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1 ***Research paper***

2 Evaluation of microbial purity, acute and subchronic toxicities of a 3 Nigerian commercial polyherbal formulation used in the treatment 4 of diabetes mellitus

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ABSTRACT

Objective: This study evaluated acute and sub-chronic toxicities in rodents and microbial purity of a polyherbal formulation, Bobwell® popular among the natives for the management of diabetes mellitus (DM). It was prepared with unspecified quantities of the following plant materials viz. *Gongronema latifolium. Garcinia kola, Vernonia amgydalina*, *Sphenocentrum jollyanum*, and *Kigelia Africana* leaves.

Material and Methods: Microbial purity was evaluated on some bacterial and fungal organisms using appropriate diagnostic media. Toxicity of the polyherbal preparation was evaluated in Swiss albino mice by administering to the animals graded oral doses of the lyophilized preparation in the ranges of 1.0 to 20.0 g/kg body weight and observed for changes. Wistar rats were also fed with different doses of the lyophilized formulation for 25 days and the effects on the biochemical profiles and haematological parameters were evaluated.

Results: The purity evaluation test revealed presence of some bacterial organisms with the load within officially acceptable limits except *Escherichia coli* having a load of 1.50×10^{2} cfu/ml while no fungal organisms were observed. The median acute toxicity value (LD_{50}) of the polyherbal medicine was determined to be 15.2 g/kg body weight. Significant increase (p 0.05) was observed in the body weight in the group treated with the highest dose of the formulation compared to the control. The biochemical parameters showed marked decrease in the plasma glucose level compared to the control. Increase in creatinine level was observed only in the animals that received the highest dose of the formulation while aspartate aminotransferase (AST) decreased significantly. Alanine aminotransferase (ALT) on the other hand increased significantly at the highest dose. The photomicrograph of hepatic tissue showed focal necro-

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inflammation around the portal hepatics. There was marked increase in the haemoglobin level and in the RBC count at the highest doses. There was also significant increase in WBC.

Conclusion: The high LD_{50} value indicated that the polyherbal preparations could be safe for use but its safety was negated by high presence of *E coli* load. Although the formulation showed good hypoglycaemic activity and beneficial effects on cardiovascular risk factors, at the highest dose, the formulation exhibited deleterious effect on the hepatic tissue.

Key words: Microbial purity, acute, subchronic, toxicity, polyherbal formulation.

1 INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disordered diseases resulting from absolute or relative defects in insulin secretion or action or both. It is defined as “a state of premature cardiovascular death which is associated with chronic hyperglycemia and also associated with blindness and renal failure” (Barnett and O’Gara, 2003). Oral hypoglycemic drugs including suphonylureas, biguanides and thiazolidinediones, have been employed in the treatment of the disease without achieving a total cure and are without some undesired side effects. Attention is, therefore, now focused on the use of alternative therapy for the disease treatment with plant and plant derived medicines as the best option. Plant derived medicine is known as herbal medicine and is currently being used by about 60% of the world population both in the developing and in the developed countries where modern medicines are predominantly used (Rickert *et al.,* 1999; Ogbonnia *et al.,* 2008). It is also believed to be patronized and is popularly employed in the treatment of various disease conditions especially those disease states that could not be effectively treated with orthodox medicines including DM.

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70 The increasing popularity in the use of herbal remedies could be attributed to their advantages
71 of being efficacious and a cheap source of medical care. Secondly, there is a growing
72 disillusionment with modern medicine and also misconception that herbal remedy being natural
73 may be devoid of adverse and toxic effects associated with allopathic medicines. Herbal
74 preparations could be contaminated with microbiological and foreign materials, such as heavy
75 metals, pesticide residues or even aflatoxins. Contaminants when present in an herbal
76 preparation may lead to serious health defects underscoring the claimed safety. An increase in
77 the morbidity and mortality associated with the use of herbal or the so called traditional
78 medicines has raised universal attention in the last few years (Bandaranayake, 2006; Ogbonnia
79 *et al.,* 2010). Upon exposure, the clinical toxicity may vary from mild to severe and even life
80 threatening making the safety and toxicity evaluations of these preparations imperative. Also
81 lack of standardization is a major concern regarding the use of medicinal herbal medicines
82 (Angell and Kassier, 1998).
83
84 Herbal medicine is most often a 'polyherbal' preparation from mixtures of many plant parts
85 obtained from various plant species and families and may contain multiple bioactive
86 constituents that could be difficult to characterize (Ogbonnia *et al.,* 2010). The bioactive
87 principle(s) in most herbal preparations are not always known and there could be possibilities of
88 interaction with each other in solution. The quality as well as the safety criteria for herbal drugs
89 may be based, therefore, on a clear scientific definition of the raw materials used for such
90 preparations. Also herbal medicine may have multiple physiological activities and an herbal
91 preparation could be used in the treatment of a variety of disease conditions (Pieme *et al.,*
92 2006), and may be administered in most disease conditions over a long period of time without
93 proper dosage monitoring and consideration of toxic effects that might result from such

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94 prolonged usage. The danger associated with the potential toxicity of such therapy and other
95 herbal therapies used over a long period of time demand that the practitioners be kept abreast
96 of the reported incidence of renal and hepatic toxicity resulting from the ingestion of medicinal
97 herbs (Tédong *et al.,* 2007).
98
99 The aim of the study was to evaluate the safety of a polyherbal preparation, Bobwell,® an
100 antidiabetic, formulation sold openly in the markets and widely consumed locally for the
101 treatment of DM in most Nigerians south western states for microbial purity and also to carry
102 out the acute and sub-chronic toxicity studies in rodents. Bobwell® was claimed to be prepared
103 with unspecified quantities of the following plant material constituents: *Gongronema latifolium*.
104 (fam. Asclepiadaceae), *Garcinia kola* Heckle.(fam.Guttiferaceae), *Vernonia amgydalina*
105 Del.(fam. Asteraceae), *Sphenocentrum jollyanum* (fam Menispermaceae), and *Kigelia africana*
106 (Lam). Benth (fam. Bignoniaceae). The individual plant material has various ethno-botanical
107 uses and has been used singly or in combination with other herbs in the treatment of many
108 other disease conditions.
109

110 **2. MATERIAL AND METHODS**

111 **2.1 Material**

112 The antidiabetic polyherbal formulation Bobwell® (a liquid dosage form, 1.5L) a slightly thick,
113 non viscous brownish coloured liquid was selected on the basis of its local consumption rate
114 and was procured from the Mushin market in Lagos suburb. The un-tampered procured
115 polyherbal formulation bottle was stored in a refrigerator at 4- 6°C until the quantity needed for
116 the purity test was aseptically taken. 1000 ml of the formulation was filtered and the resulting
117 875 ml was freeze dried which yielded 37.5 g gel. The prescribed dose for human adult was 30

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118 ml daily. The label indicated only the plant material constituents used in the formulation without
119 specifying the quantity of each used, and also there was no indication of the batch number, the
120 manufacturing and expiring dates.
121

122 **2.2 Animals**

123 Swiss albino mice (20 – 25 g) of either sex were used for the acute toxicity study, while adult
124 Wistar rats (130±15g) were used for the sub-chronic toxicity profiling. The animals were
125 obtained from the animal house of the College of Medicine of the University of Lagos. They
126 were fed with a standard animal diet (Pfizer Feeds Ltd, Nigeria) and had access to water *ad*
127 *libitum*. They were maintained in spacious polypropylene cages in well ventilated animal house
128 with 12 hrs dark and light cycle and were acclimatized for a week before the commencement of
129 the study. The use and care of the animals, and the experimental protocol were in strict
130 compliance with the Institute of Laboratory Animal Research (ILAR) guidelines on the use and
131 care of animals, in experimental studies (ILAR, 1996).
132

133 **2.3 Determination of microbial purity**

134 The microbial load of the preparation was determined using the standard plate method
135 (Fontana *et al.,* 2004). Various diagnostic media-Tryptone Soya Agar (TSA), Salmonella-
136 Shigella Agar (SSA), Eosin Methylene Blue Agar (EMBA), MacConkey Agar (MAC), Nutrient
137 Agar (NA), Manitol Salt Agar (MSA), Sabouraud Dextrose Agar (SDA) - were used to culture
138 the test products. Each of the media was prepared according to manufacturers' instruction and
139 sterilized at 121 ^{0}C for 15 minutes.
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141 Three fold serial dilutions ($10^{-1,}$ 10^{-2} and 10^{-3}) were made using sterile distilled water. The media
142 were allowed to cool to 45 ^{0}C and 1ml each of the dilutions seeded in 25 ml each of the sterile
143 culture media swirled and left to solidify. The bacterial media were incubated at 37 ^{0}C for 3 days
144 while the fungal medium (SDA culture) was incubated at ambient temperature for 7 days. They
145 were examined 24 hourly during this period for the colonies and the results recorded (Table 1).
146 The purity of the formulations for proteus organisms was evaluated using the 1/10 dilution, a
147 loopful was taken and dropped aseptically at the centre of nutrient agar plate. The site of
148 inoculation was swabbed. The triplicate plates were prepared, covered and incubated in
149 inverted position at 37 ^{0}C and observed daily for 3 days for swarming of proteus.
150
151 **2.4 Assay of antimicrobial activity**
152 The antimicrobial activity of the preparation was investigated using the cup diffusion method on
153 Mueller Hinton Agar for bacterial organisms and Sabouraud Dextrose Agar (SDA) for fungal
154 organisms (Raghavendra, 2006). 10^6 cfu/ml of the overnight clinical cultures of *Escherichia coli,*
155 *Pseudomonas aeruginosa, Klebsiella species, Shigella species* was seeded in 25 ml Mueller
156 Hinton Agar respectively while *Candida albican* was seeded in Sabouraud Dextrose Agar. Wells
157 were bored in each of the culture media using a sterile 12 mm cork borer and various dilutions
158 (100 %, 50 %, 25 % and 12.5 %) of the test material were prepared using sterile water. 0.5 ml
159 of each dilution was respectively seeded in wells made in inoculated plates with a blank well in
160 each of the plates seeded with 0.5 ml sterile distilled water to serve as a control standard. The
161 cultures were incubated at 37 ^{0}C for 24 hrs for bacterial cultures and at ambient temperature for
162 7 days for fungal cultures and observations were made for zones of inhibitions (NCCLS, 1997).
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164 **2.5 Acute Toxicity Study**

165 The toxicity study was carried out using thirty-five (35) male and female Swiss albino mice
166 (weighing 20 – 25 g) obtained from the Laboratory Animals Center, College of Medicine,
167 University of Lagos. The animals were randomly distributed into: one control group and six
168 treated groups, containing five animals per group. They were maintained on animal cubes
169 (Feeds Nigeria Ltd), provided with water *ad libitum* and were allowed to acclimatize for seven
170 days to the laboratory conditions before the experiment. After the overnight fasting, the control
171 group received 0.3 ml of acacia solution (2 %) orally. The doses 1.0, 2.5, 5.0, 10.0, 15.0 and
172 20.0 g/kg were respectively administered orally to the groups from acacia solution of the extract
173 gel which was prepared by dispersing 16 g of the gel with 7 ml of the acacia solution in a 100 ml
174 beaker and transferred to a 20 mL volumetric flask. The beaker rinsed with the acacia solution
175 was transferred to the volumetric flask and the volume made to mark with the acacia
176 solution.The animals were observed continuously for the first 4 hrs and then for each hour for
177 the next 24 hrs and at 6 hourly interval for the next 48 hrs after administering the extract to
178 observe any death or changes in general behaviour and other physiological activities (Shah *et*
179 *al.,* 1997; Bürger *et al.,* 2005).

180

181 **2.6 Determination of LD_{50}**

182 The LD_{50} of the extract by oral route was estimated using the method of Lorke (1983). The LD_{50}
183 was calculated as the geometrical mean of the maximum dose producing 0 % mortality and the
184 minimum dose producing 100 % mortality.

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187 **2.7 Sub-chronic study**

188 Male and female Wistar rats weighing 130 g ± 20 g were used. They were allowed to
189 acclimatize to the laboratory conditions for seven days. The animals were maintained on
190 standard animal feeds and provided with water *ad libitum.* The animals were weighed and
191 divided into four groups of five animals each and after the overnight fast of the animals the
192 control group received a dose of 0.6 ml of acacia solution (2 %) orally once a day for 30 days.
193 The three treated groups respectively received the following doses: 200 mg/kg, 300 mg/kg and
194 600 mg/kg bwt of the gel orally once a day for 25 days (Pieme *et al*., 2006; Joshi *et al*., 2007;
195 Mythilypriya *et al*., 2007). The gel suspension (12 %w/v) was prepared by dispersing the gel (12
196 g) with 45 ml of acacia (2 %) solution in a beaker, and transferred to a 100 mL volumetric flask.
197 Then the beaker was rinsed with the solution and the content transferred to the volumetric flask
198 and volume made to mark with the acacia solution.

199

200 The animals were weighed every five days, from the start of the treatment, to note any weight
201 variation. At the end of the experiment, the animals were starved overnight and on the 26th day,
202 they were made unconscious by cervical dislodgement. The blood was collected via cardiac
203 puncture in three tubes: one with EDTA for analysis of hematological parameters and the blood
204 chemistry, Fluoride oxalate tube for glucose analysis and with heparin to separate plasma for
205 biochemical profiles. The heparinized blood was centrifuged within 5 min of collection at 4000 g
206 for 10 min to obtain plasma which was analyzed for total cholesterol, total triglyceride, and HDL-
207 cholesterol levels by modified enzymatic procedures from Sigma Diagnostics (Wasan *et al.,*
208 2001). LDL-cholesterol levels were calculated using Friedwald equation (Crook, 2006). Plasma
209 was analyzed for Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and
210 creatinine by standard enzymatic assay methods (Sushruta *et al.,* 2006). Plasma glucose

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211 contents and protein contents were determined using enzymatic spectroscopic methods
212 (Hussain and Eshrat, 2002). Haematocrit was estimated using the method as described by
213 (Ekaidem *et al.,* 2006). Haematocrit tubes were filled with whole blood to the mark by capillary
214 action and the bottom of the tubes sealed with plasticide and centrifuged for 4-5 minutes using
215 haematocrit centrifuge. The percentage cell volume was read by sliding the tube along a
216 “critocap” chart until the meniscus of the plasma intersected the 100 % line. Hemoglobin
217 contents were determined using Cyanmethaemoglobin (Drabkin) method (Ekaidem *et al.,*
218 2006).
219
220 **2.8 Tissue histology**
221 The organs were fixed in 10 % formal saline for ten days before embedding in paraffin wax.
222 Each organ tissue was sectioned at 5 μm and stained with Haematoxylin and Eosin (H and E)
223 stain (Mbaka *et al.,* cf2012). The slide specimens were examined under light microscope at
224 high power magnification for changes in organ architecture and photomicrographs were taken.
225
226 **2.9 Statistical analysis**: Significant differences were determined using a Student’s t-test.
227 Differences were considered significant if $p < 0.05$. All data were expressed as mean ± standard
228 error of the mean.
229
230 **3.0 RESULTS AND DISCUSSION**
231 There is a renewed and increasing interest in herbal and plant derived medicines.
232 Consequently herbal medicines have received greater attention as alternative to clinical therapy
233 in recent times leading to subsequent increase in their demand (Sushruta *et al.,* 2006). In rural

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234 communities, the exclusive use of herbal drugs, prepared and dispensed by herbalists without
235 formal training, for the treatment of diseases is still very common requiring that experimental
236 screening method be established to ascertain the safety and efficacy of these herbal products
237 as well as to establish their active components (Ogbonnia *et al.,* 2010). The microbial purity
238 evaluation of the formulation (Table 1) showed no growth of bacterial and fungal organisms in
239 the first 24 hrs in the various diagnostic media used. The growth of *Bacillus subtilis* (1.0×10^2
240 cfu/ml) were observed in Tryptone Soy Agar culture and of other coli forms (2.25×10^2 cfu/ml)
241 observed in MacConkey agar culture after 72 hrs but all were within acceptable official limit
242 (Fontana *et al.,* 2004). There was no growth observed on Sabouraud Dextrose Agar even on
243 the 6th and 7th day of incubation indicating the absence of fungal organisms. In the Eosine
244 Methylene Blue Agar medium showed the growth of *E. coli* (1.5×10^2 cfu/ml) which was above
245 the acceptable microbial limit for aqueous herbal formulations according to World Health
246 Organization guidelines for the assessment of the safety, efficacy, and quality of herbal
247 medicines as a prerequisite for global harmonization (W H O, 1966).
248
249 In the acute toxicity study (Table 2) of the formulation no changes in the behaviour and in the
250 sensory nervous system responses were observed in the animals. Also no adverse
251 gastrointestinal effects were observed in the male and female mice used in the experiment. All
252 the animals that received 5.0 g/kg bwt dose survived beyond the 24 hrs of observation while
253 four of the mice that received 20.0 g/kg dose of the extract died within 4 hrs. The median acute
254 toxicity value (LD_{50}) of the formulation was determined to be 15.2 g/kg bwt. According to World
255 Health Organization (WHO) toxicity index of 2 g/kg (Ghosh.1984; Klassen *et al*., 1995) the
256 extract could be classified as being non toxic, since the LD_{50} was found to be above 15.0 g/kg
257 translating to 1064 g equivalence dose in human adult. This is a very high value making the

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preparation relatively safe for use. The viscera of the dead animals did not show any macroscopic changes that could point to the cause of the death neither did the animals convulse before dying. It could therefore, be postulated that the formulation did not kill the mice by the action on the nervous system (Ogwal- Okeng *et al.,* 2003).

The effects of the formulation on the body weight of the animals treated with various doses are summarized in Table 3. Significant (*p* 0.05) increase in the body weight was observed only in animals that received the highest dose (600 mg/kg bwt) of the lyophilized extract while no significant (*p* 0.05) increase occurred in the weight of animals treated with lower doses of the polyherbal formulation compared to the control. The appreciable weight gain of the animals that received the highest dose of the formulation suggested the ability of the polyherbal formulation to have the tendency to stimulate appetite at high dosage.

Effects of the polyherbal formulation on the biochemical profiles were summarized in Table 4. There was a remarkable decrease in the plasma glucose levels especially in the animals treated with the highest dose of the formulation compared to the control. This clearly indicated the presence of hypoglycaemic components in the formulation and gives credence to the use of the polyherbal formulation as a hypoglycaemic agent. Significant (*p* 0.05) increase in creatinine level was observed only in the animals that received the highest dose of the formulation (600 mg/kg bwt). The elevation in the plasma creatinine concentration could suggest inflammatory activity in the kidney, specifically by renal filtration mechanism (Wasan *et al.,* 2001). There was significant decrease in AST level at all the doses. The ALT level except at the lowest extract dose showed progressive increase that was significant at the highest dose compared to the control. An elevation in plasma concentration of ALT is usually due to liver

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282 damage while increase in AST level could be linked to damage to either cardiac or hepatic
283 tissues or damage to both (Wasan *et al.,* 2001; Crook, 2006). The marked increase in ALT at
284 the highest dose was therefore indicative of inflammatory challenge of the formulation on the
285 liver. The decrease in the plasma total cholesterol (TC) and triglyceride (TG) levels might be
286 attributable to the presence of hypolipidaemic agents in the extract. A significant increase in
287 HDL-cholesterol levels and a reduction in LDL-cholesterol levels observed in all the treated
288 animals was an indication that the formulation has the tendency to reduce the cardiovascular
289 risk factors which contribute to death of diabetic subjects (Barnett and O'Gara, 2003). The
290 ability of the formulation to exert a decrease in cardiovascular risk factors lent further support
291 for its use as a hypoglycaemic agent.
292
293 The photomicrograph of hepatic tissue of the animals administered with the highest dose of the
294 formulation showed focal necro-inflammation around the portal hepatics. It was obvious the
295 inflammatory changes in the hepatic tissue precipitated an increase in the ALT level. The
296 photomicrograph of renal and testicular tissues on the other hand showed normal appearance.
297 Therefore, the cause of marked increase in creatinine level at the highest dose of the
298 formulation is likely due to other remote factors.
299
300 The observed increase in the haemoglobin levels might be as a result of increased absorption
301 of iron. Although the haematocrit level at the highest dose of treatment showed no significant
302 variation compared to the control, the marked increase in the haemoglobin level and in the RBC
303 count at that dose may suggest that the polyherbal formulation has active principle that can
304 stimulate erythropoietin release in the kidney known to enhance RBC production
305 (erythropoiesis) (Polenakovic and Sikole, 1996; Sanchez-Elsner *et al.,* 2004). This potential

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306 haematinic property emphasized the beneficial effect of the formulation to the general well
307 being of the animals. The haematinic effect of the formulation could be due to the activity of
308 *Sphenocentrum jollyanum,* a constituent of the formulation reported to be rich in haematinic
309 property (Mbaka *et al.,* 2010; Mbaka and Adeyemi, 2010; Mbaka and Owolabi, 2011). This
310 study showed that there was no significant change in MCHC in the treated animals compared to
311 the control. Low MCHC is associated with iron deficiency anaemia where microcytic
312 hypochromic red cells are produced as a result of lack of iron to support haemoglobin synthesis
313 (Agbor *et al.,* 2005). There was also no significant change in MCV thus signifying that the
314 polyherbal medicine did not regenerate anaemia. However, there was a significant increase in
315 WBC count which is known to rise as body defense in response to toxic environment (Ngogan,
316 2005). Also, lymphocyte, the main effector cell of the immune system (Mc Knight *et al.,* 1999;
317 Teguia *et al.,* 2007) recorded marginal increase suggesting that the formulation might not have
318 exerted challenge on the immune system of the animals.
319

320 **4.0 CONCLUSION**

321 The high LD_{50} value (15.2 g/kg) obtained clearly indicated that the polyherbal preparations
322 could be safe for use but its safety was negated by the presence of *E coli* load above officially
323 accepted limit for liquid herbal preparation. The study showed that the formulation had some
324 hypoglycaemic activity and good reducing effects on cardiovascular factors and did not provoke
325 toxic effects to the animals' heart tissues. Furthermore, the presence of haematinic agent
326 emphasized the beneficial effect of the formulation. However, at highest dose used, the
327 formulation exhibited deleterious effect on the hepatic tissue thus necessitating for a cautious
328 use.

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329 **Ethical approval**

330 All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-
331 23, revised 1985) were followed, as well as specific national laws where applicable. All
332 experiments have been examined and approved by the appropriate ethics committee of our
333 Institution". All authors hereby declare that all experiments have been examined and approved
334 by the appropriate ethics committee and have therefore been performed in accordance with the
335 ethical standards laid down in the 1964 Declaration of Helsinki.

336

337 **Competing interest:** There is no conflict of financial interest in connection with the submitted
338 manuscript.

339

340 **Authors' Contributions:** Author A designed the experiment, the protocol for the study and also
341 partook in the manuscript preparation and statistical analysis. Author B undertook the tissue
342 processing and analysis as well as partook in the write up and editing of the manuscript. Author
343 C conducted the laboratory work and did part of the literature search. Authors D, E&F evaluated
344 the microbial purity of the formulations and did part of the literature search.

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352 **References**

353 Agbor, G. A., Oben J. E., Ngogang J. Y. (2005). Haematinic activity of *Hibiscus cannabinus*.
354 Afri. J. Biotech. 4, 833-837.
355 Angell, M. and Kassier, J. P. (1998). Alternative medicine –the risk of untested and unregulated
356 remedies. N. Engl. J. Med. 339, 839-841.
357 Bandaranayake, W. M., (2006). Modern Phytomedicine. Turning medicinal plants into drugs.
358 Ahmad, I., Aqil F. and Owais M. Edn. Wiley-VCH Verlag GmbH and Co. KGaA, Weinheim.

359 Barnett, H. A. and O'Gara, G. (2003). Diabetes and the heart. clinical practice series. Churchill
360 Livingstone. Edinburgh UK, p. 11-32.

361 Bürger, C., Fischer, D. R., Cordenunzzi, D. A., Batschauer de Borba, A. P., ,Filho, V. C, Soares
362 dos Santos A. R. (2005). Acute and sub-acute toxicity of the hydro alcoholic extract from
363 *Wedelia paludosa* (Acmela brasilinsis) (Asteraceae) in mice. J. Pharm. Sci.
364 (www.cspsCanada.org) 8(2), 370-373.
365 Crook, M. A. (2006). Clinical chemistry and metabolic medicine. 7th Edition. Hodder Arnold,
366 London: p. 426.
367 Ekaidem, I. S., Akpanabiatu, M. I., Uboh, F. E, Eka, O.U. (2006). Vitamin b12 supplementation:
368 effects on some biochemical and haematological indices of rats on phenytoin administration.
369 Biokemistri. 18 (1), 31- 37.
370 Fontana, R., Mendes, M. A., de Souza, B. M., Konno, K, Cesar, L. M. N. (2004). Jelleines, a
371 family of antimicrobial peptides from the royal jelly of honey bees (Apis mellifera) peptides 25,
372 919-928.

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373 Ghosh, M. N. (1984).Toxicity studies .In: Fundamentals of experimental pharmacology, 2nd ed.
374 Scientific Book Agency, Calcutta: p. 153-158.

375 Hussain, A and Eshrat, H. M. (2002). Hypoglycemic, Hypolipidemic and Antioxidant properties
376 of combination of Curcumin from *Curcuma longa* Linn and partially purified product from
377 *Abroma augusta* Linn in streptozotocin induced diabetes. Indian J. Clin. Biochem. 17 (2), 33-43.

378 ILAR, 1996 (Institute of Laboratory Animal Research). Commission on life science. National
379 research council. www.edu/openbook. php?record_id=5140.

380 Joshi, C. S., Priya, E. S, Venkataraman, S. (2007). Acute and sub acute studies on the
381 polyherbal antidiabetic formulation Diakyur in experimental animal model. J. Health Sci. 53(2),
382 245-249.

383 Klassen, C. D., Amdur, M. O., Doull, J. (1995). Casarett and Doull's Toxicology: The basic
384 science of poison. 8th ed. Mc Graw Hill, USA.p. 13-33.

385 Lorke, D. (1983). A new approach to practical acute toxicity testing. Arch. Toxicol. 54, 275-287.

386 Mbaka, G. O., Adeyemi, O. O., Oremosu, A. A. (2010). Acute and sub-chronic toxicity studies of
387 the ethanol extract of the leaves of *Sphenocentrum jollyanum* (Menispermaceae). Agric. Biol. J.
388 N. Am 1 (3), 265-272.

389 Mbaka, G. O. and Adeyemi, O. O. (2010). Toxicity study of ethanol root extract of
390 *Sphenocentrum jollyanum* (Menispermaceae) Pierre. Asian J. Exp. Biol. Sc .1 (4), 860-874.

391 Mbaka, G. O. and Owolabi, M. A. (2011). Evaluation of haematinic activity and subchronic
392 toxicity of *Sphenocentrum jollyanum* (Menispermaceae) seed oil. Euro. J. Med. Plant. 1(4), 140-
393 152.

UNDER PEER REVIEW

394 Mbaka, G.O., Ogbonnia, S. O., Oyeniran, K. J., Awopetu, P. I. (2012). Effect of *Raphia hookeri*
395 seed extract on blood glucose, glycosylated haemoglobin and lipid profile of alloxan induced
396 diabetic rats. British J. Med. & Med. Res., 2(4), 621-635.
397
398 Mc Knight, D. C., Mills, R. G., Bray, J. J., Crag, P. A. (1999). Human Physiology. 4th Edition,
399 Churchill Livingstone.
400 Mythilypriya, R., Shanthi, P., Sachdanandam, P. (2007). Oral acute and sub acute toxicity
401 studies with Kalpaamruthaa, a modified indigenous preparation, on rats. J. Health Sci. 53(4),
402 351-358.
403 Ngogang, J. Y. (2005). Haematinic activity of *Hibiscus cannabinus*. Afr. J. Biotech. 4, 833-837.
404 National Committee for Clinical Laboratory Standards (NCCLS) (1997). Methods for dilution
405 antimicrobial susceptibility tests for bacteria that grow aerobically. 4th Edition. Approved
406 standard M7-A4, 17, No. 2, Villanova PA.
407 Ogbonnia, S. O., Odimegwu, J. I., Enwuru, V. N. (2008). Evaluation of hypoglycaemic and
408 hypolipidaemic effects of aqueous ethanolic extracts of *Treculia africana* Decne and
409 *Bryophyllum pinnatum* Lam. And their mixture on streptozotocin (STZ)-induced diabetic rats.
410 Afr. J. Biotech. 7(15), 2535-2539.
411 Ogbonnia, S. O., Mbaka, G. O., Igbokwe, N. H., Anyika, E. N., Alli, P., Nwakakwa, N.
412 (2010). Antimicrobial evaluation, acute and subchronic toxicity studies of Leone Bitters, a
413 Nigerian polyherbal formulation, in rodents. Agric. Biol. J. N. Am., 1(3), 366-376.
414 Ogwal-Okeng, W. J., Obua, C., Anokbonggo, W. W. (2003). Acute toxicity effects of the
415 methanolic extract of *Fagara zanthoxyloides* (Lam.) root-bark. Afr. Health Sci. 3(3), 124-126.
416 Pieme, C. A, Penlap, V.N, Nkegoum, B., Taziebou, C. L., Tekwe, E. M., Etoa, F. X., Ngongang,
417 (2006). Evaluation of acute and subacute toxicities of aqueous ethanol extract of leaves of
418 *Senna alata* (L.) Roxb (Ceasalpiniaceae). Afr. J. Biotech., 5: (3), 283-289.

UNDER PEER REVIEW

419 Polenakovic, M. and Sikole, (1996). Is erythropoietin a survival factor for red blood cells? J Am
420 Soc. Nephrol. 7, 1178-1182.
421 Raghavendra, M. P., Satish, S., Raveesha. K. A. (2006). In-vitro evaluation of anti-bacterial
422 spectrum and phytochemical analysis of *Acacia nilotica*. J. Agric. Tech. 2(1): 77 – 88.
423 Rickert, K., Martinez, R. R., Martinez, T. T. (1999). Pharmacist knowledge of common herbal
424 preparations. Proc. West. Pharmacol. Soc., 42, 1-2.
425 Shah, L. P., Patil, S. P., Patil, J. (1997). Observations on clinical evaluation of indigenous
426 herbal drugs in the treatment of mental illness. Indian J. Pharmacol., 29, 347-349.
427 Sanchez-Elsner, T., Ramirez, J. R., Rodriguez-Sanz, F., Varela, E., Bernabew, C., Botella, L.
428 M. (2004). A cross talk between hypoxia and TGF-beta orchestrates erythropoietin gene
429 regulation through SPI and SMADS. J. Mol. Biol. 36, 9-24.
430 Sushruta K., Satyanarayana S., Srinivas N., Sekhar R. J (2006). Evaluation of the blood–
431 glucose reducing effects of aqueous extracts of the selected Umbellifereous fruits used in
432 culinary practice. Trop. J. Pharmaceutical Res. 5(2), 613- 617.
433 Tédong, L., Dzeufiet, P. D. D., Dimo, T., Asongalem, E. A., Sokeng, S. N., Flejou, J. F., Callard,
434 P., Kamtchouing, P. (2007). Acute and Sub chronic toxicity of *Anacardium occidentale* Linn
435 (Anacardiaceae) leaves hexane extract in mice. Afr. J. Tradit. Altern. Med. 4(2), 140-147.
436 Teguia, A., Telefo, P. B., Fotso, R. G. (2007). Growth performances, organ development and
437 blood parameters of rats fed graded levels of steeped and cooked taro tuber (*Colocasia*
438 *esculenta var esculenta*) meal. Livestock Res. Rural Dev. 19 (6), 1-7.
439 Wasan, K. M., Najafi, S., Wong, J., Kwong, M. (2001). Assessing plasma lipid levels, body
440 weight, and hepatic and renal toxicity following chronic oral administration of a water soluble

UNDER PEER REVIEW

441 phytostanol compound FM-VP4, to gerbils. J Pharmaceutical Science (www.ualberta.ca/~csps)
442 4(3), 228-234.
443 WHO, (1966). Specifications for identity and purity and toxicological evaluation of food
444 colours. WHO/Food Add/66.25 Geneva, WHO.
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Table 1: Microbial Purity test of the polyherbal formulation

MEDIA	*S. typhi*	*Bacillus* species x 10^2	*Shigella* species	Other Coliforms x 10^2	Proteus species	*P aeruginosa*	*S. aureus*	*E. coli* x 10^2	TMYC	TAC x 10^2	TOTAL
SSA	0	-	0			-	-	-	-	-	0
MAC	-	-	-	2.25	-	-	-	-	-	-	$2.25x10^2$
NA	-	-	-	-	0	-	-	-	-	-	0
CA	-	-	-	-	-	0	-	-	-	-	0
MSA	-	0	-	-	-	-	0	-	-	-	0
EMBA	-	-	-	-	-	-	-	1.50	-	-	1.50x10
SDA	-	-	-	-	-	-	-	-	0	-	0
TSA	-	$1.0x10^2$	-	-	-	-	-	-	-	9.93	$1.093x10^3$

Targeted Organisms: *Salmonella typhi 0, Shigella species* 0, Other Coli forms $2.25x10^2$, Proteus species 0, *Pseudomonas aeruginosa* 0, *Staphylococcus aureus* 0, *Escherichia coli* $1.50x10^2$, Mould and Yeast 0 and *Bacillus species* $1.0x10^{2.}$

CA - Cetrimide Agar, EMBA - Eosine Methylene Blue Agar, MAC- MacConkey Agar , NA- Nutrient Agar, SDA- Sabouraud Dextrose Agar, SSA - Salmonella Shigella Agar, TSA- Trytone Soya Agar, TNTC-To numerous to count TYMC Total yeast and mould count

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473 **Table 2: Acute toxicity evaluation of the polyherbal formulation in mice**

474

Doses of drugs g/kg	Number of Animals	Number of animals dead	% cumulative Death
Control	0	0	0
0.5	0	0	0
1.0	5	0	0
2.5	5	0	0
5.0	5	0	0
10.0	5	1	14.3
15.0	5	2	42.8
20.0	5	4	100.0

475 476 477 478 479 480 481 482 483 484 485

486 Control received 0,3ml of Acacia (2%w/v) solution

487 488 489 490 491 492 493 494 495

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497 **Table 3.The effects on weight variations of animals treated with different doses of**

498 **polyherbal formulation for 30 days in the sub-chronic toxicity study.**

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Dose	Day 1	Day 7	Day 14	Day 21	Day 28	Day 31
GPI	130.6±2.7	133.2±1.8	135.2±1.0	138.1±2.5	140.5±2.2	143.5±2.3
GPII	150.5±2.1	153.4±0.4	153.5±1.2	156.3±1.8	158.2 ±0.2	160.6±0.2
GPIII	130.2±0.3	133.7±1.5	135.6±1.3	136.7±1.9	137.2±1.7	140.7±0.4
GPIV	120.5±2.2	125.6±4.2	128.7±2.5	130.3±4.1	138.5±1.2	139.2±1.5

500

501 N=5 m ± sem**p* <0.05; ** *p* <0.01 vs. control group. Control group received 0.5 Acacia

502 (2 %w/v) solution

503 KEY GP I (Control), GPII (200mg/kg), GP III (300mg/kg), GP IV (600mg/kg)

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515 **Table 4: Plasma glucose level and other biochemical profiles of animals treated**

516 **respectively with various doses of polyherbal formulation extract for 30 days and the**

517 **control**

PARAMETER	Group I	Group II	Group III	Group IV
Glucose(mmol/l)	4.1±0.2	3.2±0.4**	2.7±0.2*	2.2±0.3*
Cholesterol(mmol/l)	2.0±0.3	1.4±0.1*	1.6±0.2**	1.3±0.5*
Triglyceride(mmol/l)	0.5±0.0	0.4±0.0	0.3±0.0*	0.2±0.0*
HDL(mmol/l)	1.5±1.0	2.0±0.5**	2.0±0.6**	2.0±0.7**
Creatinine(mmol/l)	59.6±0.3	67.6±0.1	57.8±0.0	130.9±2.5*
AST(IU/L)	304.0±0.5	244.7±5.0**	208.7±0.4*	205.3±0.0*
ALT(IU/L)	66.7±2.0	59.9±0.2	68.8±0.3	79.4±0.2*
LDL(mmol/l)	1.3±0.5	0.8±0.3**	1.0±0.2**	0.6±0.2*

518 N=5, values = m ± sem *p <0.05; ** p <0.01 vs. control group. Control group received 0.5 ml

519 Acacia (2 %w/v) solution.

520 GP I (Control), GPII (200mg/kg), GP III (300mg/kg), GP IV (600mg/kg)

521 HDL- High density lipoprotein; LDL - Low density lipoprotein; AST - Aspartine

522 aminotransferease; ALT- Alanine aminotransferease

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523 **Table 5: Haematological and blood differential profiles of animals treated respectively**
524 **with various doses of polyherbal formulation extract for 30 days and the control**
525

PARAMETER	Group I	Group II	Group III	Group IV
RBC x 10^3	6.0±0.0	6.7±0.3	6.3±0.2	8.1±0.4*
WBC(10^6)	4.5±0.1	9.0±0.1*	7.0±0.3*	8.9±0.2*
MCV(fl)	64.1±0.3	52.8±0.1*	54.6±0.2*	53.9±0.5*
HCT (%)	44.0±1.0	39.7±0.5	38.9±0.7	44.6±0.6
PLT (%)	451.0±2.0	527.0±5.0	292.1±0.5	502.0±0.7
PCT (%)	0.3±0.0	0.3±0.1	0.2±0.0	0.3±0.0
Hb (g/dl)	12.8±0.2	61.9±1.5	14.9±0.1	15.9±0.5**
MCH(pg)	21.6±1.0	19.8±0.5	20.5±0.7	19.7±0.6
MCHC (g/dl)	33.7±0.5	37.4±0.6	37.5±1.0	36.6±0.5
LYM (%)	69.2±2.0	61.9±1.5	81.3±0.5	70.2±0.3

526
527 N=5 m ± sem*p <0.05; ** p <0.01 vs. control group. Control group received 0.5 Acacia (2
528 %w/v) solution
529 KEY GP I (Control), GPII (200mg/kg), GP III (300mg/kg), GP IV (600mg/kg)
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534 **Table 6: The effects on the weights on kidney, heart, liver and brain of animals treated**
535 **with various doses of the polyherbal formulation extract for 30 days and the control in**
536 **the sub-chronic toxicity study.**
537

ORGAN g/kg bwt	GPI	GPII	GPIII	GPIV
Heart(g)	0.5±0.2	0.5±0.1	0.5±0.0	0.5±0.0
Liver(g)	3.4±0.1	3.6±0.5	3.6±0.5	3.7±0.6
Kidney(g)	0.8±0.0	0.9±0.0	0.7±0.0	0.9±0.0
Brain(g)	1.5±0.1	1.5±0.1	1.3±0.0	1.5±0.0

538
539 N=5 values= (m ± sem)*p <0.05; ** p <0.01 vs. control group. Control group received 0.5
540 Acacia (2 %w/v) solution
541 GP I (Control), GPII (200mg/kg), GP III (300mg/kg), GP IV (600mg/kg)
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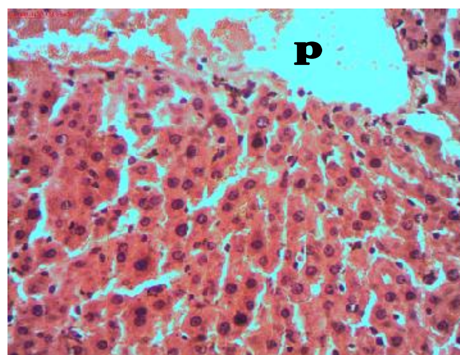

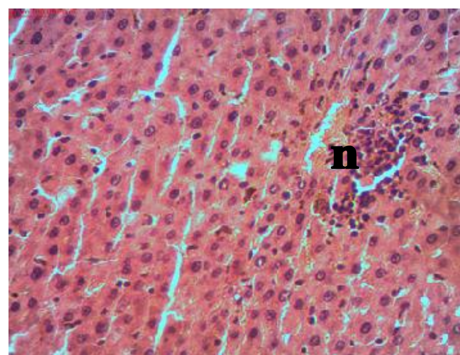

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553 Fig. 1a: Photomicrograph of normal hepatic tissue indicating portal area (p) and radially arranged cords of hepatocytes separated by sinusoids. (H&E stain) Mag. X400

Fig. 1b: Photomicrograph of hepatic tissue treatment with 600mg/kg of the formulation indicating focal necro-inflammation (n) around portal hepatics. (H&E stain) Mag. X400

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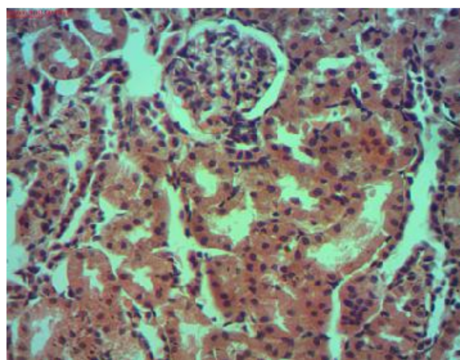

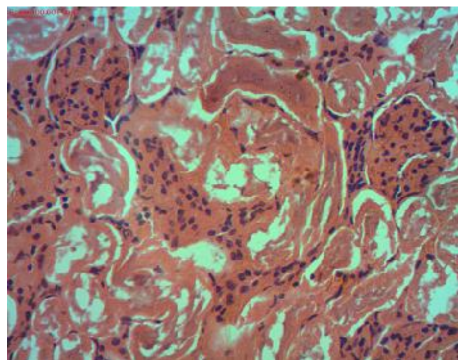

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560 Fig. 2a: Photomicrograph of a cross section of cortical region of the renal tissue of the control indicating renal corpuscles and convoluted tubules. . (H&E stain) Mag. X400

Fig. 2b: Photomicrograph of a cross section of cortical region of the treated animals (600mg/kg) showing normal appearance. (H&E stain) Mag. X400

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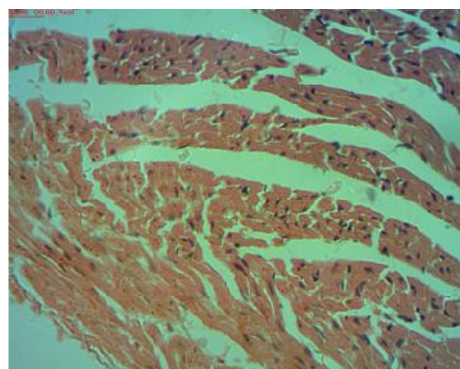

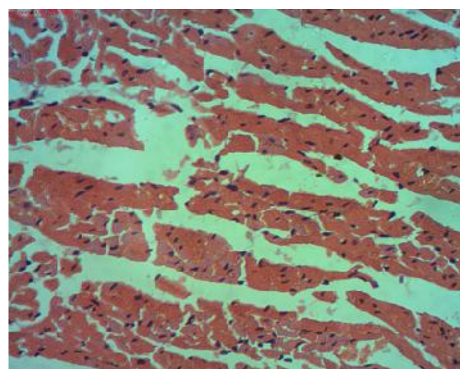

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565 Fig. 3a: Photomicrograph of a cross section of cardiac muscle of the control group showing the branched network of muscle fibres. (H&E stain) Mag. X400

Fig. 3b: The cross section of cardiac muscle of animal treated with 600mg/kg of the formulation indicating no abnormality. (H&E stain) Mag. X400

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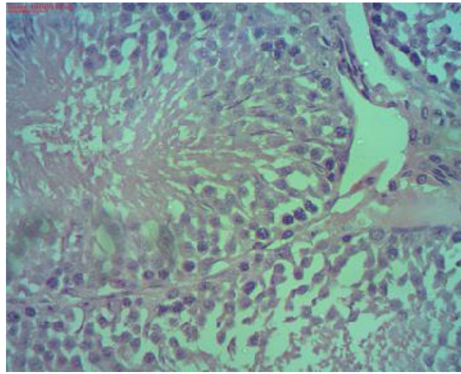

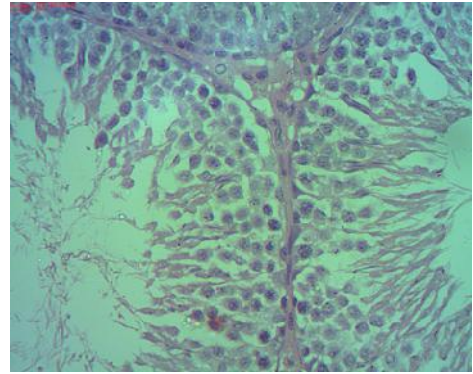

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Fig. 4a: The histology of testes of the control group showing cross sections of seminiferous tubules and interstitial cells. (H&E stain) Mag. X400

Fig. 4b: The cross section of seminiferous tubules of testis treated with 600mg/kg of the polyherbal drug showing no abnormality. (H&E stain) Mag. X400