Acute and Sub- chronic Toxicity Study of Encapsulation of Combine Plants Extract of *Ficus deltoidea* and *Gynocthodes sublanceolata* in Balb/c Mice Model

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Abstract

This study aimed to evaluate the safety of dose range of encapsulated extract with Nano hydroxyapatite from a combination of *Ficus deltoidea* and *Gynocthodes sublanceolata* leaves by inducing "acute and sub-chronic toxicity" based on an animal model. A single dose (300, 2000 and 4000 mg/kg) of encapsulated combination extracts were administrated orally in the acute toxicity trial and the toxic effects were assessed up to 72 h post-treatment. While, in the sub-chronic study, the encapsulated combination extract was given orally at doses of 600 12 and 1000 mg/kg for 28 days. Hematological and histopathological analysis of some vital organs were evaluated. Neither in the acute toxicity trial nor the sub-chronic toxicity groups, was mortality observed throughout the experimental period. Significant increasing was seen in the SGOT and SGPT level following the administration of tested encapsulated plant extract (600, 1000 mg/kg) and histological evaluation showed the normal tissue limit of liver, kidney and spleen. It was concluded that the oral treatment with encapsulated plant extract did not appear adverse side effect and it is safe to use for therapeutic purposes.

Key Words: Toxicity, Medicinal herb, Safety, *Ficus deltoidea* and *Gynocthodes sublanceolata*

Significance | The use of encapsulation of combine plants extract and its mechanism of safety are meeting requirements on the level of approach chosen in this study which might be applied for future human pre- clinical studies.

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Edited by Md. Shamsuddin Sultan Khan, University of Western Sydney. And accepted by the Editorial Board Dec 30, 2019 (received for review Aug 19, 2019)

Introduction

People in Malaysia prefer to use traditional medicine before modern medicine is introduced. A complete report on the Malay traditional medicinal plants was compiled in a book, entitled "A Dictionary of the Economic Products of the Malay Peninsula". This book has inspired both phytochemists and ethno-botanists to conduct research on medicinal plants, which helps add knowledge to Malaysian medicinal plants (R. Renuka et al., 2001, Al-Sokanee et al., 2009). *F. deltoidea* and *G.sublanceolata* leaves are used as traditional medicine. These plants are used to treat various sickness and diseases. Malaysia is famous for *F.deltoidea* called "Mas Cotek". This plant belongs to *Moraceae* family. *F.deltoidea* leaves have two kinds: female and male. Female leaves have higher active ingredients, so this kind of leaves are used for medical purposes compared to male ones. Hail of *Rubiaceae* family includes *G. Sublanceolata* leaves called Pitang. These leaves turn brown when they are dry. It is very difficult to find these leaves because they are very rare; however, we can find a huge number of these plants in Thailand. These plants have a diversity of

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Please cite this article:
Rafida Kh. Ahmed A, M. Syarhabilahmad A, Midhat Nabil A, Fouad Saleih R. Al –Suede B,* (2019). Acute and Sub- chronic toxicity study of encapsulation of combine plants extract of *Ficus deltoidea* and *Gynocthodes sublanceolata* in Balb/c mice model., Journal of Angiotherapy, 3(1), pages 147-155.

Pigments, namely xanthophyll, chlorophyll, flavonol, flavones, carotene and anthocyanin (Mucalo et al., 2004). The biochemical ingredient of the plants has a significant role as the natural antioxidants and the activity of the extracted plant polymers is higher when they consumed as crude and the combination of different extracts (R. Renuka et al., 2001). However, the major disadvantage is that the quantity of herbal extract necessary for the treatment is higher because the degradation of different plant compounds such as alkaloids, flavonoids, phenols, steroids, and anthraquinon in the gastrointestinal tract. It is due to the acidic pH in the stomach, which increase their breakdown, loss of the effect and increment with the duration of treatment with the decreased absorption of these compounds in the intestine (Al-Sokanee et al., 2009). Currently, several researchers and studies are concentrated on encapsulation/capping of the plant extracts to increase the sustained release of active compounds in the intestine for the maximal absorption (Mucalo et al., 2004, Hench et al., 1991, Ivone 2001, Kavitha et al., 2017, Walum et al., 1998, Yehya et al., 2019, Gopinath et al. 2016). Recently, nanotechnological methods have activated the advanced delivery systems, which include the controlled drug delivery to the site of action by developing hydroxyapatite and other nanoparticles (Anniebell and Gopinath, 2018, Suk et al., 2018. Hydroxyapatite [Ca10(PO4)6(OH)2] (HAp), is the main constituents of bone and teeth, a biomaterial in view of its excellent bioactivity, biocompatibility, non-toxicity and non-inflammatory. HAp nanoparticles can be prepared by the direct precipitation method possess the advanced important characteristics such as, large pore volumes, high surface area and reactive surfaces for post-functionalization and pure powder which make them ideal as potential carriers for the plant extract as drugs (Cao et al., 2014). Moreover, it has also been confirmed that HAp nanoparticles with the size lesser than 100 nm could be up taken by the cells efficiently. The biocompatibility and bioactivity of HAp nanoparticles represent a choice for the controlled drug delivery (Matouskova et al., 2016). Using nanotechnology with phyto-extracts has explored a beneficial strategy for herbal drugs including the enhancement of bioavailability, bioavailability, solubility, sustained delivery pharmacological activity, physical and chemical degradation and protection from toxicity (Arunachalam et al., 2017, Theivasanthi et al., 2018). This is the first study were conducted on the ingestion of these encapsulated combine of plant extract with nanoHAp at high doses. Therefore, there is a need to explore the systemic for evaluating their efficacy and safety properties. Hence, this study aims to evaluate the safety of this extract with acute and sub-chronic toxicity tests in BALB/c mice model.

Materials and methods

Samples Collection and Extraction

Ficus deltoidea (female leaves) and *Gynochthodes sublanceolata* were collected from the covered green house garden of School of Bioprocess Engineering and University Malaysia Perlis. Then, they were cleaned with a running tap water to remove debris and contamination. Each set of the collection was dried at room temperature for two weeks. The dried leaves were ground, then 100 grams (50 grams from each plant leaves) were mixed grams were mixed with 2000 ml of a methanol: distilled water (60:40 % v/v) was put in a beaker and covered with aluminum foil at an ambient temperature about 24 h and shaken during the extraction. The extract was filtered through Whatman No.1 filter paper. Then, the solvent was removed from samples using a rotary evaporator then freeze dryer was used to transform the sample into the powder form. Finally, the extract was placed in air-tight amber bottles and stored in a freezer to prevent the oxidation of damage until further use (Kavitha et al., 2017).

Hydroxyapatite (HAp) Preparation by direct precipitation

In this experiment, HAp was obtained by a direct precipitation. According to Song et al. the Stoichiometric reaction of calcium nitrate tetrahydrate ($Ca(NO_3)_2.\ 4H_2O$ and ammonium phosphate ($NH_4H_2PO_4$) will yield the hydroxyapatite as below;

$$5Ca(NO_3) + 3(NH_4)_2HPO_4 + H_2O \rightarrow Ca_5(PO_4)_3OH + 6NH_4NO_3 + 4HNO_3$$

Calcium nitrate tetrahydrate ($Ca(NO_3)_24H_2O$ and ammonium phosphate ($NH_4H_2PO_4$) were dissolved in 100 ml distilled water at room temperature. In this experiment, calcium nitrate tetrahydrate act as a calcium source, while ammonium dihydrogen phosphate acts as a phosphorus source. The concentration 100 mM of ($Ca(NO_3)_24H_2O$ and 60 mM of ($NH_4H_2PO_4$) was applied to give the molar ratio of calcium to phosphate is 1.67. The solution then was poured and mix into a set up magnetic stirrer beaker on the hot plate at 60 °C while the stirring rate at 550 rpm the mixing was conducted for 1 hour. After that, the suspension will be filtered by using Whatman filter No.1 paper, the drying process was carried out in an oven at a temperature of 60 °C for 24 hours. Finally, the dried sample was crushed by using pestle and mortar for 5 minutes.

Preparation of Encapsulated Phyto-extract Nano-hybrid with Hap

Nanoparticles containing the phyto-components combination from the test plants were prepared by solvent evaporation method described before with the additional modifications (Bernard, S. A., &Olayinka, 2010. Accurately weighed amount of HAp and plant extract with mass ratio were chosen, which includes 1:5. They were separately dissolved in distilled water containing ethanol for plant extract and ethanol for HAp, then mixed and stirred for 2 hr to evaporate the organic solvent and left for 24 hr at room temperature. The nanoparticles formed were isolated by centrifugation for 15 minutes at 10000 xg. Finally, the nanoparticles were washed with deionized water to remove the residual solvent.

Test animal

The animal study was approved and conducted in strict guidance according to *Eman Research Animal Ethics Committee Malaysia* (Reference #: 112074A2212130719. Healthy BALB/c mice in a weight of 20–30 g were kept in an animal house in EMAN Biodiscoveries, Malaysia. Plastic cages (34 × 47 × 18 cm3) at animal house are used to keep animals. There were five mice in each cage which is in an air conditioned environment at room temperature of (25 ± 2)°C with relative humidity (60% ± 10%) under 12 h night and light cycle. The animals were fed with commercially available standard pellet chow and unlimited supply of filtered drinking water.

Acute toxicity study

The oral acute toxicity study of encapsulated extract was evaluated according to Organization for Economic Cooperation and Development (OECD) guideline 423 on BALB/c mice (20–30 g) (Walum, 1998), where the test doses of 300, 2000 and 4000 mg/kg were used. Before the experiment was done all the animals free excess to water, andwere kept at overnight fasting. The mice were divided into four groups of five animals for each group each (n=5). The 1st group belongs to the control, whereas the 2nd, 3rd and 4th groups are experimental groups that received orally encapsulated extract (in normal saline) at dose of 300 mg/kg, 2000 mg/kg and 4000 mg/kg respectively. Before dose administration, the body of each animal was weighed, and there was a calculation of the dose based on the body weight An animal was not observed any toxic effect for the first 4 hour after a period of treatment More animals were observed and investigated within 3 days for if any toxic effect. It was found that there were behavioral changes and other parameters such as body weight, urinations, food intake, water intake, respiration, convulsion, tremor, temperature, constipations. Also, their eye and skin colors changed etc (Yehya et al., 2019).

Sub-chronic toxicity study

According to OECD guideline 407 (Gopinath et al., 2016), oral sub-chronic toxicity study was conducted. Fifteen healthy BALB/c mice (20–30 g) were divided into three groups five animal each and kept under standard conditions. The control group belongs to Group I; whereas the experimental groups are the other two groups which received the encapsulated extract at a dose of 600 and 1000 mg/kg, respectively within 28 consecutive days (Anniebell, and Gopinath, 2018). The control group was received normal saline. The second and third groups were given with a single dose of 600 mg/kg and 1000 mg/kg of body weight of encapsulated extract, respectively. Gavage dosing was performed using a curved, ball-tipped intubation needle affixed to a 5 ml syringe. Fresh preparation of solutions was made prior to dosing and they were kept chilled and tightly capped.

Hematological and biochemical examination

At the end of study, animals should be anaesthetized with a ketamine and xylazine with 0.05-0.1mL/10g body weight . 1mL of ketamine (100mg/mL) and 0.5mL xylazine (20mg/mL) single dose. after anovernight fasting (8 h). Test tube contains blood sample with and ethylene diaminetetra acetic acid as an anticoagulant, and without it respectively for biochemical and hematological parameters. Biochemical analysis was done through the evaluation of blood without the ethylene diamine tetra acetic acid, allowed to clot after centrifugation at 2 500 r/min for 15 min to obtain serum and stored at −20°C until it was assayed for biochemical estimation. After blood was collected, all important organ, namely liver, kidney, lung, heart, pancreas and small intestine were harvested. Each organ was weighed on electronic balance to observe any changes in organs weights of treated animals compared to control group)(Suk et al., 2018). The relative organ weight (ROW) of each organ was calculated as follows (Anniebell, and Gopinath, 2018):

$$ROW = (\text{Absolute organ weight (g)} / \text{mice body weight on sacrifice day}) \times 100$$

Effect of encapsulate plant extract on hematological parameters

Red blood cell count, hematocrit, mean cell volume, hemoglobin, white blood cell count, mean corpuscular hemoglobin concentration, mean corpuscular volume, monocyte, neutrophil, lymphocyte and platelet count of the control and treated groups were determined and compared with control group using an automatic

hematology analyzer (Sysmex K21, Tokyo, Japan).

Effect of encapsulate plant extract on serum biochemical parameters

There was an biochemical analysis on serum after collected blood was centrifugated, and the following parameters such as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, high density lipoprotein, total bilirubin (TBIL), total protein, albumin, urea and creatinine level were determined for both control and extract treated groups. All analyses were done through the analyzer of clinical chemistry.

Histopathological studies

After the experimental stage, the animals were sacrificed and necropsied. Different visceral organs including kidney, liver and lung were collected and fixed in 10% formaldehyde solution. The tissues were further processed with the Automatic Tissue Processor and sectioned at 5 µm thickness using the Rotary Microtome (Heitz 150 Rotary Microtome, Cambridge model) and embedded in paraffin wax to prepare blocks. Sections were stained according to Haematoxylin and Eosin (H and E) technique for microscopically examination (Suk et al., 2017). Subsequently, the sections were examined using Swift Binocular

in-built lighting system and photographed using a microscope-digital-camera with an Olympus photomicroscope.

Statistical analysis

Statistical analysis was done as mean of variance ± SEM (n = 5),

Table 1. General appearance and behavioral observations of acute toxicity study for control and treated groups.

Observation	Control	300 mg/kg	2000mglkg	4000mglkg
Digestiondisturbances	NO	NO	NO	**NO**
Food intake	Normal	Normal	Normal	**Normal**
Temperature	Normal	Normal	Normal	**Normal**
Rate of Respiration	No effect	No effect	No effect	**No effect**
Urination disturbances	Normal	No effect	No effect	**No effect**
Change in skin	No effect	No effect	No effect	**No effect**
Drowsiness	Not present	Not present	Not present	**Not present**
Eye color	No effect	No effect	No effect	**No effect**
Sedation	Normal	No effect	No effect	**No effect**
Diarrhea	Not present	Not present	Not present	**Not present**
General physique	Normal	Normal	Normal	**Normal**
Coma	Not present	Not present	Not present	**Not present**
Death	**NO**	**NO**	**NO**	**NO**

NO: Not observers

Table 2. Effects of the encapsulation on body weight of mice of sub-chronic toxicity study at different times.

Groups	1st Day	9th Day	18th Day	28 th Day
Control	25.12 ± 0.395[a]	26.67 ± 0.593[a]	27.80 ± 0.588[a]	**28.77 ± 0.600[a]**
600 mg/kg	25.22 ± 0.555[a]	26.98 ± 0.233[a]	27.99 ± 0.899[a]	**28.98 ± 0.898[a]**
1000 mg/kg	**25.34 ± 0.764[a]**	**27.77 ± 0.674[a]**	**28.16 ± 0.190[a]**	**29.11 ± 0.180[a]**

Values are presented as mean ± SEM; N = 5. were not significantly different ($p > 0.05$). Statistical comparisons were made within a column and values with the same superscript letter are not significantly different.

Table 3. Effect of oral administration of encapsulated combine extracts on average organ weight (g) of mice of sub-chronic toxicity study for control and treated groups.

Organ weight	Normal	600mg/kg	1000mg/kg
Heart	0.159 ± 0.001[a]	0.158 ± 0.010[a]	**0.156 ± 0.022[a]**
Liver	0.772 ± 0.015[a]	0.776 ± 0.014[a]	**0.780 ± 0.043[a]**
Pancreas	0.135 ± 0.016[a]	0.137 ± 0.013[a]	**0.130 ± 0.007[a]**
Kidney	0.333 ± 0.011[a]	0.329 ± 0.020[a]	**0.331 ± 0.030[a]**
Intestine	0.660 ± 0.017[a]	0.662 ± 0.019[a]	**0.662 ± 0.032[a]**
Average body weight on the sacrifice	**26.430 ± 0.130[a]**	**26.370 ± 0.007[a]**	**27.300 ± 0.001[a]**

Values are expressed as mean ± SEM. $P > 0.05$ when compared to control group. Statistical comparisons were made within a column and values with the same superscript letter are not significantly different.

Table 4. Effect of oral administration of encapsulated combine extracts on relative organs weight (g) of mice of sub-chronic toxicity study for control and treated groups.

Organ weight	Normal	600mg/kg	1000mg/kg
Heart	0.629 ± 0.002[a]	0.619 ± 0.019[a]	**0.617 ± 0.011[a]**
Liver	3.210 ± 0.016[a]	3.243 ± 0.016[a]	**3.250 ± 0.053[a]**
Pancreas	0.514 ± 0.021[a]	0.524 ± 0.011[a]	**0.570 ± 0.017[a]**
Kidney	1.310 ± 0.015[a]	1.312 ± 0.029[a]	**1.298 ± 0.023[a]**
Intestine	2.580 ± 0.014[a]	2.565 ± 0.023[a]	**2.523± 0.012[a]**
Lung	**0.91±0.15[a]**	**0.90±0.32[a]**	**0.89±0.057[a]**

Values are expressed as mean ± SEM. P > 0.05 when compared to control. . Statistical comparisons were made within a column and values with the same superscript letter are not significantly different.

Table 5. Effect of encapsulated combine plants extract on hematological parameters of sub-chronic study.

Parameters	Normal group	600 mg/kg	1000 mg/kg
Total RBC (10^{12}/L)	8.620 ± 0.514[a]	9.160 ± 0.923[a]	**9.040 ± 0.722[a]**
Hemoglobin (g/L)	11.446 ± 0.612[a]	11.230 ± 1.520[a]	**12.230 ± 1.200[a]**
PCV (L/L)	41.445 ± 2.020[a]	40.520 ± 3.101[a]	**40.420±2.83[a]**
MCV (fL)	46.230 ± 1.115[a]	45.199 ± 3.830[a]	**45.340±2.420[a]**
MCH (pg)	15.000 ± 1.000[a]	15.350 ± 0.545[a]	**15.670±0.555[a]**
MCHC (g/dL)	33.500 ± 2.700[a]	34.420 ± 0.900[a]	**35.000 ± 0.300[a]**
Platelet count (10^9 /L)	308.000 ± 32.500[a a a]	311.000 ± 29.300[a a]	**310.013 ± 32.500[a]**
WBC (10^9 /L)	11.550 ± 1.031[a]	10.000 ± 2.0324[a]	**10.210 ± 1.345[a]**
Neutrophil (%)	26.250 ± 2.570[a]	26.200 ± 1.736[a]	**27.100 ± 2.689[a]**
Lymphocyte (%)	53.210 ± 5.140[a]	54.133 ± 3.172[a]	**55.250 ± 3.510[a]**
Monocyte (%)	**4.000 ± 1.020[a]**	**4.101 ± 1.050[a]**	**4.750 ± 0.885[a]**

Table 6. Effect of oral extract on serum biochemical parameters of sub-chronic study

Parameters	Normal group	600 mg/kg	1000 mg/kg
Urea (mg/dL)	32.40 ± 2.22[b]	30.82 ± 0.30[b]	**32.90± 0.16[b]**
Creatinine (mg/dL)	1.05 ± 0.11[b]	0.95 ± 0.10[b]	**0.89 ±0.83[b]**
Uric acid (mg/dL)	0.51 ± 0.33[b]	0.45 ± 0.01[b]	**0.43 ±0.01[b]**
Total cholesterol (mg/dL)	115.90 ± 12.06[b]	106.00 ± 0.32[b]	**107.60± 13.21[b]**
Total protein (g/dL)	6.30 ± 0.30[b]	6.27 ± 0.79[b]	**6.85± 0.23[b]**
Bilirubin (g/dL)	0.35 ± 0.01[b]	0.35 ± 0.15[b]	**0.33 ±0.12[b]**
Albumin (g/dL)	2.80 ± 0.22[b]	2.57 ± 0.14[b]	**2. 93 ±0.37[b]**
Globulin (g/dL)	34.00 ± 2.00[b]	32.00 ± 5.00[b]	**31.00 ± 0.01[b]**
SGOT (AST) (IU/L)	114.00 ± 12.00[b]	118.46 ± 33.40[b]	**125.33 ± 11.20[b]**
SGPT (ALT) (IU/L)	**36.40 ± 2.11[b]**	**38.12 ± 23.50[b]**	**40.29 ±57.11[b]**

Values are expressed as mean ± S.E.M. P > 0.05 when compared to normal control group. A spartate aminotransferase (*AST* or *SGOT*) and Alanine aminotransferase (*ALT* or *SGPT*) were not significantly different (p > 0.05). . Statistical comparisons were made within a column and values with the same superscript letter are not significantly different.

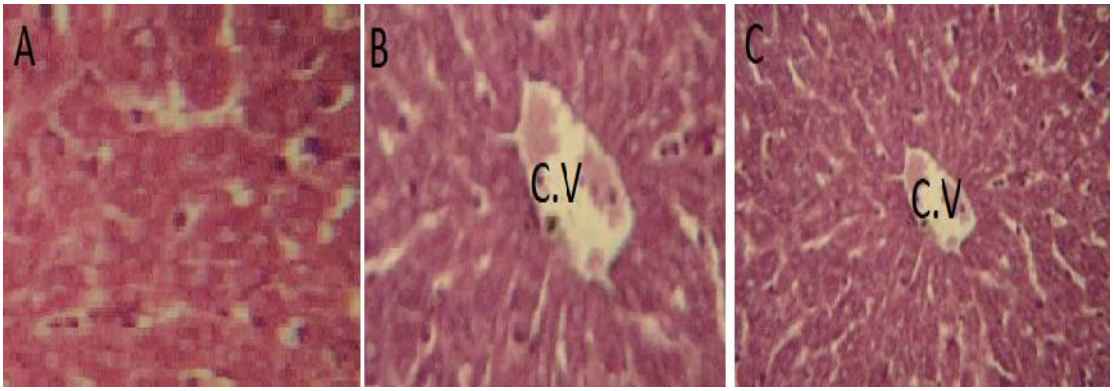

Figure 1. photo graphics of liver sections of sub-chronic toxicity A = control mice showing no histopathological change , B = the mice treated with 600 mg/kg and C the mice treated with 1000 mg/kg of encapsulated extract showing no histopathological changes (H & E stain).c.v central vein.

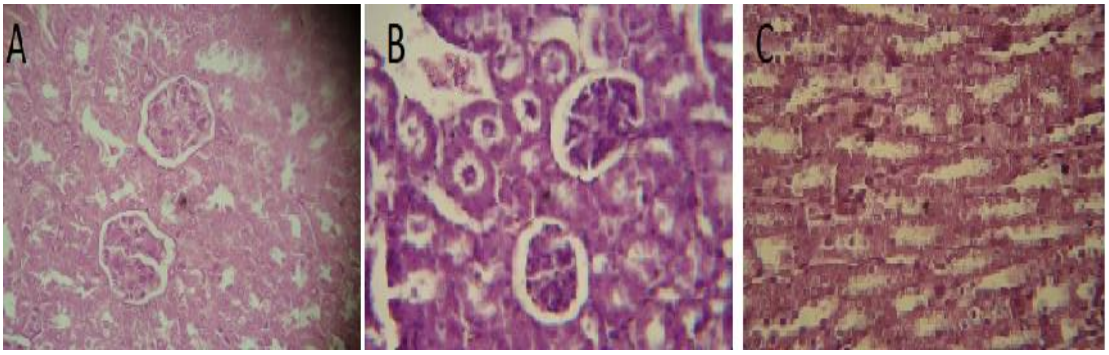

Figure 2. photo graphics of kidney sections sub-chronic toxicity A = control mice showing no histopathological change, B = the mice treated with 600 mg/kg and C= the mice treated with 1000 mg/kg of encapsulated extract showing no histopathological changes in renal tubules (H & E).

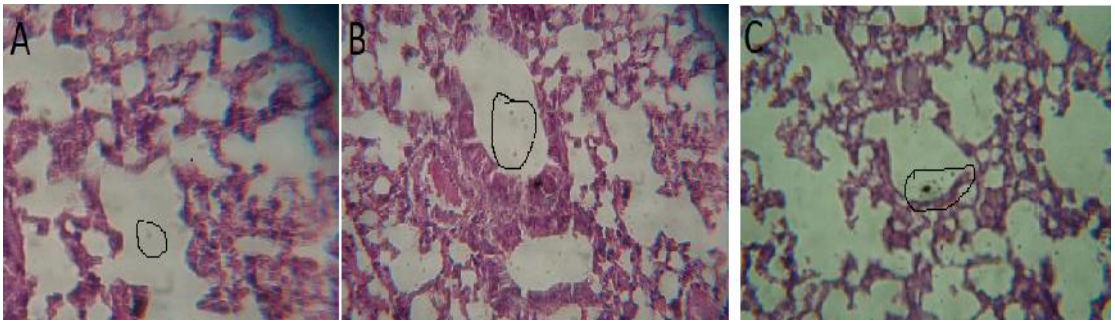

Figure 3:. photo graphics of Lung sections sub-chronic toxicity. A)control mice showing no histopathological change, B) the mice treated with 600 mg/kg of encapsulated combination extracts C) The mice treated with 1000 mg/kg of encapsulated combination extracts showing mild inflammation changes the lungs were unaffected (H & E, x100).

followed by ANOVA test using Graph Pad Prism and for multiple comparison test among the groups, Bonferroni test was performed. A probability level 0of $p < 0.001$ was accepted statistically (Cao et al. 2014).

Results

Acute toxicity study

The acute toxic effect of encapsulated combination extracts was determined as per the OECD guideline 423, where the limit test dose of 4000 mg/kg was used. No treatment related toxic symptom or mortality were observed after oral administration of the tested encapsulated combination extracts at a dose of 300, 2000 and 4000 mg/kg. The behavior of the treated groups with encapsulated combination extracts and the control group was first observed for a short period (4 h), followed by a long period (72 h). The results showed no changed behavior, breathing, skin effects, water consumption, impairment in food intake and temperature. Therefore, the extract is safe at a dose level of 4000 mg/kg, and the LD50 was >4000 mg/kg. 161 The parameters were observed for acute toxicity study after the test plant extract was 162 administered compared to the control group (Table 1).

Sub-chronic toxicity study

The sub-chronic toxic study on the tested plant extract was conducted as per OECD guidelinen407. All the tested group animals treated with encapsulated extract at a dose of 600 and 1000 mg/kg daily survived throughout the 28 days. There were no clinical toxicity signs in the experimental group compared to the control.

Effect of extract on relative organ body weight

Weight was no significantly different. The results showed that the vital organs such as liver, kidney, heart, pancreas and small intestine were not adversely affected throughout the treatment by the encapsulated combine extracts. There was no statistically significant difference between the average and relative organ weight of control group and treated groups ($p > 0.05$).

Effect of encapsulated combine plants extract on hematological parameters

Table 5 show the results of the hematological tests. All the tested hematological parameters such as total blood count, hemoglobin, red blood cell, total white blood cell, neutrophil, monocyte, lymphocyte, packed cell volume, and platelet count of the treated groups were within normal limits compared to the control group. There was no toxicologically significant difference ($P > 0.05$) between treated animals with the encapsulated combination extract and control. The hematological parameters between the control and treated groups were not significantly different.

Effect of encapsulated combine plants extract on biochemical parameters

Table 6 shows the results of the various biochemical tests on the experimentally treated animals with the plant extract and the control group. There was no impact of oral administration of the encapsulated treated at a dose of 600 and 1000 mg/kg on serum biochemical parameters such as albumin, total protein, globulin, T-BIL, urea, sodium, creatinine and uric acid levels and SGOT (AST) and SGPT (ALT) when compared to control group. There was no significant

Table 5 values are expressed as mean ± S.E.M. $P > 0.05$ when compared to normal control group. 226 PCV=packed cell volume, MCV=Mean cell volume or mean corpuscular volume, MCH= Mean cell 227 haemoglobin and MCHC= mean cell haemoglobin concentration and WBC=white blood cells. Statistical comparisons were made within a column and values with the same superscript letter are not significantly different.

Histology of selected organs

Histopathological examination of formalin fixed paraffin embedded tissues of liver (Fig. 1), kidney (Fig. 2), and lung (Fig. 3) of all treatment groups and negative control group. Histopathology studies showed no adverse effect and potential of encapsulated combination extracts. The result suggests that there is no histopathological abnormality in the tested 9 groups. The effect of encapsulated combination extracts was investigated in selected tissues. The H & E sections were studied under photomicrographs was performed at 100 x magnification using Olympus light microscope.

Discussion

This study aimed to evaluate the encapsulation combination of *F.deltoidea* and *G.sublanceolata* leaf extracts with a Nano hydroxyapatite for exploring acute and sub-chronic toxicity and identifying the range of dose that could be used for further studies. The oral acute toxicity of the tested plant extract was explored on BALB/c mice at a single dose of 300, 2000 and 4000 mg/kg body weight, and it was monitored for the first 4 h, followed for a period of 72 h for any toxic effect after a period of treatment. The results revealed no major changes in behavior, mortality and appeared normal for general anatomical appearance in all groups).

Extract don't showed any toxic at a dose level of 4000 mg/kg, and the LD50 is >4 000 mg/kg when used with sub-chronic study. Critical toxicological effects such as teratogenesis and reproductive disruption have not been explored. Therefore need more investigations on toxic effects. (Arsad et al., 2018). It is suggested that the encapsulation extract is practically non-toxic in a single dose of level 4000 mg/kg body weight. However, study on sub –chronic toxicity showed that multiple doses is used to treat chronic disorder such as cancer, diabetes or hyperlipidemia safely. It does not affect the weight of relative organ, hematological and biochemical parameters.

Study on a sub-acute toxicity was done with a dose of 600 and 1000 mg/kg of extract as per OECD guideline (Donko et al., 2014). There was a toxic effect of chemicals and 289 drugs on decreasing or increasing the weight of body. However, scientific evidence showed increase or decrease in the weight of body when fat is accumulated and physiological adaptation responds the extract of plants rather than the toxic effects of chemicals or drugs that lead to decreasing appetite, hence, lower caloric intake by the animal (Kausar et al., 2010). The relative weight of the vital organs, namely liver, kidney,

heart, pancreas and small intestine was normal, which indicated that there was no significant difference in toxic effect between the control and the experimental group ($p > 0.05$). The results show that this no significant difference in the liver, kidney, heart and small intestine weight and in general anatomical appearance (Figure 1). After 28 days of treatment with tested extract, the hematological parameters were not significantly different, at $p > 0.05$, compared to the control group.

The bone marrow impacts the production of blood cell, and some phytochemicals isolated from plants affect the level of blood cell. Thus, the extract of tested plant does not affect the function of bone marrow. It was indicated that all doses of encapsulate extract do not induce anemia, making it safe. Similarly, the estimation of serum biochemical parameters in treated animals was not significantly different ($p > 0.05$), compared to the control group. When the plasma membranes of liver cells are damaged, various enzymes in the cytosol are released into the stream of blood. The extent and type of Hepatocellular damage is quantitatively measured based on the levels of these enzymes in the serum. The lack of alteration in liver parameters (the transaminases enzyme SGOT (AST) and SGPT (ALT) showed that the administration of extrat for 28 days is not any abnormalities to the liver. The results also showed that the indicators of kidney tests (creatinine, uric acid, urea, albumin, globulin, bilirubin, and total protein) are not affected. Thus, livers or kidneys are not damaged by the sub-chronic administration of extract. Researcher reported that no overt signs of acute toxicity or death were observed in mice and rats treated with a methanol extract of *F. deltoidea* up to the dose of 6400 mg/kg (Ilyanie et al., 2011).

However, the differential leukocyte counts for monocyte and eosinophils around within the reference value range (Harkness et al., 1993), which strongly suggests that there is no effects to treatment with encapsulated combination extracts. All biochemical parameters analyzed remained within the reference levels for the species (Elham Farsi et al., 2013). Beside that these results take indicate on adverse effects of *Gynocthodes sublanceolata* and hydroxyapatite. The histopathological examination of selected organs (liver, kidneys and lung) harvested from treated and control animals confirm these results. This analysis revealed normal architecture for these vital organs. In the liver parenchyma of animals treated with extrat at doses up to 1000 mg/kg, normal-sized cells with a centrally located euchromatic nucleus and a very prominent nucleolus were observed. The hepatic vascular distribution was homogeneous when compared with that of the control group (Figure 2) with a normal hepatic portal triad. The results showed no significant difference between the kidney sections of the control and the experimental groups, where urinary pole, vascular pole, glomerulus, convoluted tubules are normal and clearly visible (Figure 3). The lungs, which showed mild irritation of in the air spaces for both the treated group with 1000, 600 mg/kg and control. These morphological changes in the lungs were due to by the daily oral gavage ordue to the anesthetized procedure and not by encapsulated extract itself because these changes were also showed in the control group. All vital organs have a normal histological architecture and do not have any precipitation of nano hydroxyapatite (Rhiouaniet al., 2008, Muhammad et al., 2015). The histological studies revealed no obvious detrimental effects or morphological disturbances of the daily oral administration of extract for 28 days, even at the highest tested dose of 1000 mg/kg.

Conclusion

Overall, this study provides preliminary scientific evidence about the safety profile of Encapsulation of combine plants extract of *Ficus deltoidea and Gynocthodes sublanceolata* for long-term use. The result confirms that the use and development of encapsulation of combine plants extract as a therapeutic agent seems to be quite safe. The use of encapsulation of combine plants extract and its mechanism of safety are meeting requirements on the level of approach chosen in this study which might be applied for future human pre- clinical studies. Critical toxicological effects such as teratogenesis and reproductive disruption have not been explored. Therefore need more investigations on toxic effects.

Author Contributions

R.K.A. and M.S. run the in vitro assay, M.N. assisted with experimental protocol and drafted the paper, F.S.A.S. designed the study, drafted and revised the article.

Acknowledgment

Authors would like to acknowledge School of Bioprocess Engineering, University Malaysia Perlis for the identification of the plant.

Competing financial interests

The author(s) declare no competing financial interests.

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