

ACUTE TOXICITY STUDIES AND PHYTOCHEMICAL CONSTITUENTS OF DIFFERENT SOLVENTS EXTRACTS OF *CARICA PAPAYA* SEEDS

7 ABSTRACT

8 **Aim:** To investigate the effect of five extraction solvents of varying polarity, namely aqueous,
9 methanol, ethyl acetate, chloroform and n-hexane on phytochemicals yield and composition of *Carica*
10 *papaya* seed. The acute toxicity test of each solvent fraction was also carried out and the average
11 weight of rats in each group was measured before and after the experiment.

12 **Methodology:** The phytochemical screening, both qualitative and quantitative was carried out using
13 standard methods and procedures. Acute toxicity study was conducted by determining the LD₅₀ of
14 each extract.

15 **Place and Duration of Study:** Department of Biochemistry Laboratory, Faculty of Basic Medical
16 Sciences, Bayero University Kano, Nigeria, from April 2018 to August 2018.

17 **Results:** The results shows that the higher the solvent polarity, the better the yield of extract thus the
18 extract yield was higher in aqueous followed by methanol, ethyl acetate, chloroform and n-hexane in
19 that order. Furthermore, the phytochemical analyses of all the five extracts of *Carica papaya* seed
20 showed the presence of various compounds. The phytochemicals include flavonoids, alkaloids,
21 tannins, saponins and cardiac glycoside in varying amounts. Anthraquinones was not detected in all
22 the five extracts. The LD₅₀ of the aqueous, methanol, ethyl acetate, chloroform and n- hexane extracts
23 of *Carica papaya* seed in rat was greater than 5000 mg /kg body weight while the results of the weight
24 changes shows that there is no statistically significant ($p>0.05$) difference in weight gain or weight loss
25 in rats in most of the experimental groups administered with either of the five extracts of *Carica*
26 *papaya* seed as compared with the control rats.

27 **Conclusion:** It was concluded that *Carica papaya* seed contain bioactive phytochemicals which yield
28 is highest when extracted with water and that the plant material could have clinical potential with safe
29 therapeutic application.

30 Keywords: Extraction, Phytochemical, Acute toxicity, *Carica papaya* seed

31 1. INTRODUCTION

32 In Nigeria today, most rural communities depend on plant based products for phytochemicals to
33 satisfy medicinal requirements. Plant products are generally considered safe and proven to be
34 effective against various human ailments [1]. The world Health Organization (WHO) reported that
35 more than 80% of the world's populations are believed to be dependent mainly on traditional
36 medicine, which is largely obtained from plant [2]. This upsurge in the use of traditional medicine has
37 given the practice a significant place in healthcare delivery particularly in developing countries and
38 thus has led the WHO to advocate the application of scientific criteria and methods for proof of safety
39 and efficacy of medicinal plants. The two resolutions of the WHO on essential drugs for member
40 states from Africa namely resolutions AFR/RC49/R5 and AFR/RC50/R5 are classical examples aimed
41 at encouraging medicinal plant research and promotion of its use in health care delivery systems [3].
42 Despite this, and many other interventions, data regarding phytochemical characteristics, safety and
43 efficacy of medicinal plants are only available for few plants and these has limited its potentials in
44 drug development by pharmaceutical companies [4].

45 Phytochemical analyses and acute toxicity test are important processes aimed at understanding the
46 nature and safety of medicinal plants and information obtained from these techniques can provide
47 data about plant that have shown to be efficacious. Phytochemicals are extracted from plants using
48 different solvents in processes that are dependent on solvent polarity and solubility of bioactive
49 compounds in the plant material (5). However the safety of the crude extracts cannot be ascertained
50 since plants are known to produce toxic compounds as well [2] thus the need for toxicity analyses. In
51 acute toxicity test, a single oral dose of the test substance is administered to animals to determine the
52 gross behavior and the dose that can cause the death of 50% of the animals, called the LD₅₀. It is
53 usually expressed as the amount of chemical administered (e.g. Milligrams) per 100 g (for small

54 animals) or per kilogram (for bigger subjects) of the body weight of the test animal [6]. LD₅₀ is the first
55 step in the assessment and evaluation of the toxic characteristics of a substance [7].

56 *Carica papaya* is a fast growing tree-like herbaceous plant in the family *caricaceae* with four genera.
57 The genus *Carica linn* is the most common and is the most widely cultivated species [8]. *Carica*
58 *papaya* is believed to have originated from the lowlands of East-Central America [1] but presently
59 grown in all tropical countries and many sub-tropical regions of the world including Nigeria. *Carica*
60 *papaya* is a soft wooden perennial plant that has a life span of about 5-10 years, although commercial
61 plantations are usually replanted sooner. Researchers have reported that *Carica papaya* seed has
62 several therapeutic uses and is being used for centuries in folk medicine across Nigeria. Previous
63 studies have documented anthelmintic activity of *Carica papaya* seed. Sapaat and co-workers
64 reported that over 90% efficacy percentage against *Hymenolepis diminuta* in rats was observed
65 following administration of 1.2g/kg body weight of *Carica papaya* seed [9]. Aqueous extract of *Carica*
66 *papaya* seed at 100mg/ml concentration was reported to have significantly inhibited bacterial activity
67 against *Salmonella typhi* and other bacteria [10]. This research aimed to investigate the effect of
68 different solvents on phytochemicals yield and composition and also determine the LD₅₀ of each
69 extract of *Carica papaya* seed. This is important as it will provide data on the type of bioactive
70 compounds available in the plant material and the most desirable solvent for its extraction. It will also
71 provide toxicity and/or safety information on the plant material

72 **2. MATERIALS AND METHODS**

73 **2.1 Plant Sample and Collection**

74 Forty five matured unripe *Carica papaya* was bought in April, 2016 from Na'ibawa fruit market Kano,
75 Nigeria. Taxonomic authentication of the plant was done by the department of Plant Biology, Bayero
76 University Kano, Nigeria and was given accession number BUKHAN 0012.

77 **2.2 Experimental Animal**

78 Sixty six apparently healthy young male Wistar rats, each weighing between 120-150g were used for
79 the study. The study was carried out at the animal house unit of the department of Biological
80 Sciences, Bayero University Kano, Nigeria. All animal procedures were performed according to the
81 guide for the care and use of laboratory animals of the National Institute of Health as well as the
82 Animal Welfare Act. Prior to the experiment, the animals were acclimatized in the laboratory for one
83 week and were maintained on standard pellet rat diet with free access to water.

84 **2.3 Sample Preparation and Extraction**

85 Each of the *Carica papaya*, was cut to remove the seeds which was washed with tap water, shade-
86 dried and ground into fine powder with an electric blender. Maceration as described by Azwanida [11]
87 was used. 500 g of the powdered dried *Carica papaya seed* was suspended in 1500 ml of each of the
88 five solvents namely n- hexane, chloroform, ethyl acetate, methanol and water for 24 hours and
89 shaken at regular intervals. Each of the extract was then sieved first with cheese cloth and then with
90 Watman filter paper No 1. The filtrate in each case was concentrated to dryness in a water bath
91 preset at 45°C and was kept in the refrigerator at 4°C until required.

92 **2.4 Phytochemical Analyses**

93 Qualitative phytochemical analyses of aqueous, methanol, ethyl acetate, chloroform and n-hexane
94 extracts of *Carica papaya* seed were carried out using standard methods to detect which
95 phytochemical is present (results not shown). In each case, where the presence of a given
96 phytochemical was established, then the amount of that phytochemical was determined quantitatively.
97 The tests are as shown below;

98 **(i) Test for flavonoids**

99 1.0g of each of the five extracts of *Carica papaya* seed was diluted with 200µL of distilled water
100 separately followed by the addition of 150 µL of sodium nitrate (5%) solution. The mixture was then
101 incubated for 5 minutes and 150 µL of ammonium chloride (10%) solution was added and made up to
102 5ml with distilled water. The mixture was shaken well and left for 15 minutes at room temperature.
103 The absorbance was measured at 510nm. The total flavonoids were expressed as rutin equivalent
104 (mg RE)/g extract on a dry weight basis using the standard curve [12].

105 **(ii) Test for alkaloids**

106 A total of 100ml of 20% acetic acid was added to 2.5g of each extract of *Carica papaya* seed in a
107 250ml beaker and covered to stand for 4hours. The mixture was then filtered and the volume reduced
108 to one-quarter using a water bath. A concentrated ammonium hydroxide was then added drop-wise to
109 the sample until the precipitate was complete. The whole solution was then allowed to settle and the
110 precipitate was collected by filtration and weighed [13]. The percentage of total alkaloid content was
111 calculated as;

112 Percentage of total alkaloids (%) = $\frac{\text{weight of residue (g)} \times 100}{\text{Weight of sample taken}}$
113

114 (iii) Test for saponins

115 This was estimated based on vanillin-sulphuric acid colorimetric reaction with some modifications.
116 50µg of each extract was added with 250 µL of distilled water. To this, 250 µL of vanillin (800mg of
117 vanillin in 10 ml of 99.5% ethanol) was added. Then 2.5 ml of 72% sulphuric acid was added and
118 mixed thoroughly and the solution was kept in a water bath at 60°C for 10 minutes after which it was
119 cooled in ice-cold water and the absorbance was read at 544 nm. The values were expressed as
120 diosgenin equivalents (mg DE/g extract derived from the standard curve [12]

121 (iv) Test for tannins

122 500 µg of each of the five extract of *Carica papaya* seed were taken in a test tube separately and
123 treated with 100mg of polyvinyl polypyrrolidone and 500 µL of distilled sample was centrifuged at
124 5000rpm for 5 minutes and 20 µL of the supernatant was taken. This supernatant has only simple
125 phenolics free of tannins (the tannins would have been precipitated along with the polyvinyl
126 polypyrrolidone). The phenolics content of the supernatant was measured at 725 nm and expressed
127 as the content of free phenolics on a dry matter basis. From the above results, the tannin content of
128 the extract was calculated as;

129 Tannins (mg/g) extract) = total phenolics (mg GAE/g extract) – free phenolics (mg GAE/g extract) [14]

130 (v) Test for Cardiac glycosides

131 Cardiac glycosides of each *Carica papaya* seed extract were quantitatively determined according to
132 Solich *et al* and was based on vanillin-sulphuric acid colorimetric reaction with some modifications
133 [14]. 50µg of each extract was added with 250 µL of distilled water. To this, 250 µL of vanillin (800mg
134 of vanillin in 10 ml of 99.5% ethanol) was added. Then 2.5 ml of 72% sulphuric acid was added and
135 mixed thoroughly and the solution was kept in a water bath at 60°C for 10 minutes after which it was
136 cooled in ice-cold water and the absorbance was read at 544nm. The values were expressed as
137 diosgenin equivalents (mg DE/g extract derived from the standard curve [15].

138 (vi) Test for anthraquinones (qualitative)

139 *Carica papaya* seed extracts (0.5 g) were shaken with 5 mL of chloroform. The chloroform layer was
140 filtered and 5.0 cm³ of 10% ammonia solution was added to the filtrate. The mixture was shaken
141 thoroughly and the formation of a pink-violet or red, yellow colour in the ammoniacal phase indicates
142 the presence of anthraquinones [12].

143 2.5 Experimental Design for the Acute Toxicity

144 The acute toxicity study was conducted in accordance with Lorke's method [16]. According to the
145 method, LD₅₀ is given by the square root of the highest dose that did not kill, multiplied by the lowest
146 dose that killed. The first stage involved the oral administration of three different doses of 10, 100 and
147 1,000 mg/kg body weight of the crude extract of each of the five extracts, to three different groups of
148 adult male albino rats. In a fourth group, three adult male albino rats were administered with
149 equivalent volume of distilled water to serve as control. All the animals were orally administered the
150 extract using a curved needle to which a catheter had been fixed. The animals were monitored closely
151 every 30 minutes for the first 3 hours after administration of the extracts and hourly for the next 6
152 hours for any adverse effects. Then the animals were left for 72 hours for further observations.

153 When no death occurred, the second stage of the method [16] was employed. For this stage, only one
154 animal was required in each group. Groups 1-3 animals for each extract were orally given 1,600,
155 2,900 and 5,000mg/kg body weight dose of the crude extract while group 4 animal, was administered
156 distilled water. All the animals were left for observation as in stage one.

157 3. RESULTS

158 3.1 Extraction and Percentage Yield of *Carica papaya* Seed

159 The crude extracts of *Carica papaya* seed from five different solvents of varying polarity were all
160 brownish in colour with an offensive odour; it dissolves partially in distilled water. A total of 2500g of
161 the ground seed powder of *Carica papaya* with 500g for each test was used and the cumulative
162 weight of the extracts was 426.45g. (Table 1) For all the five extracts, polarity index of solvents is
163 proportional to the yield of the extracts thus the extract yield was in the following order; aqueous>
164 methanol > ethyl acetate > chloroform > n-hexane.

165 Table 1: Percentage yield of five extracts of *Carica papaya* Seed

Solvent used	Polarity index	Weight (g)	% Yield
n-Hexane	0.0	60.25	12.05
Chloroform	4.1	80.45	16.09
Ethyl acetate	4.4	90.85	18.17
Methanol	5.1	96.34	19.27
Aqueous	9.0	98.56	19.71

166 3.2 Phytochemical Contents of Different Solvent Extracts of *Carica papaya* Seed

167 The crude seed extracts of *Carica papaya* seed extracted using water, methanol, ethyl acetate,
168 chloroform and n- hexane screened for the presence of some classes of phytochemicals (flavonoids,
169 alkaloids, saponins, cardiac glycosides, tannins and anthraquinones) showed that flavonoids,
170 alkaloids and saponins are prominently present in all the extracts in varying composition while other
171 phytochemicals are present in relatively small quantities. Anthraquinones were not detected in all the
172 extracts.(Table 2)

173 Table 2: Phytochemical Contents of Five Different Solvent Extracts of Crude *Carica papaya* Seed

Solvent	Polarity index	Flavonoids	Alkaloids	Saponins	Tannins	Cardiac glycoside
n-Hexane	0.0	34.04 ±0.08	16.20 ±0.02	26.78±0.04	0.04±0.02	1.97±0.02
Chloroform	4.1	36.76 ±1.02	21.62 ±0.06	36.76 ±1.04	0.14 ±0.06	1.96 ±0.02
Ethyl acetate	4.4	23.50 ±0.04	19.88 ±0.06	23.50 ±0.02	0.91 ±0.02	2.18 ±0.04
Methanol	5.1	38.68 ±0.42	27.62 ±0.24	28.64 ±0.02	0.03 ±0.01	1.20 ±0.04
Aqueous	9.0	35.85 ±1.02	27.26 ±0.04	25.86 ±0.04	0.09 ±0.02	0.84 ±0.60

174 n = mean of 3 tests±SD;

175 Anthraquinones = Negative

176 3.3 Acute Toxicity (LD₅₀) of *Carica papaya* Seed Extracts

177 All the animals orally administered with the crude extracts of *Carica papaya* seed obtained from the
178 following solvent; n-hexane, chloroform, ethyl acetate, methanol and water up to 5000mg/kg body
179 weight showed no signs of distress and were physically active, even 72 hours post administration. No
180 death of animal was observed throughout the study.(Table 3)

181

182 Table 3: Determination of LD₅₀ dose following oral administration of *Carica papaya* seed extracts

Solvent	Dose (mg/kg bw)	Mortality ratio	% Survival
Aqueous		0/3	100
	10	0/3	100
	100	0/3	100
	1000	0/3	100
	1600	0/1	100
	2900	0/1	100
Methanol	5000	0/1	100
	10	0/3	100
	100	0/3	100
	1000	0/3	100
	1600	0/1	100
	2900	0/1	100
Ethyl acetate	5000	0/1	100
	10	0/3	100
	100	0/3	100
	1000	0/3	100
	1600	0/1	100
	2900	0/1	100
Chloroform	5000	0/1	100
	10	0/3	100
	100	0/3	100
	1000	0/3	100
	1600	0/1	100
	2900	0/1	100
n-hexane	5000	0/1	100
	10	0/3	100
	100	0/3	100
	1000	0/3	100
	1600	0/1	100
	2900	0/1	100

183 **3.4 Weight Changes**

184 Table 4 shows the result of weight changes in rats following the administration of aqueous,
 185 methanol, ethyl acetate, chloroform, n- hexane seed extract of *Carica papaya*. Generally, there were
 186 increases in weight of the experimental animals at the end of the each experiment

187

188 Table 4: body weight of experimental animals before and after oral acute administration of *Carica*
 189 *papaya* seed extracts

Extract	Weight (g)	Treatment groups/ doses						
		Control (g)	10mg/ Kg	100 mg/kg	1000 mg/kg	1600 mg/kg	2900 mg/kg	5000 mg/kg
Aqueous	Before	123±2.45	133±3.22	143±2.47	137±3.44	145±4.21	138±2.11	147±2.67
	After	126±3.22	137±2.17	145±3.01	140±2.13	149±1.55	141±2.11	150±3.17
Methanol	Before	142±3.21	143±2.78	133±2.43	151±2.55	148±1.54	141±2.04	145±1.26
	After	145±2.25	151±2.34*	143±2.37*	153±2.04	149±1.32	146±2.13*	149±2.30
Ethyl acetate	Before	139±2.11	147±2.28	137±1.45	141±2.10	139±2.34	143±2.15	143±2.03
	After	142±1.47	151±2.33	142±1.07*	146±1.86*	140±2.65	145±2.40	145±2.00
Chloroform	Before	145±1.33	145±2.11*	143±1.68	151±2.12	137±3.45	141±2.14	151±4.33
	After	148±2.33	138±2.33	147±2.30	147±2.08	141±2.44	145±1.56*	147±2.88
n- Hexane	Before	137±1.88	145±4.21	152±2.06	147±3.05	138±3.04	145±2.12	147±2.19
	After	142±2.33*	146±2.45	151±2.76	148±2.36	141±2.07	147±3.45	151±3.21

190 *significantly different (P<0.05) between before and after

191 4. DISCUSSION

192 *Carica papaya* is a known medicinal plant reported to possess health benefits against many diseases
 193 [17]. This health benefits are attributed to the presence of many bioactive phytochemicals in various
 194 parts of the plant [1]. In traditional herbal practice, water being a universal and more readily available
 195 solvent, is used to extract plant constituents however this practice was found to be scientifically
 196 inadequate as it was reported that chemical properties of solvents used in extraction of plant material
 197 influences the phytochemical composition of extracts [2]. Solvent polarity plays important role in
 198 determining the yield and chemical constituents of extract [4]. In the present study, five solvents
 199 namely, water, methanol, ethyl acetate, chloroform and n-hexane were used for the extraction of seed
 200 of *Carica papaya* and it was discovered that water gave the highest yield of extract followed by
 201 methanol, ethyl acetate and chloroform. Hexane, the most non polar of all the solvents, had the
 202 lowest yield. This finding is consistent with Ogbuehi *et al.* [18] who observed a proportional
 203 relationship between polarity of extraction solvent and the yield of extracts. Methanol is the best
 204 solvent for the extraction of flavonoids, alkaloids and saponins in *Carica papaya* seed however, this is
 205 not so with tannins which produced the highest yield when the highly non- polar n- hexane was used.
 206 This therefore could provide hint for researchers who wish to choose extraction solvent based on the
 207 targeted phytochemical of interest. For example, it has been reported that medicinal plants with
 208 nephroprotective properties mediates their protective effect via antioxidant and/or free radical
 209 scavenging activities due to the high concentration of flavonoids and alkaloids [19]. Therefore if a
 210 researcher requires *Carica papaya* seed extract with high amount of these phytochemicals, then the
 211 most desirable solvent is methanol.

212 Previous studies have reported the non-toxic nature of aqueous seed extract of *Carica papaya* with
 213 LD₅₀ above 5000mg/kg body weight of rat [20]. This is similar to the findings of this research however
 214 this study went further to determine the LD₅₀ for methanol, ethyl acetate, chloroform and n- hexane
 215 extracts which was found to be same as that of the aqueous extract of *Carica papaya* seed. This
 216 shows that all the five seed extracts of *Carica papaya* are safe for use in clinical practice.

217 There were generally slight increases in the calculated body weight of experimental animals before
 218 and after administration of extracts in all the treatment groups and control. Losses in body weight of
 219 experimental animals following administration of drug or toxicant are regarded adverse effect of drug
 220 and chemicals [21]. However, the present study recorded non-significant increases (P>0.05) in weight

221 of animals in most of the experimental groups and this further suggest the safety of *Carica papaya*
222 seed extracts. These slight increases in the weights of rats could be due to normal effect of food on
223 animal since the animals were allowed free access to water and food following administration of the
224 seed extracts of *Carica papaya*.

225 5. CONCLUSION

226 This study has provided data on the most efficient solvent for the extraction of *Carica papaya* seed to
227 obtain a higher extract yield or the desired phytochemical for any biological or pharmacological study.
228 It has also determined the LD₅₀ of all the solvent extracts studied. This can serve as a prelude for
229 further studies on the sub-acute and chronic toxicity effect of *Carica papaya* seed.

230 COMPETING INTERESTS

231 Authors have declared that no competing interests exist.

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UNDER PEER REVIEW