				
M	1956.00 ± 229.18	1624.17 ± 198.95	1821.33 ± 98.14	1607.67 ± 98.14
F	659.17 ± 112.11	536.50 ± 59.28	486.33 ± 84.23	473.67 ± 59.63
60				
M	1532.83 ± 160.42	1075.67 ± 104.44	891.83 ± 158.90*	855.83 ± 127.96*
F	922.00 ± 68.73	719.67 ± 62.75	531.50 ± 78.08*	590.67 ± 54.97*
90				
M	1086.67 ± 183.67	1031.17 ± 103.42	974.17 ± 169.28	712.0 ± 120.90
F	576.00 ± 64.07	528.67 ± 73.16	487.33 ± 43.31	471.33 ± 25.17

Data are presented as mean ± SEM. *p < 0.05 significant differences were observed between groups respect to the control group, Bonferroni test, (n=6). M: Male; F: Female; CPH: Cocoa pod husks.

Table 6. Urinalysis results at termination of treatment (day 90).

Parameter	Control	CPH pectin (mg/kg)		
		0.714	7.14	71.4
pH				
M	6.167 ± 0.17	6.17 ± 0.28	6.17 ± 0.28	6.17 ± 0.17
F	6.250 ± 0.17	6.42 ± 0.20	6.00 ± 0.00	6.67 ± 0.21
Sp gravity				
M	1.028 ± 0.001	1.027 ± 0.002	1.027 ± 0.002	1.027 ± 0.002
F	1.028 ± 0.002	1.025 ± 0.003	1.030 ± 0.000	1.023 ± 0.003
Urine output				
M	4.53 ± 0.73	6.67 ± 1.92	7.76 ± 1.83	6.52 ± 1.88
F	12.06 ± 4.17	8.15 ± 2.60	7.15 ± 1.14	7.67 ± 2.24

Data are presented as mean ± SEM. *p >0.05 no significant differences were observed between groups respect to the control group, Dunnett's test, (n=6). M: Male; F: Female; CPH: Cocoa pod husks; Sp gravity: Specific gravity.

Table 7. Percentage organ weight to body weight ratio for rats at termination of treatment (day 90).

CPH pectin (mg/kg)	Organ				
	Kidney	**Spleen**	**Heart**	**Liver**	**Lungs**
0 (Control)					
M	0.586 ± 0.038	0.171 ± 0.015	0.272 ± 0.008	2.94 ± 0.130	0.600 ± 0.050
F	0.611 ± 0.017	0.250 ± 0.011	0.366 ± 0.021	2.73 ± 0.090	0.756 ± 0.029
0.714					
M	0.582 ± 0.018	0.177 ± 0.015	0.278 ± 0.010	2.78 ± 0.130	0.600 ± 0.030
F	0.621 ± 0.016	0.244 ± 0.019	0.377 ± 0.017	2.66 ± 0.064	0.890 ± 0.032
7.14					
M	0.600 ± 0.037	0.175 ± 0.016	0.283 ± 0.014	2.94 ± 0.157	0.597 ± 0.024
F	0.587 ± 0.014	0.205 ± 0.008	0.349 ± 0.014	2.81 ± 0.068	0.842 ± 0.074
71.4					
M	0.596 ± 0.036	0.185 ± 0.017	0.293 ± 0.009	2.95 ± 0.16	0.606 ± 0.034
F	0.535 ± 0.014*	0.177 ± 0.012*	0.307 ± 0.015	2.58 ± 0.017	0.757 ± 0.048

Data are presented as mean ± SEM. *p < 0.05 significant differences were observed between groups respect to the control group, Dunnett's test, (n=6). M: Male; F: Female; CPH: Cocoa pod husks.

Fig. 1 presents sections of lung tissue showing normal alveolar spaces, lined by normal pneumocytes, or alveolar cells, as well as normal bronchioles with projections. There is neither fibrosis nor inflammatory activity. There are normal vessels. Fig. 2 shows sections of tissues from liver with regular hepatocytes radiating from the portal tract, with nuclei. There is neither ballooning nor loss of nuclei, which could progress to fibrosis. There is no ballooning and giant cell formation. Fig. 3 presents sections of tissues from kidney showing regular glomeruli and tubules with their lumen made up of cells with nuclei. The spaces around the glomeruli had no attachment to capsule. Fig. 4 presents photomicrographs of transverse section of normal heart cardiac muscle, showing spindle shaped bodies, with unit cardiomyocytes, with characteristic nuclei and occasional vessels. There is neither fibrosis nor inflammatory activity.

The pentobarbital induced sleeping time, which is indicative of the effect of CPH pectin on microsomal enzymes remained unaltered after the 90 days study (p>0.05) (Table 8).

Table 8. Pentobarbital induced sleeping time of Sprague Dawley rats.

CPH pectin (mg/kg)	Duration of sleep (min)	
	Males	**Females**
0 (Control)	93.17 ± 4.19	190.17 ± 46.50
0.714	89.00 ± 3.34	200.17 ± 21.50
7.14	86.33 ± 2.90	250.33 ± 4.98
71.4	82.83 ± 4.13	228.17 ± 26.82
P value	0.769	0.465

DISCUSSION

The use of animal models in pre-clinical toxicity studies, which are predictive of human effects, are necessary to evaluate the safety or otherwise in the development of new pharmaceutical excipients such as CPH pectin.

Food and water intake could indicate an adverse effect of an excipient at an early stage of its development. Water intake is evaluated for any potential effects of the test material, especially on the kidneys

(Poole and Leslie, 2009). As indicated earlier, reports of reduced food consumption in some toxicity studies involving copolymer excipients, associated with the swelling capacity has been made (Eisele et al., 2013). CPH pectin with similar swelling, and viscous properties did not elicit any treatment related effects associated with food or water intake with the doses administered.

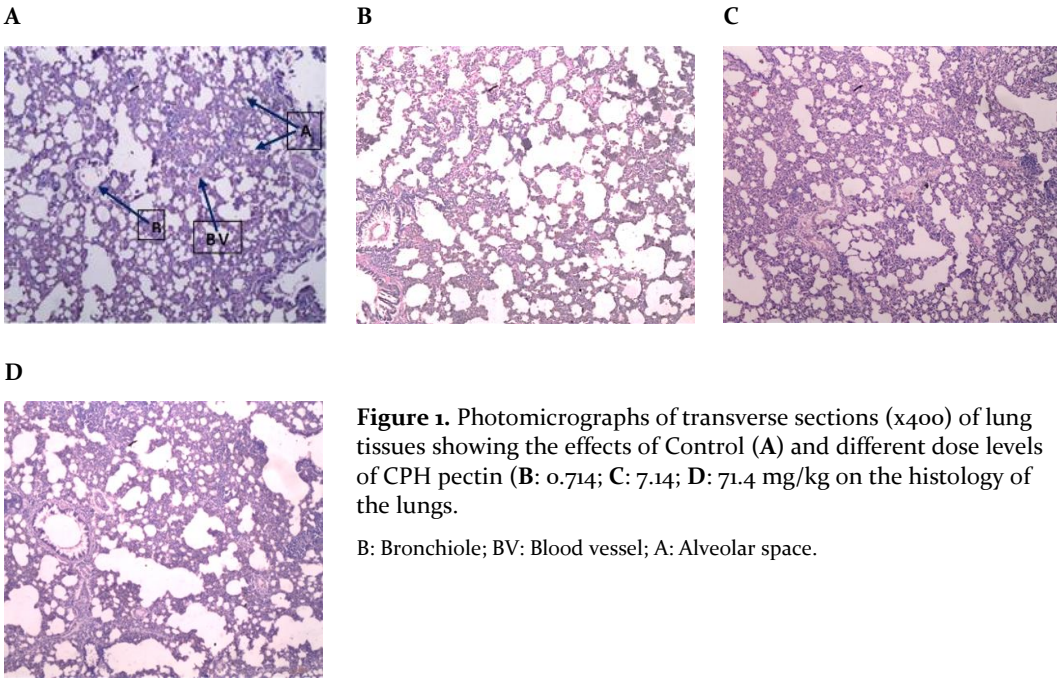

Figure 1. Photomicrographs of transverse sections (x400) of lung tissues showing the effects of Control (**A**) and different dose levels of CPH pectin (**B**: 0.714; **C**: 7.14; **D**: 71.4 mg/kg on the histology of the lungs.

B: Bronchiole; BV: Blood vessel; A: Alveolar space.

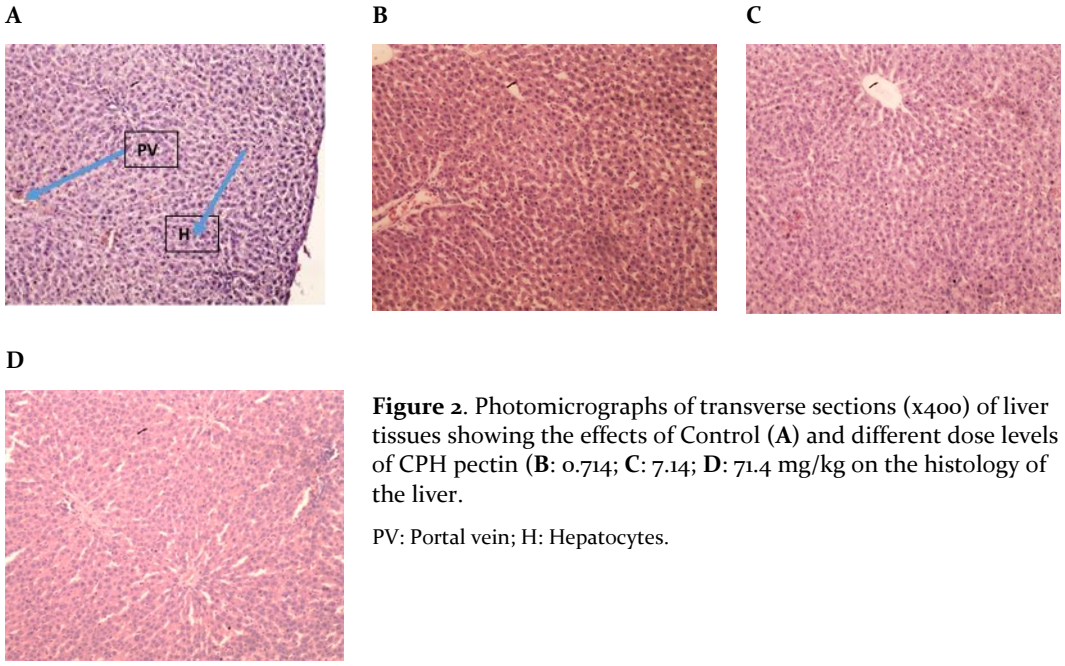

Figure 2. Photomicrographs of transverse sections (x400) of liver tissues showing the effects of Control (**A**) and different dose levels of CPH pectin (**B**: 0.714; **C**: 7.14; **D**: 71.4 mg/kg on the histology of the liver.

PV: Portal vein; H: Hepatocytes.

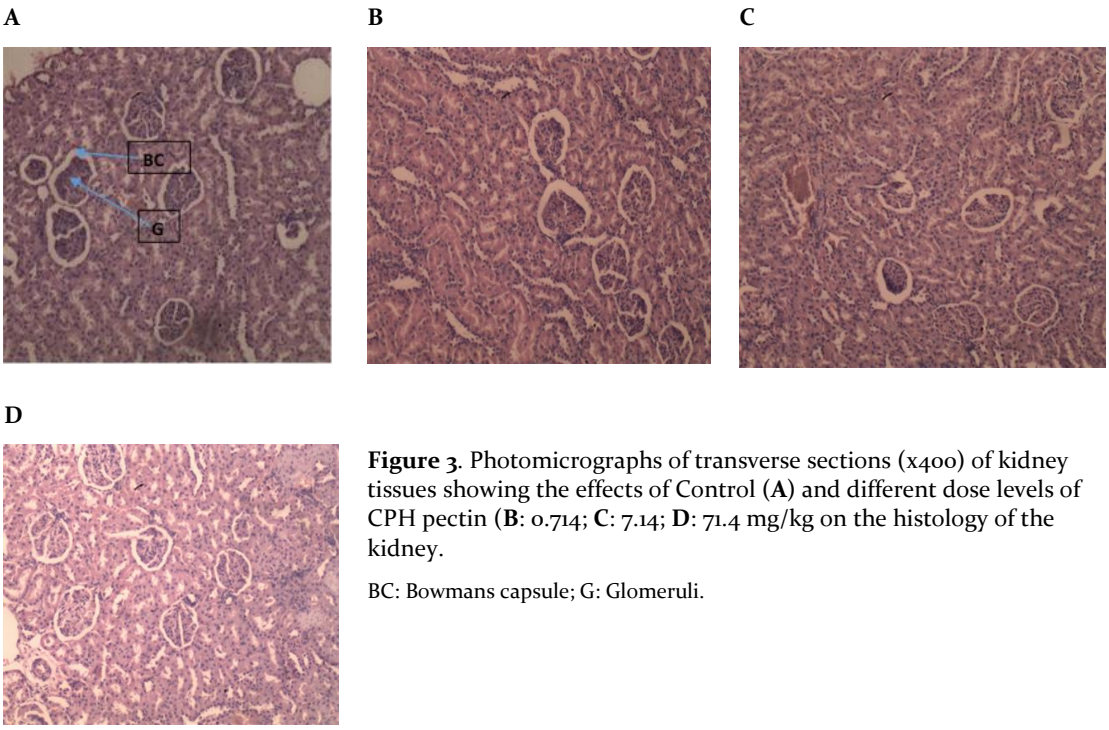

Figure 3. Photomicrographs of transverse sections (x400) of kidney tissues showing the effects of Control (**A**) and different dose levels of CPH pectin (**B**: 0.714; **C**: 7.14; **D**: 71.4 mg/kg on the histology of the kidney.

BC: Bowmans capsule; G: Glomeruli.

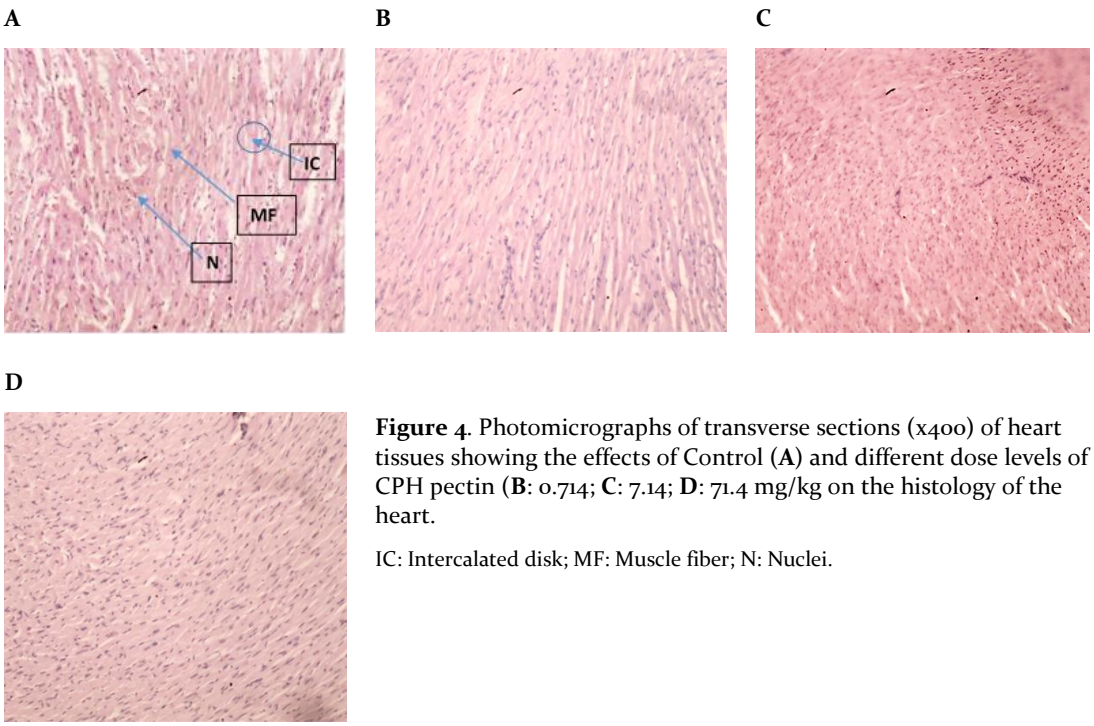

Figure 4. Photomicrographs of transverse sections (x400) of heart tissues showing the effects of Control (**A**) and different dose levels of CPH pectin (**B**: 0.714; **C**: 7.14; **D**: 71.4 mg/kg on the histology of the heart.

IC: Intercalated disk; MF: Muscle fiber; N: Nuclei.

Urinalysis, involving an evaluation of renal metabolites and filtrates, conducted in toxicity studies serves as an indirect measure of kidney damage (Afriyie et al., 2013). There were no significant changes observed with glucose, urobilinogen, bilirubin, red blood cells, nitrite, leukocytes, ketones, proteins, urinary pH and specific gravity and urine output when rats were treated with CPH pectin. In the investigation and monitoring of renal function the measurement of plasma creatinine levels is preferred, in addition to urea. Creatinine is more consistently elevated in glomerular disease and a sensitive indicator of overall renal function progress (Salazar, 2014). Generally, urea levels remained unaffected, though there was an isolated significant increase in urea levels associated with the high dose group after the 90th day in female rats. However, this was not considered to be toxicologically relevant, there was no dose relationship. Creatinine levels were reduced after the 90th day with no dose specific pattern. The renal profile was not adversely affected by the administration of CPH pectin. Previous studies reported that pectin exhibited a therapeutic effect on kidney dysfunction induced by octylphenol exposure resulting in oxidative stress and apoptosis (Koriem et al., 2014).

Hepatocellular damage is observed with elevated activities of ALT and AST and is useful in monitoring patients on drug associated hepatotoxicity. ALP production is increased in response to cholestasis, providing a sensitive indicator of obstructive and space-occupying lesions of the liver (Esani, 2014). There were insignificant reductions in, ALT and ALP activities for all dose levels, with no dose specific pattern, during the 90-day period with CPH pectin administration. There was however a significant increase in serum ALP levels for the medium and high dose groups in female rats after 30 days, with no apparent dose relation. However, this increment was not observed with continuous dosing. The histopathological evaluation of the liver was devoid of any evidence of toxicological relevance. This finding was therefore regarded as spontaneous and of no toxicological significance. AST activity remained unaffected by the administration of CPH pectin.

The CPH pectin extract did not alter total protein and albumin levels, indicating the absence of any disorders with protein synthesis. There was no indication of hepatocellular injury and the findings show no evidence of obstructive jaundice or cholestatic liver disease. Serum activity of creatinine kinase-MB is an indirect biochemical marker of the integrity of cardiac muscle (Baird et al., 2012). A significant concentration of CKMB isoenzyme is found almost exclusively in the myocardium, and the appearance of elevated CKMB activity in serum is highly specific and sensitive for myocardial cell wall injury (Baird et al., 2012). Remarkable reductions in CKMB activity associated with the medium and high dose groups of both male and female rats, after day 60 was observed. This suggests a possible cardioprotective effect, indicative of the absence of cardiac muscle damage. Cardioprotective effects of cocoa have been widely studied (Aprotosoaie et al., 2016). This effect of cocoa pod husk pectin requires further investigation.

Dosing of laboratory animals with various doses of a new drug for a period of time together with histopathological examination of the tissues has withstood the test of time. Hence the evaluation of pathological changes induced in laboratory animals by test compounds is the cornerstone of their safety assessment (Greaves, 2011).

Histopathology reports of some excipients, useful for oral drug formulation have elicited treatment-related incidence of hepatocellular injury after repeated oral dose toxicity studies (Weber et al., 2012). The sections showed features consistent with normal tissue from liver, lung, heart and kidney. There was no treatment or dose-related microscopic pathological changes after administration of CPH pectin.

Moreover, wet organ weight can be the most sensitive indicator of an effect of an experimental compound. Preclinical toxicity experiments involve a comparison of organ weights in treated and untreated animals as a prediction of organ specific toxicity (Wolfsegger et al., 2009). The organ to body weight ratio (%) for the heart, liver, kidney, lungs and spleen was unaffected in the male SDRs. Generally, wet organ weights remained unaltered after

the 90-day study, except for the kidney and spleen of the female high dose group, which would require further investigation. Organ weight data is usually interpreted in an integrated manner, with the gross pathology, clinical pathology and histopathology findings (Sellers et al., 2007). This observation was not considered to be of any toxicological significance as the changes were not associated with the findings of biochemical, gross and clinical pathology and histopathological examination of the spleen and kidney. There was no evidence of cell proliferation or damage associated with treatment.

Drug metabolism by the cytochrome P450 system plays a role in many drug-drug interactions. A prediction of drug interaction is done by the identification of P450 isozymes and some of the drugs that share them. Information about the effects of certain drugs on enzyme-mediated biotransformation has led to the identification of enzyme inducers and inhibitors. Test materials that cause CYP450 metabolic drug interactions are referred to as either inhibitors or inducers. Inhibitors usually block the metabolic activity of one or more CYP450 enzymes. The extent to which an inhibitor affects the metabolism of a drug depends upon factors such as the dose and the ability of the inhibitor to bind to the enzyme. Inducers increase CYP450 enzyme activity by increasing enzyme synthesis (Lynch, 2007). It is reported that the effects of pre-treatment of rats with phenobarbital shortened their sleeping time due to the quantitative increase in liver microsomal enzyme activity, resulting in accelerated drug disposition. Alternatively, a prolonged effect would be observed by the administration of compounds that had an inhibitory effect on the same enzyme systems. The pentobarbital sleeping time is used as an evaluation of the influence of CPH pectin on liver microsomal enzyme activity (Quaye et al., 2017).

There have been reports of hepatocellular and thyroid injury associated with repeated high doses of oral excipients, resulting from hepatic microsomal enzyme induction (Weber et al., 2012). With respect to CPH pectin, there were no significant differences between the control and treated groups for the duration of sleeping time. Consequently, there was no effect on the microsomal enzymes and no potential for drug interactions.

Previous reports have indicated that the complex nature of excipients of natural origin, and their inherent biological variations, warranting the investigation of their safety and quality (Park et al., 2010). However, biological activity of biopolymer excipients like chitosan, exhibited in its hemostatic action and macrophage activation has been exploited in biomedical and wound healing applications (Baldrick, 2010). Although CPH pectin has potential biological activity such as lowering of cholesterol (Brouns et al., 2012), the polymer was also devoid of any adverse effects on food and water intake, biochemical and histopathological parameters evaluated. Pectin is associated with negligible absorption in the gastrointestinal tract and can be too complex to be absorbed (Naqash et al., 2017), suggesting an enhanced safety margin for oral administration.

CONCLUSIONS

There were no treatment related effects on urinalysis, biochemical markers, target organs, food and water intake, wet organ weight, and no abnormal histopathological findings. The duration of treatment with CPH pectin showed no adverse effect on the various parameters. These findings suggest that the safety of CPH pectin in pharmaceutical formulation, in the oral doses administered and under the experimental conditions, for long-term continuous administration is assured.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Concepts or ideas		X					
Design		X					
Definition of intellectual content	X	X	X	X	X		
Literature search	X					X	X
Experimental studies	X			X			
Data acquisition	X			X			
Data analysis	X	X	X		X	X	X
Statistical analysis	X					X	
Manuscript preparation	X	X	X				
Manuscript editing		X	X	X	X		X
Manuscript review	X	X	X	X	X	X	X

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