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Research Article

Safety Evaluation of Oral Toxicity of Potential Anticancer Agents: An Acute and Sub-Chronic Toxicity Studies of Combinations of *Carica papaya* Linn, *Vernonia amygdalina* Delile and Dihydroartemisinin

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ABSTRACT

The increase in cancer prevalence rate worldwide is a public health concern. Studies have recognized the use of phytomedicines in cancer treatment but their safety data is deficient. *Vernonia amygdalina* Delile (VA), Carica papaya Linn (CP), and dihydroartemisinin (DHA) have been previously reported to have anti-cancer activities but their safety profiles when combined have not been validated. Pilot toxicity study was carried out to determine doses for acute and sub-chronic toxicity studies of the two combined plant extracts and compound in albino mice (n=10) and Wistar rats (n=18). The experiment was done based OECD guidelines. Animals were dosed with 500, 1,000 and 2,000 mg/kg of drug combinations and observed for 24 hours and 90 days. Hematological, biochemical parameters and histopathological changes of liver and kidney were evaluated. Drugs administration up to 5, 000 mg/kg did not cause mortality (LD_{50} > 5,000mg/kg) and concentrations of 2,000 mg/kg (acute study) did not show observed toxicity. There was no significant difference in body weight between the treated and control animals in 90 days study although there were observed significant differences in some hematology and biochemistry parameters. Histopathological examination of the liver and kidney did not reveal organ alterations. Although there were significant changes in hematological and biochemistry parameters, based on all results analysis and OECD classification of formulas as category 5, studied combinations might not cause known toxicity.

Keywords: *acute, sub-chronic toxicity, Carica papaya Linn, Vernonia amygdalina Delile, Dihydroartemisinin, biochemical analysis, hematological parameters, histopathology assessment*

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INTRODUCTION

In the last two decades, the use of natural-based medicines has gained growing popularity worldwide. According to the report by the World Health Organization (WHO), about 80% of people in the developing world rely on herbal medicines as part of their primary health care needs (Modak *et al*, 2007). Cancer has been a constant public health challenge globally accompanied by upsurge in research for cures and prophylactic therapies. The disease is characterized by uncontrolled growth of abnormal cells in the human body. Consequently, forming tumors of malignant cells with the potential to be metastatic (Sherr, 1996). The treatment and prevention of cancer has facilitated the mounting curiosity in research for using plants as medicine. In view of the natural origin of such products, they are associated with low toxicity. This is a false assumption as the plants in their solitary application, combinations, or administration with pure compounds or drugs are xenobiotic and may lead to toxicity through their metabolites. Furthermore, various drug interactions may take place leading to serious adverse events. Medicinal plants can be misused due to assumption that a natural product cannot cause toxic or adverse effects (Wang, 2012). The efficacy of these medicines is validated by their use in many communities around the world yet toxicological studies showed that plants, in some cases, can be harmful or even fatal, if given in high doses (ref). Therapeutic, medicinal and toxic substances are evident in the plants which can be harmful to humans and animals (WHO, 2015; Ferlay *et al*., 2013). Increasing pervasiveness of cancer has stimulated the interest in investigating medicinal value of plants and their possible application in cancer prevention and treatment.

Carica papaya (CP) is an evergreen, tree-like herb that has been recognized to have more than 5,000 compounds linked to anticancer properties such as phenolics, carotenoids and glucosinolates (Noor Atiqah, 2014). The plant is rich in

papain, which is effective against cancer cells. Papain breaks down the fibrin of cancer cell wall and protein into amino acid. In addition, C. papaya contains lycopene, a very reactive compound to oxygen and free radicals as well as isothyocynate that is effective against several cancers (eg lung, colon, pancreas, prostate cancer, leukemia and breast cancer). These compounds are proficient in inhibiting both formation and development of cancer cells (Rumiyati, 2006; Murukami *et al*., 1994; Rashed and Fouche, 2013). Carica papaya has been documented as being used in African traditional medicine to treat several forms of cancer including breast cancer (Omara, *et al*., 2020).

On the other hand, V. amygdalina (VA) is a bushy shrub or small tree of 2–5 m with petiolate, elliptic shape leaf of about 6 mm diameter. It is documented to have diverse therapeutic effects including anti-cancer properties. Similarly, the use of V. amygdalina in the management of cancer in African traditional medicine has been documented by Ayele (2018) particularly in Ethiopia. Chemical constituents such as coumarins, flavonoids, sesquiterpene lactones and edotides have been reported to be responsible for its anticancer activity (Ijeh *et al*., 2013; Wong *et al*, 2013).

Artemisinin is a sesquiterpene lactone containing an unusual peroxide bridge. According to Singh *et al*. (2006) in the Traditional Chinese Medicine, artemether injection was used to inhibit proliferation in advanced breast cancer cases. This was confirmed by Rowen (2002), that artemisinin has been used for about 30 years in Vietnam and China for cancer treatment and the knowledge of artemisinin for this purpose is growing (Tin *et al*., 2012; Lai *et al* 2013; Crespo-Ortiz and Wei, 2011). Dihydroartemisinin (DHA), which is the main active metabolite of ARTs, has been proven to be the most effective anti-cancer compound and has been established to subdue breast tumor-induced osteolysis through inhibiting the propagation, migration and incursion of MDA-MB-231 cells (Kongpatanakul, *et al*., 2009; Jiang, *et al*., 2018; Feng, *et al*., 2016). Several studies have also yielded positive results on various combinations of DHA with other compounds (Zhao, *et al*., 2017; Wu, *et al*, 2013), which has inspired its consideration in this study and thus the combination of CP, VA and DHA on animal model.

This study aimed to assess the safety profile of combined extracts of Carica papaya (CP) with Vernonia amygdalina (VA) and CP, VA with dihydroartemisinin (DHA) on animal model. To consider combinations of studied plant extracts and compound as potential anticancer agents in human cancer treatment, toxicity studies on animal models in accordance to Organization for Economic Co-operation and Development (OECD) guidelines is required. As stated above, all botanicals and compound that are part of studied combinations have been reported to have various health promoting as well as anticancer properties but safety profile of their combinations has not been assessed as yet (Wong *et al*, 2013; Lai *et al* 2013; Noor Atiqah, 2014).

MATERIALS AND METHODS

Collection and identification of plant material: Fresh leaves of V. amygdalina and C. papaya were collected in the morning (September 2017) from an organic certified farm in Luwero and taken to Makarere University Herbarium (MHU) for authentication with the Voucher specimens' numbers JN/001 and JN/ 002.

Preparation of methanol extract: The plant (leaves) were air dried under shade at ambient temperature of 25 to 30 °C for 5 to 10 days to constant weight. The dried material was pulverized into coarse powder by using mortar and pestle and then kept in air tight container at room temperature. One kilogram of each plant powder was soaked in 5 litres of methanol for 3 days with occasional shaking after every 8 hours. The acquired extract of each plant was filtered using Whatman filter paper (No.1) and concentrated in vacuum below 45 °C using a rotary evaporator. Extracts were left standing in a sterile hood until all methanol evaporated to obtain dry crude methanol extracts that were subsequently stored in - 4°C for further use.

Preparation of extracts and dihydroartemisinin combinations: Plant extracts and dihydroartemisinin were separately dissolved in DMSO to obtain stock solutions that were later dissolved in Phosphate-Buffered Saline (PBS) to achieve the following concentrations; VA= 1056 µg/mL, CP= 1600 µg/mL, DHA=118.289 µg/mL. The different concentrations were combined and mixed in a ratio of 1:1 (CP+VA) and 1:1:1 (CP+VA+DHA). The body weights (BW) of the animals were used to calculate the doses used in the toxicity study.

Experimental Animals: A total of 10 healthy female non-pregnant albino mice weighing 30–36 g and 18 female Wistar rats weighing 180–200 g, were used for this study. According to OECD 420 guidelines, female rodents can be used as they are more sensitive when being expose to hot thermal, chemical, inflammatory and mechanical nociception than the males. Female rodents were maintained at standard conditions of 25 ± 3 °C and relative humidity with a 12 h light/dark cycle. During 2 weeks, animals were randomized into experimental and control groups and kept to allow for their acclimatization to the laboratory conditions. Each group was housed in a separate, sanitized cage with sterile paddy husk as bedding. Free access to standard pellet diet and water ad libitum were given to the animals. All experimental procedures were in compliance with the Institutional Ethics Committee (Animal Models) of Makerere University, Kampala, Uganda.

Acute Oral Toxicity Study: An acute toxicity study was performed in compliance with the Organization for Economic Co-operation and Development (OECD) guidelines 420 for testing of chemicals (OECD, 2001). Albino mice (26.5-30 g) of about 3 months old, fed with commercially formulated rodent feed and water ad libitum were randomly selected. Ten female mice were divided into three groups (2 tested groups n= 4 and 1 control group n=2). Each group (CP+VA and CP+VA+DHA) was kept in a separate cage that was cleaned daily while food and water were changed every 24 h. The animals were allowed to acclimatise for 3 weeks. The mice were fasted overnight and their body weights determined in the morning. A preliminary pilot toxicity test was done using a single dose of 5 000 mg/kg BW for this study. Based on pilot

results as well as on OECD guidelines 420 (OECD, 2001), the single tested dose of 2,000 mg/kg was used for the acute study (i.e. according to the approximation that was done for mg/kg BW). The drugs were administered orally using intragastric tube. The negative control group received PBS/DMSO solution (in ration 3:1) that was used as a solvent in formulations at a dose rate of 10 ml/kg BW. After treated animals received food and water at 25 ± 3 °C in 12 h light/dark circadian cycle and were under special observation for 24h, with care given to first 4 h for any signs of acute toxicity like mortality, gross toxicity and behavioral changes.

Sub-chronic Toxicity Studies: Sub-chronic oral toxicity studies were performed with adaptations according to the Organization of Economic Co-Operation and Development (OECD) guidelines 452 for testing of chemicals (OECD 2008). A total of 18 female Wistar rats, weighing 180–200g were randomly divided into 4 groups of 4 rats per treatment group. The rats were grouped based on two different treatment doses of combinations with one control group (n=2) for both combinations. The animals were daily treated with CP+VA and CP+VA+DHA at doses of 500 and 1000 mg/kg for 90 days. At the end of treatment period, the rats were abstained of feed but left with drinking water ad libitium for 24 h and were sacrificed by decapitation under inhaled diethyl ether anaesthesia. Cardiac puncture into EDTA was used to collect blood samples from rats using capillary tubes into heparinised and non-heparinised centrifuge tubes for the haematological and biochemical studies respectively.

A deep longitudinal slit was made into the ventral surface of the abdomen and thorax of the sacrificed rats and by blunt dissection of the muscles and fasciae; vital organs such as liver and kidneys were made visible and harvested.

General observations, mortality, body weight, food and water intake: Observations for mortality, moribund and ill health, or reaction to treatment were carried out twice daily. These observations comprise changes in fur, skin, eyes, mucus membranes, salivation, diarrhea, sleep, behavioral pattern, tremors and coma. The observations were carried out in compliance to the Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (OECD, 2000). Individual rat was weighed before and during experimental period every two weeks. Mean body weight change was calculated for each dose level during the entire test period. Animals were allowed access to food throughout the study without any limit and food and water intake was monitored daily.

Hematological Parameters: Animals were anesthetized with light ether and blood samples were collected every two weeks from rats tail vain as well as via direct cardiac puncture at the end of sub-chronic study into EDTA containing tubes and analyzed using an automated analyzer (BC-3200). White blood cells (WBCs), lymphocyte (LYMPH), erythrocyte counts (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean cell volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were evaluated (Yuet *et al*., 2013).

Serum biochemical analysis: Blood serum was acquired by coagulation and centrifugation of the blood sample in the non-heparinized centrifuge tubes. Serum samples were analyzed for albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBili), total protein, creatinine and Urea (Das *et al*., 2015).

Histopathological assessment: The liver and kidney were carefully excised and fixed in 10% buffered formalin solution for 24 h in branded bottles in order to observe for any histopathological changes. Following fixation, the tissues were exposed to routine processing, embedded in paraffin and section at 3–5 µm. Tissue sections were stained with hematoxylin and eosin using UIS2/UIS Series Olympus BX51 microscope (Shinjuku Monolith, 3-1, Nishi Shinjuku 2-chrome, Shinjuku-ku, Tokyo, Japan).

Statistical Analysis: All values were expressed as the mean ± SD (standard deviation) and the results were analyzed statistically by one-way Analysis of Variance (ANOVA), followed by F-test and Fisher's Least Significant Difference (LSD) comparison tests using statistical IBM SPSS 21 (IBM SPSS 21 Software, Inc., USA) version 21.0. Level of significance was considered at values $p < 0.05$.

RESULTS

Acute toxicity study of both combinations: Methanolic extracts of CP+VA and CP+VA+DHA at a dose of 2,000 mg/kg lacked any lethal or toxic effect on the behavioral responses of the treated mice observed first for short period of 4 hours then followed by long period of 24 h. There were no indications of changes in breathing, skin, water consumption, body temperature of treated animals and impairment in food intake and temperature of treated animals. Therefore, both drugs seemed to be safe at a dose level of 2 000 mg/kg, and the LD50 was considered to be >2 000 mg/kg. Preliminary pilot study, where dose was tested up to 5000 mg/kg, did not induce any lethal effect.

Effect of CP+VA and CP+VA+DHA combinations on body weight in sub-chronic study: Daily administration of the combined extracts of CP+VA and CP+VA+DHA at a dose of 500 and 1000 mg/kg to the treated animals caused non-significant ($p>0.05$) changes in body weight throughout the 90 days period (Table 1).

Table 1
Effects of CP+VA and CP+VA+DHA combinations on animal weight in subchronic study

Study	Group	Dose (mg/kg)	Weight (g)
Subchronic (90 days)		Control	183.4 ± 10.6
	CP+VA	500	201.6 ± 2.7
		1000	179.7± 19.9
	CP+VA+DHA	500	165.4 ± 32.9
		1000	199.8 ± 9.1

Effect of CP+VA and CP+VA+DHA combinations on hematological parameters in the 90-days studies: The effect of sub-chronic administration of CP+VA and CP+VA+DHA combinations on hematological parameters are

presented in Table 2. Most hematological parameters (lymphocyte, erythrocyte counts, hemoglobin concentration, hematocrit, mean corpuscular hemoglobin, mean cell volume, and mean corpuscular hemoglobin concentration) in the treated animals were not significantly different from the control, except for white blood cells with statistical significance ($p<0.05$) during the 90 days of treatment period the in group treated with CP+VA at 500 mg/kg (WBC=9.2±2.4) and 1000 mg/kg (WBC=7.8±2.0) compared to control (WBC=13.9±4.8), while the group receiving CP+VA+DHA at 500mg/kg (WBC=8.8±2.4) but at 1000 mg/kg the values came back to normal.

Effect of CP+VA and CP+VA+DHA combinations on serum biochemical parameters in 90-days studies: As shown in Table 3, the sub-chronic administration of CP+VA combination had no effect on the serum electrolytes after the 90-day treatment period, while for animals treated with CP+VA+DHA, only total protein level had changed for both concentrations, 500 mg/kg (70.6±10.6) and 1 000 mg/kg (69.5±8.3) compared to controls (86.0±5.8).

Effect of CP+VA and CP+VA+DHA combinations on histopathological assessment in sub-chronic studies: The light microscopic examinations of the transverse section of tissues of the kidneys and liver of the control and treated animals are shown in Plates 1 and 2. Histopathological examination of the control group and CP+VA and CP+VA+DHA combinations treated animals showed normal cellular appearances with no alteration and absence of any gross pathological lesion in organs.

Table 2.
Effect of CP+VA and CP+VA+DHA combinations on hematological parameters in rats in the sub-chronic toxicity studies

Parameter	Control	CP+VA		CP+VA+DHA	
		500mg/kg	1000mg/kg	500mg/kg	1000mg/kg
Total white blood cells (x 10^9/L)	13.9±4.8	9.2±2.4*	7.8±2.0**	8.8±2.4*	9.8±2.9
Lymphocyte (%)	4.2±2.2	3.5±1.1	2.8±1.3	3.4±1.5	3.0±1.1
Erythrocyte counts (x 10^{12}/L)	9.5±0.1	9.0±1.6	8.8±1.3	9.1±0.9	8.8±0.6
Hemoglobin (g/dL)	16.1±0.5	15.5±1.8	15.0±1.2	16.0±1.4	15.4±1.0
Hematocrit (%)	55.7±2.4	57.1±8.7	54.9±5.0	56.9±6.8	56.5±5.4
Mean corpuscular volume (fL)	58.6±3.6	64.1±4.2	62.8±6.5	62.2±2.9	64.1±4.7
Mean cell hemoglobin (pg)	16.9±0.8	17.5±1.2	17.1±1.5	17.6±2.2	17.5±0.8
Mean cell hemoglobin concentration (g/dL)	28.8±0.4	27.3±1.6	27.3±1.4	28.4±3.4	27.4±1.7

Values are mean ± SD (n= 4).
**p<0.05; ** p<0.01 significantly different from control (One-way ANOVA followed by Least Significance Difference (LSD) posthoc tests).*

Table 3.
Effect of CP+VA and CP+VA+DHA combinations on biochemical parameters in rats in the sub-chronic toxicity studies

Parameter	Control	CP+VA		CP+VA+DHA	
		500mg/kg	1000mg/kg	500mg/kg	1000mg/kg
Albumin (g/L)	35.0±0.0	30.0±4.8	29.6±4.9	29.4±5.0	28.9±2.4
Alanine aminostranferase (U/L)	95.5±8.7	88.5±18.2	76.4±20.3	74.3±17.4	114.6±52.1
Aspartate aminostransferase (U/L)	251.0±18.5	168.5±36.0	159.3±42.4	162.8±45.6	190.6±123.7
Total bilirubin (mg/dL)	1.3±0.3	1.4±0.8	2.1±1.1	2.1±1.2	1.1±0.7
Total protein (g/L)	86.0±5.8	71.8±10.4	74.4±9.9	70.6±10.6*	69.5±8.3*
Creatinine (μmol/L)	53.2±1.4	44.3±5.7	48.8±7.5	50.7±8.2	44.2±7.9
Urea/Bun (Mmol/L)	7.7±0.3	8.6±1.7	8.1±1.9	10.0±3.1	9.0±1.1

Values are mean ± SD (n= 4).
**p<0.05; ** p<0.01 significantly different from control (One-way ANOVA followed by Least Significance Difference (LSD) posthoc tests).*

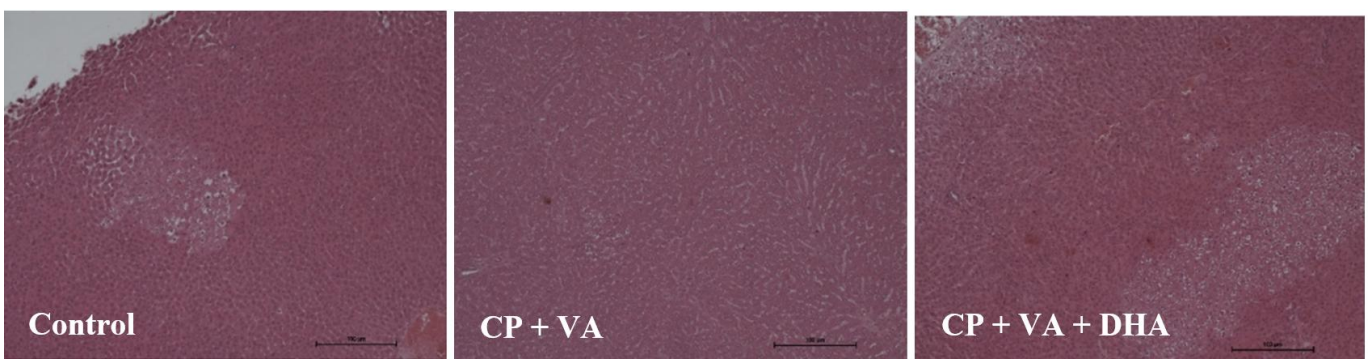

Plate 1
Photomicrographs of sections of the liver in control rats and rats treated with 1000mg of CP+VA and CP+VA+DHA extracts daily for 90 days. No significant damage was observed in all treated groups (x 10 magnification).

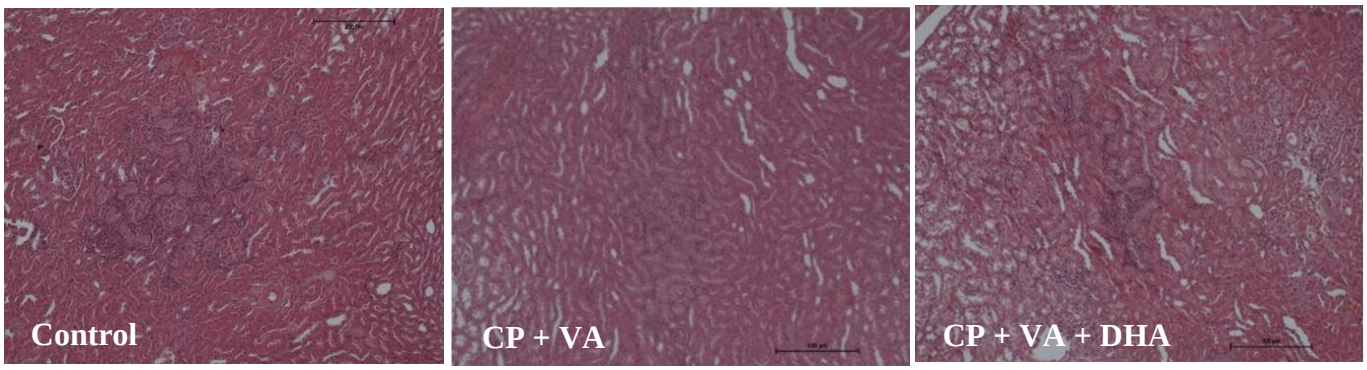

Plate 2
Photomicrographs of sections of the kidneys in control rats and rats treated with 1000mg of CP+VA and CP+VA+DHA extracts daily for 90 days. No significant damage was observed in all treated groups (x 10 magnification).

DISCUSSION

For several centuries, medicinal plants have been the basis for the treatment of various ailments (Wang *et al*., 2014). Carica papaya has been recognized to have more than 5,000 compounds linked to anticancer properties such as phenolics, carotenoids and glucosinolates (Noor Atiqah, 2014). On the other hand, Vernonia amygdalina has been reported to contain chemical constituents such as coumarins, flavonoids, sesquiterpene lactones and edotides which are responsible for its anticancer activity (Ijeh *et al*., 2013). Artemisinin has been used for years in Vietnam and China to treat cancer (Rowen, 2002; Singh *et al*., 2006). The use of plant extracts in combinations with other plants together with or without pure compounds may provide an advantage over using one isolated or synthetic compound as many formulations contain several active components with different possible intracellular targets. According to Yang *et al*. (2014), the mechanisms fundamental to synergistic therapeutic actions of herbal medicines revealed that different agents may determine either the same or different targets in numerous pathways. In terms of cooperating in an agonistic or synergistic way; regulating the enzymes and transporters that are included in hepatic and intestinal metabolism to enhance oral drug bioavailability; overcoming the drug resistance mechanisms of microbial and cancer cells; and lastly eliminating the adverse effects and enhancing pharmacological potency of agents by "processing" or by drug-drug interaction. Although chosen botanicals and compounds have potentially proven to prevent and /or treat cancer and are relatively safe when used individually (Tarkang *et al*., 2012; Teo *et al*., 2002; Tin *et al*., 2012; Wang *et al*., 2012; Wang *et al*., 2014; Singh *et al*., 2020; Akowuah *et al*., 2015; Jiang *et al*., 2018; Galal *et al*., 2008; Hossain *et al*., 2020), a thorough toxicity study of their combinations is important to ascertain their safety profile as it may differ to their individual application based on their mechanism of actions. C. papaya leaves extracts modulate the immune system by improving the production of Th1 type cytokines, such as interleukin (IL-12), interferon (IFN-gamma) and tumor necrosis factor (TNF-alpha) and fractions with molecular weight less of than 1000 are most active in constraining tumor cell growth (Otsuki *et al*., 2010). V. amygdalina include mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) activity induced attenuation (Izevbigie *et al*., 2004) but the synergetic effect of the two combined extracts may affect their biological activities.

During the toxicological assessment of any herbal drug, the determination of LD50 is generally a preliminary step to be conducted in order to detect the nature and implication of adverse effects as well as to determine limits of exposure level where the effect has occurred (Ifeoma and Oluwakanyinsola, 2013). For example, according to Halim *et al*. (2011), Sprague dawley rats treated with 2,000 mg/kg dose of the leaf extract of CP did not produce mortality or significant changes in the body weight, food and water consumption. This indicated that the plant extract is reasonably safe as classified by the Organization for Economic Cooperation and Development (OECD) Guidance Document for Acute Oral Toxicity Study (OECD, 2001; Tarkang *et al*., 2012). But acute toxicity data are of limited clinical application since cumulative toxic effects could occur even at very low doses over longer period of time. Hence, multiple dose studies are usually invaluable in evaluating the safety profile of phytomedicines that target management of chronic disorders (Abotsi *et al*., 2011; Ismail *et al*., 2014). In this present study, the acute and sub-chronic toxicity profiles of combined leaves extract of C. papaya, V. amygdalina and dihydroartemisinin were evaluated in mice and rats using measurement of body weights, haematological, biochemical and histological assessment.

Body weight changes observed are indicators of adverse effects of drugs and chemicals (Raza *et al*., 2002; Teo *et al*., 2002; Santos *et al*., 2009). Daily oral administration of CP + VA and CP+VA+DHA combinations over the period of 90-days showed insignificant changes in body weight gain or loss pattern in the treated animals compared to the control rats. Thus, the results indicated that there was no effect on normal growth of animals at the administration of oral doses of CP + VA and CP+VA+DHA throughout the study.

Assessment of hematological parameters can be used to determine the extent of deleterious effect of foreign compounds including plant extracts (Daradka, 2016). The extensive sensitivity of the hematopoietic system to toxic compounds serves as an important index of the physiological and pathological status (Kulkarni and Veeranjaneyulu, 2012). Such toxicity testing is pertinent for variations in the

hematological system which has a higher predictive value for human toxicity, when extrapolated from animal studies. The hematology results attained from this study showed significant decrease in WBC parameters compared to the controls after 90-days oral administration of studied combinations. No significant changes were observed in other parameters.

Hematopoiesis is the lifelong process of continuous formation and turnover of blood cells to meet everyday demands as well as respond to increased demand (Hoggatt and Pelus, 2013). The significant ($p<0.05$) decrease of WBC may imply the impact of CP+VA leaves extracts combination in decreasing the immune system of the treated rats (Okonkwo *et al*., 2019). In the case of CP+VA+DHA combination, significant change of WBC was observed only at lower concentration of 500 mg/kg, with WBC parameter normalizing at higher concentration of 1000 mg/kg, leading to conclusion that the change may not be dose dependent or not induced by drug administration but related to other unknown factors, such as infection or tissue damage (Olusola *et al*, 2015).

The biochemical analysis were carried out to assess the possible changes in the hepatic and renal functions influenced by the combinations compared to control rats in order to identify possible pathological changes. Combinations of CP + VA and CP+VA+DHA did not show any significant changes in all measured serum biochemical parameters in the 90 days of treatment except for significant decrease in plasma total protein (for CP+VA+DHA) levels. Liver and kidney are the most sensitive and main target organs of body detoxification. The most important parameters used to assess the function of the liver are ALT, AST, albumin and alkaline phosphatase (Lakshmi et al., 2013; Mossa et al., 2015) and lack of significant changes in these parameters may indicate formulas being non-toxic to this organ. Measurement of total protein parameter can represent nutritional status and may also be used to screen for and help diagnose a liver disorder or kidney disease (Patrick-Iwuanyanwu et al., 2012), but with other liver parameters being in norm it can be concluded that changes in TP values are nutritional rather than liver disease-related. Histopathology assessments are performed in order to confirm possible toxic influence of tested drug on animal vital organs and are important indicators of general drug toxicity (Kaid et al., 2019). Although studied combinations of plant extracts and compound exerted significant changes in blood parameters of tested animals, they did not cause significant histopathological changes in the liver and kidneys of the treated rats. Preliminary pilot study, where there was no loss of animal once treated with both combinations up to 5,000 mg/kg, together with unaltered histopathology results, strongly supports the non-toxic activities of both studied combinations and their safety profile can be classified as category 5 in accordance to OECD guidelines (OECD, 2001).

In conclusion, the combinations of CP+VA and CP+VA+DHA administrated in two different doses provided valuable data in the acute and sub-chronic studies. The two different combinations have been reported in this manuscript to be non-toxic in sub-chronic administration with regard to the body weights and histological assessment but significant effects have been revealed on the hematological and biochemical parameters of rats. Further studies are needed for experimentation on the reproductive capacity, fetuses and pregnant rats to complete the safety profile of these combined plants and compound.

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