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Original Research Article

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Preclinical safety assessment and mutagenicity of the hydroethanolic extract of *Syzygium campanulatum* leaves.

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Abstract

Syzygium campanulatum (Myrtaceae) is a species indigenous to Southeast Asia, a widely consumed medicinal herb and rich in phytochemical content and notable antiangiogenic and anti-colon cancer. Safety reports of administration of *S. campanulatum* are however lacking.

In this study, we investigated the quality of dried leaves, chemical composition analyzed by FTIR and HPLC, phytochemicals content and repeated doses toxicity and mutagenicity effect of the hydroethanolic extract of *S. campanulatum* leaves (HESCL). The rats were divided into experimental and control groups and fed with 500, 1000, 2000 mg/kg/day of "HESCL" for 28 and 90 days and with a single dose of 5000 mg/kg in acute study.

The obtained results showed the dry leaves of *S. campanulatum* were devoid of any heavy metal and microbial contamination. The major components of HESCL were, respectively betulinic acid (60.43 mg/g.), total glycol saponins, total phenolics, total proteins, total tannins, and total flavonoids. No mutagenicity was detected in *S. typhimurium* auxotroph and no signs of clinical toxicity and mortality were observed in the experimental groups after 28 and 90-day experiment. However, significant ($p < 0.05$) statistical deviations were observed in hematological, and biochemical parameters but they were within the normal clinical range for rat, therefore not considered treatment-related.

Based on these findings the fifty percent lethal dose was > 5000 mg/kg and the NOAEL was up to 2000 mg/kg for 90 days, as such oral administration of HESCL is a relatively safe.

Keywords : *Syzygium campanulatum*, Myrtaceae, Mutagenicity, Acute toxicity; Subacute; Subchronic toxicity.

Introduction

In many countries, traditional medicine employs the use of plants and herbal products to combat numerous human illnesses. This practice has become increasingly popular in recent decades due to rising healthcare cost, easier access and availability of a wide range of finished products, relatively low cost, and mainly perceived absence of toxic side effects as a result of its source of origin [1]. Despite popular public opinion that herbs are safe, consumption of plant or natural products can lead to adverse reactions, toxicity, and fatal drug-herb interactions even if the intended therapeutic endpoints are achieved [2,3]. Chronic consumption of herbal medicines also may only manifest toxic effects much later in life. Most often these potential hazards can be related to improper dose administration, poor quality of raw material, the presence of contaminants and also long-term mutagenic properties. It is not surprising that the safety of plant products has come under immense scrutiny. Considering the complexity of herbs in general and their inherent biological variation, it has become necessary to evaluate the safety, efficacy, and quality of herbal medicines for human consumption [4].

The Organisation for Economic Co-operation and Development (OECD) adopted guidelines for *in vitro* and *in vivo* preclinical toxicological studies to assess the safety of herbal medicines and uncover potential risks [5]. *In vitro* toxicity assessment relies on short-term tests such as the mutagenicity assay to evaluate the tendency of a test substance to induce mutation in DNA, and the Ames test is often the method of choice. *In vivo* studies include single and repeated dose exposure of laboratory rodents to a test sample for periods of four or/and thirteen weeks. These assays are used to evaluate the general, long-term toxicity and for hazard assessment of drug candidates. Evaluation of the pathological alterations that occur in laboratory animals exposed to novel drugs is the cornerstone of assessment of their safety [6].

Syzygium campanulatum (Myrtaceae) is an evergreen species of a shrub which originates from Southeast Asia. Our group has recently been investigating the biological and chemical attributes of

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n-Hexane and chloroform extracts of *S. campanulatum*. It was noted that inhibition of outgrowth of microvessels in rat aortic rings is a key property of the extracts [7]. Moreover, the hydroalcoholic extract of *S. campanulatum* showed *in vitro* anti-colon cancer activity via suppression of vascular endothelial growth factor in endothelial cells and inhibition of tumor angiogenesis and growth in tumor-bearing nude mice. The major phytochemical constituents related to this activity include phenolics, flavonoids, and betulinic acid [8].The hydroethanolic extract of *S. campanulatum* had a dose-dependent cytotoxic effect on colon cancer cells *in vitro*, and the activity was related to the presence of dimethyl cardamonin [9]. Although the pharmacological activities are apparent and many remain to be explored, safety studies for *S. campanulatum* extracts are more crucial and significantly lacking. The present toxicological study was thus conducted as per OECD guidelines to investigate whether acuteand repeated treatments for 28 and 90 days with an hydroethanolic extract of *S. Campanulatum* leaves are associated with any kind of toxicity in the preclinical setting. The data generated were used to identify the median lethal dose (LD_{50}) and to predict the no observed adverse effect level (NOAEL). The *in-vitro* mutagenic effect of the plant extract was tested using Ames test. The extract also was investigated for its chemical composition through FTIR and HPLC analysis. Data from the *in vitro* and *in vivo* studies were used to establish the preclinical toxicological profile of the SC *leaves*.

Material and Methods

Herbal material and extraction

The plant leaves were purchased from Herba Bagus Sdn Bhd. The sample was deposited with voucher reference number of 11047 at School of Biology Herbarium, Universiti Sains Malaysia (USM). The fine powder *S. campanulatum* (SC) leaves (500 g) macerated in 50% hydroethanolic (1 L)in 50-55 C degree water bath for 8-10 h. The solven was filtered and replaced with a fresh supply. The filtrate was concentrated under reduced pressure using rotary evaporator (Buchi, Sweden) at 40 C until the solvent was completely evaporated. The fifteen percent yield of the hydroethanolic extract of *S. campanulatum* leaves (HESCL) was preserved in powder format –20 °C until the time of use.

Analysis of physicochemical parameters

Physicochemical parameters such as total ash, acid-insoluble ash, loss on drying, and nitrogen content of ground air-dried leaves were measured using a TGA701 thermogravimetric analyzer (LECO, USA) based on standard test methods [10].

Heavy metal analysis

The heavy metal (lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg)) concentrations of ground leaves of SC were measured using an atomic absorption spectrometer (*Perkin Elmer; model: AAnalyst800*, Autosampler,USA) following United States Pharmacopeia [11].

Microbial limit test

Ground dried leaves of SC were subjected to the microbial limit test (MLT) following USP methods [11]. The sample was tested qualitatively for the presence of Enterobacteria, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella spp.*, and *Escherichia coli*. The total aerobic microbial count, total yeast and mold counts in SC leaves powder also were measured.

Ultra violet visible (UV) analysis of HESCL

The UV analysis was performed using Perkin-Elmer Lambda 45 UV/Vis Spectrophotometer. A dilute solution extracts (40 µg/mL) was subjected to UV analysis. All samples were scanned from wavelength 500-200 nm using methanol as blank.

Fourier transform infrared (FTIR) spectroscopy of HESCL

FTIR spectra were recorded using FTIR Spectrometer (Thermo Nicolet, USA) equipped with software OMNIC version 6.0 a. The analysis was carried out using a single-bounce Attenuated Total Reflection FTIR (ATR-FTIR) scan technique. The extract (3 mg) were placed directly onto the diamond crystal for data acquisition. Sample data were recorded in the mid-IR range of 4000-650 cm^{-1} at a resolution of 4 cm^{-1} for 6 replicates.

Quantification of betulinic acid

The presence of betulinic acid in HESCL was investigated by high performance liquid chromatography(HPLC). HPLC analysis was performed using Eclipse Phenyl-Hexyl (250 mm 4.6 mm) packed with 5 µm diameter particles size (Agilent, Malaysia) was maintained at 25ºC and the injection sample (20 µL) was eluted with an isocratic mobile phase comprising of Isocratic elution of Acetonitrile: 0.1% formic acid in Milli-Q water in volume ratio 90:10. Flow rate was 1 mL/min and detection was carried out at 202 nm. The identification of the compound was performed by comparing their retention time and UV absorption spectrum with those of the commercial standard. The result was derived from the mean of a peak area from three replicates injections.

Primary and secondary metabolites analysis

HESCL was analyzed for total phenolics, total flavonoids, total proteins, total glycosaponins and total tannins following previously described method[12].

Mutagenicity assay: Ames test

Mutagenicity of HESCL was evaluated by bacterial reversion assay (Ames test) employing histidine auxotrophs of *Salmonella typhimurium* (TA98 and TA100 without the S9 mixture)[13]. The experiment was performed using 500 µg of HESCL/well following the manufacturer's instructions for the Muta-Chromo Plate™ Kit (EBPI, Canada). The level of mutagenicity was scored by comparing the number of colonies formed in the HESCL well to those present in the 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) as the negative control and the 2-nitrofluorene well as the positive mutagen for TA98 and the sodium azide for TA100.

Animals

Male and female Sprague-Dawley (SD) rats7–8 weeks of age were obtained from the animal house of the school of pharmaceutical sciences, USM. Animals were acclimatized to the laboratory environment (60 ± 10% and temperature of 22 ± 2 °C) for at least 1 week prior to the study. The animals were provided *ad libitum* access to standard pellet diet (Gold Coin Holdings Sdn Bhd, Malaysia) and water. The study was approved by the animal ethics committee, USM [Protocol No: USM / Animal Ethics Approval / 2013 / (90) (566)].

Acute oral toxicity study

An acute oral toxicology study was conducted in healthy female SD rats weighing 200–220 g using the aqueous solution of HESCL at the limit dose of 5000 mg/kg against vehicle-treated (10 ml/kg) control group in accordance with the published guidelines[14]. Ten female rats (treated and control group) were monitored for14 days for signs of possible toxicity every 1 hour, 3–4 hours after administration, and daily thereafter. The animals were euthanized on the last day, and the median lethal dose values were estimated.

28-day oral toxicity study

The study was performed in accordance with OECD guideline 407 for a 28-day oral toxicity test[15]. After 1 week of acclimatization, 20 female and 20 male rats were randomly divided into four groups of five animals each: control group (0 mg/kg), low-dose group (500 mg/kg), middle-dose group (1000 mg/kg), and high-dose group (2000 mg/kg). The body weight of each rat was recorded on days 0, 7, 14, and 28 using analytical lab balance (Mettler-Toledo AX-204, Canada). At the end of the experiment, all the rats were anesthetized by carbon dioxide inhalation, and blood samples were collected via cardiac puncture. Then, the animals were euthanized by cervical dislocation, and their organs were removed, and preserved in 10% buffered neutral formalin.

Hematological parameters

The blood samples were analyzed for level of hemoglobin (Hb), total red blood cell counts (RBCs), total white blood cell counts (WBCs), neutrophils, lymphocytes, monocytes, eosinophils, basophils, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (Plt), red cell distribution width (RDW), and activated partial thromboplastin time (APTT). These parameters were measured using an automatic hematology analyzer (Sysmex-XT-1800, Germany).

Clinical chemistry parameters

The serum was examined for the levels of total protein, albumin, globulin, albumin/globulin ratio, total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), urea, potassium, sodium, chloride, creatinine, and uric acid using biochemistry autoanalyzer (Olympus 640, USA).

Relative organ weights

The weight of brain, thymus, heart, lungs, spleen, liver, kidneys, adrenal glands, stomach, gut, testes, ovaries, and uterus were recorded and expressed as relative weight.

Histopathological examination

The heart, liver, lungs, kidneys, and spleen were retrieved from 10% buffered neutral formalin and paraffinized. The paraffinized organs were sliced to 5 µm thick using microtome (Leica Microsystems Pte Ltd, Singapore), placed on the slides, hydrated using an alcohol xylene series, and stained with hematoxylin-eosin. The tissues were examined for toxicity-related changes under a light optical microscope.

90-day oral toxicity study

The study was performed following OECD guideline 408, using four groups of 20 animals each (10 female and 10 male rats) [16]. The experiment was performed as described in the 28-day study.

Statistical analysis

Statistical analysis was performed using the statistical package for the social sciences (SPSS 16.0 package, Chicago, USA). The data are shown as mean ± standard deviation, and the analysis was performed using one-way analysis of variance. Significant differences between the control and experimental groups were identified using Dunnett's test. $P < 0.05$ was considered to be statistically significant.

Results and discussion

Raw material analysis

Quality control assessment was conducted to ensure that the raw material was devoid of any impurities and contamination to avoid any confounding in the toxicological result. The result showed evidence of traceheavy metal (Pb, Cd, As, and Hg) present in the leaves powder falls within the safe levels as per World Health Organization guidelines[10].Further analysis also showedthat the powdered SC leaves contained 11.4% total ash, 7.8 % acid insoluble ash, and 1.1% nitrogen contents. The growth of any microorganism was tested by the microbial limit test.The aerobic bacterial count and yeast and mold counts were within the limits prescribed by USP [11].

UV-Vis analysis

Spectra obtained from UV scan of HESCL and reference marker showed maximum absorption (λ_{max}) peaks at 284.6 and 328.8 nm (Figure 1).The peaks at 284.6 and 328.8 nm are associated with the presence of phenolics as well as flavonoids compounds. Phenolics and flavonoids have pi electrons on their benzene ring. These peaks at 284.6 and 328.8 nm which are known as B band are related to the excitation of pi electrons in the benzene ring [17]. These peaks in HESCL are sharp with high intensity suggesting that major chemical components in the HESCL are based on phenolics and flavonoids skeletons.

FTIR spectra

The FTIR spectra of HESCL revealed a strong peak at 2929 cm^{-1} (Figure 1). This peak normally refers to the C-H stretching vibration of saturated and unsaturated hydrocarbons. Furthermore, peak at 1387 cm^{-1} is assigned to the C-H vibration. The peak at 3201cm^{-1} is attributed to a hydroxyl group. Moreover, peaks at 1600-1700 cm^{-1} are related to carbonyl group [18]. Furthermore, peaks at 1000-1200 cm^{-1} and 1600-1670 cm^{-1} are contributed to the stretching vibration of C-O-C and absorption of alkene C=C, respectively. In overall, FTIR spectra of HESCL showed the great intensity of aromatic main bands (1654-1532 cm^{-1}) and hydroxyl band (3201 cm-1). This could be due to the presence of polyphenols and phenolics component. Moreover, carboxylic C-O band at 1387 cm^{-1} could be related to the existence of triterpenoids and phenolic acid constituents.

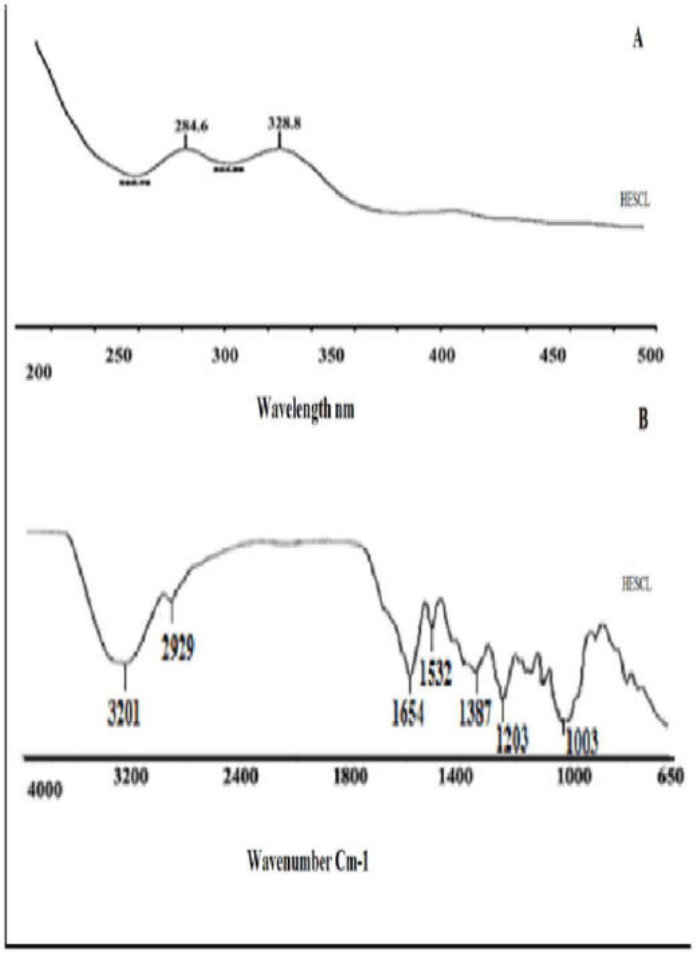

Figure 1.A: UV-Vis spectra of *S. campanulatum* extracts. UV-Vis spectra were collected in the wavelength range 500 – 200 nm. **B**: FTIR (MIR) fingerprints of *S. campanulatum*. IR spectra were collected in the wavenumber range 4000 – 650 cm-1.

Betulinic acid quantification

The betulinic acid concentration in HESCL was determined by HPLC and the chromatogram is presented in Figure 2. HESCL contains 60.43 mg/g betulinic acid.

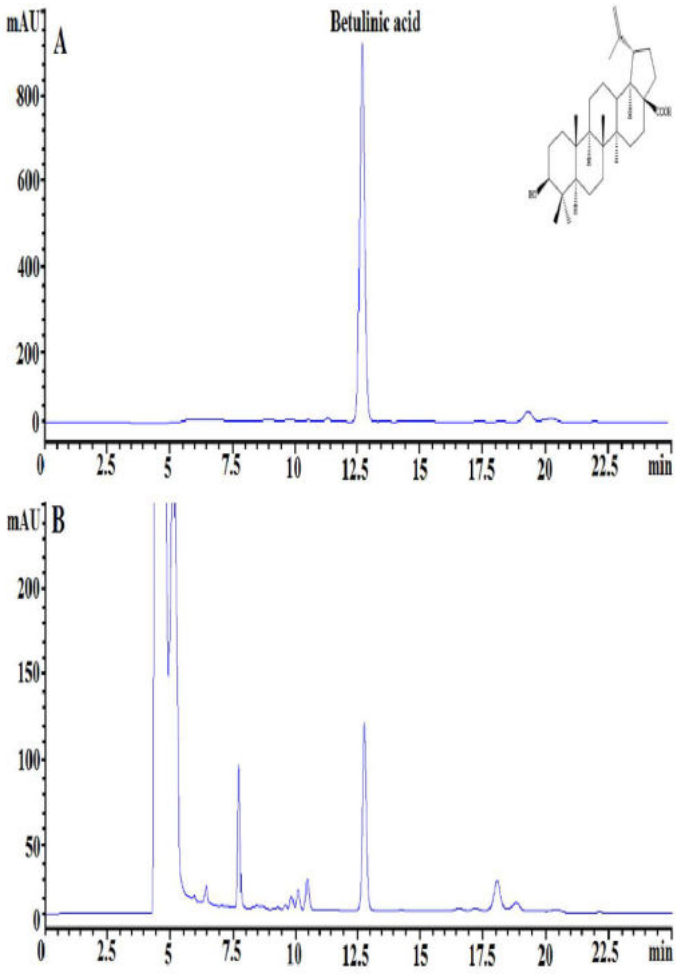

Figure 2. Representative high performance liquid chromatography profile of BA in HESCL

Phytochemical analysis

The phytochemical analysis of HESCL showed, the presence of total phenolics (15.625 ± 1.12 mg/g), total flavonoids (2.51 ± 0.11mg/g), total proteins (14.41 ± 1.33 mg/g), total glycosaponins (23.34 ± 2.45mg/g) and total tannins (8.17 ± 0.60 mg/g) in HESCL.

Mutagenic study

The results show that the number of mutation occurred in the two test strains at 500 µg/well of HESCL in the absence of S9 mixture was within the standard range introduced by Muta-Chromo Plate™ Kit compared to DMSO plate (TA98: 3 and TA100: 18 wells/plate). The 2-nitrofluoreneand sodium azide both exhibited both the highest number of mutated colonies (96 wells/plate) after 6 days of the incubation. These results indicate that the HESCL was not mutagenic (significant at $P < 0.001$). The Ames test is used to detect the potential of a test substance to induce reverse mutation in the His gene of modified *S. typhimurium*. Most of the research reports proved that there is a countable relationship between mutagenicity in *S. typhimurium* and carcinogenicity of tested compounds [19]. In this study, HESCL did not have mutagenic effects on the tested bacterial strains. The obtained result suggests the administration of HESCL is safe and not carcinogenic.

Acute oral toxicity study

Animals showed no signs of toxicity after single oral administration of 5000 mg/kg HESCL. Changes in body weight and water and food consumptions also were not statistically or clinically significant (data not shown). Thus, under the conditions of this study, HESCL at 5000 mg/kg was not acutely toxic; this indicates that the LD_{50} is greater than 5000 mg/kg. Based on the Hodge and Sterner scale the test sample was classified as category 5, and consider non-toxic [20].

28 and 90-day toxicity studies

Hematological parameters

The results showed, administration of HESCL to the male rats for 28 days resulted in significant ($P < 0.05$) reduction in WBCs (6.48 ± 0.31×10^9 /L), and neutrophil (18.20 ± 1.64 %) values in the middle-dose group (1000 mg/kg) (Table 1).The percentages of monocytes (4.50 ± 0.53 %) also significantly decreased in male rats treated with 1000 mg/kg of HESCL for 90 days comparing to untreated rat. Female rats showed a significant decrease in neutrophil level after administration of HESCL at 1000 mg/kg for 90 days (Table 2).The significant changes in hematological parameters after 28 and 90 days of treatment all occurred in the middle-dose groups (1000 mg/kg). The altered values fell within the normal ranges for rat WBCs (4–17 10^9/L), neutrophils (5–30 %), and monocytes (1–6%) [21, 22]. Thus, the statistically significant changes cannot be attributed to the treatment. Except for the above-mentioned significant differences, all other parameters were comparable to the control group in male and female rats after 28 and 90-day of oral treatment.

Table 1. The effect of 28-day oral administration of the hydroethanolic extract of *Syzygium campanulatum* leaves on hematological parameters male SD rats.

Male	**Unit**	**Hydroethanolic extract of *Syzygium campanulatum* leaves**			
		0 mg/kg	**500 mg/kg**	**1000 mg/kg**	**2000 mg/kg**
Hemoglobin	g/l	168.80 ± 5.76	165.20 ± 3.03	164.40 ± 3.36	164.60 ± 3.85
Total Red Blood Cell	10^{12}/l	9.65 ± 0.44	9.37 ± 0.30	9.34 ± 0.15	9.14 ± 0.52
Total White Blood Cell	10^{9}/l	8.36 ± 0.70	8.42 ± 0.43	6.48 ± 0.31 *	8.44 ± 0.27
Neutrophil	%	30.60 ± 1.34	31.20 ± 1.64	18.20 ± 1.64 *	31.20 ± 1.30
Lymphocyte	%	61.00 ± 0.71	60.80 ± 1.10	60.00 ± 0.00	60.00 ± 0.00
Monocyte	%	5.80 ± 0.45	5.00 ± 0.71	5.00 ± 0.71	6.00 ± 0.00
Eosinophil	%	1.20 ± 0.45	1.20 ± 0.45	1.40 ± 0.55	1.60 ± 0.55
Basophil	%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Packed Cell Volume	%	0.49 ± 0.02	0.48 ± 0.01	0.46 ± 0.02	0.47 ± 0.02
Mean Corpuscular Volume	Fl	50.80 ± 2.39	51.00 ± 1.87	49.00 ± 2.55	53.20 ± 2.86
Mean Corpuscular Hb	Pg	17.40 ± 0.89	17.20 ± 0.45	17.00 ± 1.00	18.20 ± 0.84
Mean Corpuscular Hb Conc	g/l	346.00 ± 6.28	342.60 ± 5.55	345.40 ± 4.56	342.60 ± 7.09
Platelet Count	10^{9}/l	1065.20 ± 63.92	838.60 ± 85.25	857.20 ± 36.09	866.40 ± 87.98
RWD	Second	18.98 ± 0.62	18.26 ± 0.57	18.88 ± 0.59	18.14 ± 0.96
APTT	Second	50.38 ± 1.11	46.78 ± 3.31	46.38 ± 4.12	46.66 ± 1.32
Female					
Haemoglobin	g/l	150.00± 4.24	152.20± 0.50	152.20± 0.45	148.20± 5.67
Total Red Blood Cell	10^{12}/l	8.20± 0.50	8.30± 0.00	8.05± 0.12	8.18± 0.58
Total White Blood Cell	10^{9}/l	5.56± 0.44	5.04± 1.05	5.70± 0.41	5.20± 0.55
Neutrophil	%	21.40± 3.13	18.60± 2.41	18.40± 0.89	19.00± 1.22
Lymphocyte	%	73.80± 1.64	74.40± 0.55	73.80± 0.45	73.80± 1.30
Monocyte	%	5.40± 0.00	5.00± 0.00	4.80± 0.45	5.60± 0.22
Eosinophil	%	1.20± 0.45	1.20± 0.45	1.20± 0.45	1.20± 0.45
Packed Cell Volume	%	0.42± 0.01	0.44± 0.01	0.43± 0.02	0.43± 0.01
Mean Corpuscular Volume	fl	53.80± 1.10	53.20± 0.45	54.00± 0.71	53.20± 0.84
Mean Corpuscular Hb	pg	18.60± 0.89	18.60± 0.55	18.80± 0.45	18.00± 0.71
Mean Corpuscular Hb Conc	g/l	347.60± 1.52	346.40± 0.55	345.80± 1.30	349.40± 8.71
Platelet Count	10^{9}/l	734.20± 3.90	726.40± 3.78	724.40± 4.28	743.80± 19.87
RDW	Second	14.92± 0.67	14.20± 0.00	14.14± 0.05	14.84± 0.93
APTT	Second	40.58± 5.15	37.94± 2.73	38.50± 3.77	41.10± 4.95

The values are mean ± SD of five rats per group. The statistical analysis was done by ANOVA followed by Dunnett's test for all groups. * P < 0.05 vs. control group (AECNL 0 mg/kg)

Table 2. The effect of 90-day oral administration of the hydroethanolic extract of *Syzygium campanulatum* leaves on hematological parameters male SD rats.

Male	Unit	Hydroethanolic extract of *Syzygium campanulatum* leaves			
		0mg/kg	500 mg/kg	1000 mg/kg	2000 mg/kg
Haemoglobin	g/l	147.60 ± 8.90	147.80±1.93	146.00±1.80	147.67±1.58
Total Red Blood Cell	10^{12}/l	8.11 ± 0.74	8.60±0.34	8.58±0.30	8.37±0.40
Total White Blood Cell	10^{9}/l	7.26 ± 1.34	8.54±1.54	8.87±2.87	9.21±1.45
Neutrophil	%	27.50 ± 3.30	29.50±2.22	33.80±10.14	31.33±5.92
Lymphocyte	%	60.10 ± 8.14	62.20±8.59	59.10±9.28	54.67±10.15
Monocyte	%	7.00 ± 1.16	5.10 ± 0.57	4.50 ± 0.53 *	5.33 ± 0.71
Eosinophil	%	1.11 ± 0.31	3.00 ± 0.32	2.10±0.32	2.11±0.33
Packed Cell Volume	%	0.46 ± 0.04	0.47±0.01	0.44±0.02	0.44±0.03
Mean Corpuscular Volume	fl	55.00 ± 2.79	54.22±1.64	53.11±2.26	53.10±1.97
Mean Corpuscular Hb	pg	18.00 ± 1.00	17.67±0.50	17.11±1.05	17.30±0.48
Mean Corpuscular Hb Conc	g/l	328.50 ± 8.95	333.67±6.46	333.00±6.50	336.30±9.27
Platelet Count	10^{9}/l	901.70 ± 76.65	790.10±52.59	874.70±91.67	847.00±56.69
RWD	Second	18.42 ± 2.39	18.54±0.55	19.38±1.13	18.44±0.84
APTT	Second	50.22 ± 5.18	147.80±1.93	146.00±1.80	147.67±1.58
Female					
Haemoglobin	g/l	145.25 ± 8.10	145.20±3.52	145.40±4.14	146.20±3.46
Total Red Blood Cell	10^{12}/l	7.89 ± 0.54	7.77±0.27	8.09±0.49	8.30±0.45
Total White Blood Cell	10^{9}/l	7.45 ± 1.83	5.38±0.51	5.94±0.78	8.52±0.77
Neutrophil	%	24.66 ± 4.87	26.20±4.83	19.90±1.73*	26.10±3.87
Lymphocyte	%	70.60 ± 6.95	69.10±4.18	71.60±4.60	69.00±6.78
Monocyte	%	4.94 ± 0.42	3.78±0.44	5.70±1.64	5.10±1.37
Eosinophil	%	2.50 ± 0.52	2.10±0.32	1.10±0.32	1.70±0.95
Packed Cell Volume	%	0.45±00.00	0.44±0.04	0.44±0.03	0.45±0.03
Mean Corpuscular Volume	fl	0.44 ± 0.03	57.70±2.11	54.10±2.88	54.80±2.49
Mean Corpuscular Hb	pg	56.10 ± 2.26	19.00±0.94	18.00±0.67	17.70±0.67
Mean Corpuscular Hb Conc	g/l	18.50 ± 0.67	327.60±4.01	332.20±5.53	322.10±8.05
Platelet Count	10^{9}/l	319.94 ± 13.87	766.30±54.72	823.70±42.65	729.70±53.60
RDW	Second	866.45 ± 95.52	14.93±0.52	14.22±0.80	15.76±1.71
APTT	Second	15.20 ± 0.82	47.52±4.75	48.62±6.12	51.21±3.35

The values are mean ± SD of ten rats per group. The statistical analysis was done by ANOVA followed by Dunnett's test for all groups. * $P < 0.05$ vs. control group (AECNL 0 mg/kg)

Clinical biochemistry parameters

Administration of HESCL at 500, 1000, and 2000 mg/kg for 28 and 90 days did not induce any significant changes in serum biochemical parameters of male and female rats with two exceptions. A significant ($P < 0.05$) increase in the level of AST (299.50±40.52 U/L) and a decrease in the level of ALP (220.22±30.05 U/L) after 90 days was detected in male rats treated with 2000 mg/kg of HESCL. However, the observed ALP level is within the normal physiological level (70–450 U/L), and the AST value is in the range of normal values in our laboratory records. Thus, compared to control male rats, the observed alterations were not considered to be clinically significant and treatment related (Table 3) [23]. ALP is a vital enzyme that is manufactured mainly by the liver, bones, kidneys, and intestines. A toxically low level of ALP is caused by malnutrition, deficiencies in vitamin B6, folic acid, and vitamin C, and excessive intake of vitamin D. Phosphorous, zinc, and protein deficiencies also can cause an abnormally low level of ALP [24-26]. Previous studies have shown that a toxicologically low level of ALP is often accompanied by massive weight loss [27]. However, in the current investigation, none of these signs were observed. AST is a non-specific liver enzyme and the toxic

changes in AST level usually seen with alterations in the ALT level, as both are the same range of sensitivity [28]. In this study, no notable changes in ALT level were observed, therefore, the observed alteration in AST level may have been due to sample collection. However, to assure the safety of HESCL the level of glutamate dehydrogenase and galactose as more sensitive liver biomarker should be investigated [29]. These findings provide sufficient evidence to consider HESCL non-toxic substance because the altered values fall within the normal physiological level [6], and are comparable to the normal value for studied enzymes in our laboratory history [30].

Table 3. The effect of 90-day oral administration of the hydroethanolic extract of *Syzygium campanulatum* leaves on biochemical parameters in female SD rats.

Male	**Unit**	**Hydroethanolic extract of *Syzygium campanulatum* leaves**			
		0mg/kg	**500 mg/kg**	**1000 mg/kg**	**2000 mg/kg**
Total Protein	g/l	70.90 ± 4.06	72.50±3.54	71.40±2.67	72.60±2.50
Albumin	g/l	34.10 ± 2.77	29.00±1.41	34.30±1.16	34.10±2.64
Globulin	g/l	37.60 ± 1.74	42.00±2.83	37.10±2.60	41.20±4.44
Albumin / globulin ratio		0.95 ± 0.03	0.81±0.11	0.93±0.07	0.84±0.12
Total Bilirubin	µmol/L	<2.00 ± 0.00	<2.00 ± 0.00	<2.00 ± 0.00	<2.00 ± 0.00
Alkaline Phosphatase	U/L	263.30 ± 21.61	261.20±19.09	249.60±40.30	220.22 ± 30.05*
Alanine aminotranferase	U/L	68.90 ± 9.92	99.50±6.36	73.80±4.13	85.40±9.92
Aspartate aminotransferase	U/L	232.70 ± 34.25	212.00±9.90	207.80±27.52	299.50 ± 40.52*
Gamma glutamyl transferase	U/L	< 3.00 ± 0.00	< 3.00 ± 0.00	< 3.00 ± 0.00	< 3.00 ± 0.00
Urea	mmol/l	7.45 ± 0.34	7.60±0.00	6.87±0.64	6.92±0.23
Potassium	mmol/l	6.41 ± 0.67	6.45±0.45	6.31±0.31	6.87±0.35
Sodium	mmol/l	14130 ± 1.88	139.50±1.00	141.00±0.94	140.10±1.37
Chloride	mmol/l	103.30 ± 2.00	101.00±1.41	102.40±1.84	101.60±1.51
Creatinine	µmol/l	29.60 ± 4.45	31.50±0.71	25.70±6.73	28.00±5.10
Uric Acid	µmol/l	0.14 ± 0.03	0.82±0.96	0.15±0.03	0.17±0.03
Female					
Total Protein	g/l	78.50 ± 4.72	78.00±4.32	78.50±3.14	78.20±3.91
Albumin	g/l	37.00 ± 4.60	34.50±1.91	33.50±2.68	32.40±2.41
Globulin	g/l	41.20 ± 5.55	43.50±3.79	45.00±3.65	43.70±4.03
Albumin / globulin ratio		0.90 ± 0.26	0.82±0.08	0.75±0.10	0.75±0.10
Total Bilirubin	µmol/l	<2.00 ± 0.00	<2.00 ± 0.00	<2.00 ± 0.00	<2.00 ± 0.00
Alkaline Phosphatase	IU/L	211.10 ± 29.14	257.00±36.67	212.70±28.76	250.60±26.86
Alanine aminotranferase	U/L	65.30 ± 10.30	74.50±3.51	73.80±3.12	74.70±3.11
Aspartate aminotransferase	U/L	221.40 ± 24.68	257.00±18.67	291.30±44.86	253.80±38.40
Gamma glutamyl transferase	U/L	< 3.00 ± 0.00	< 3.00 ± 0.00	< 3.00 ± 0.00	< 3.00 ± 0.00
Urea	mmol/l	7.40 ± 1.12	8.03±0.98	6.69±0.62	7.99±0.55
Potassium	mmol/l	5.22 ± 0.18	5.40±0.23	5.47±0.25	5.47±0.33
Sodium	mmol/l	139.80 ± 1.66	139.25±0.96	139.80±2.44	138.20±1.32
Chloride	mmol/l	101.40 ± 2.20	102.50±1.73	103.50±2.07	100.20±1.03
Creatinine	µmol/l	32.70 ± 6.59	33.50±3.11	33.80±3.82	35.10±3.98
Uric Acid	µmol/l	0.16 ± 0.05	0.17±0.03	0.17±0.01	0.17±0.03

The values are mean ± SD of ten rats per group. The statistical analysis was done by ANOVA followed by Dunnett's test for all groups. * $P < 0.05$ vs. control group (AECNL 0 mg/kg)

Relative organ weights

Organ weights and terminal body weights were recorded at the end of study so that absolute and relative (to body weight) values could be calculated and used to evaluate the level of safety of HESCL. Compared with control values, a significant increase ($P < 0.05$) only in the relative not the absolute weight of the lung of male rats was found following 90 days of treatment with 500, 1000, and 2000 mg/kg of HESCL. The absolute and relative weights of the other organs in both male and female rats were not significantly different compared to control groups in 28 and 90 days study (Tables 4).

Table 4. The relative organ weight of female ratsreceivedthehydroethanolic extract of *Syzygium campanulatum* leaves for 90 days.

Male	Hydroethanolic extract of *Syzygium campanulatum* leaves			
	0mg/kg	500mg/kg	1000mg/kg	2000 mg/kg
Brain	0.51 ± 0.07	0.51 ± 0.08	0.49 ± 0.05	0.51 ± 0.06
Thymus	0.10±0.05	0.08±0.03	0.09±0.03	0.09±0.03
Heart	0.27±0.03	0.31±0.04	0.28±0.03	0.31±0.04
Lung	0.39±0.06	0.58±0.16*	0.42±0.16*	0.56±0.09*
Spleen	0.16±0.02	0.16±0.04	0.14±0.03	0.19±0.05
Liver	2.79±0.20	2.74±0.36	2.30±0.77	3.04±0.43
Kidney (R)	0.29±0.04	0.28±0.02	0.25±0.06	0.31±0.05
Kidney (L)	0.28±0.04	0.29±0.03	0.26±0.03	0.31±0.04
Adrenal gland (R)	0.02±0.01	0.01±0.00	0.03±0.07	0.01±0.00
Adrenal gland (L)	0.02±0.01	0.01±0.02	0.01±0.00	0.01±0.00
Stomach	1.43±0.30	1.26±0.40	1.04±0.26	1.55±0.35
Stomach (empty)	0.44±0.06	0.43±0.06	0.41±0.04	0.50±0.08
Gut	5.19±0.50	4.77±0.41	4.72±0.42	5.50±0.98
Gut (empty)	2.800.43	2.62±0.41	2.81±0.43	2.65±0.92
Testis (R)	0.42±0.03	0.43±0.08	0.39±0.06	0.42±0.06
Testis (L)	0.42±0.04	0.43±0.07	0.38±0.07	0.42±0.06
Female				
Brain	0.48 ± 0.04	0.48 ± 0.03	0.49 ± 0.06	0.50 ± 0.01
Thymus	0.08±0.03	0.07±0.02	0.07±0.03	0.07±0.02
Heart	0.25±0.04	0.23±0.03	0.23±0.05	0.24±0.04
Lung	0.48±0.23	0.48±0.09	0.48±0.07	0.57±0.16
Spleen	0.14±0.06	0.14±0.03	0.15±0.03	0.17±0.06
Liver	2.39±0.33	2.36±0.26	2.35±0.34	2.59±0.52
Kidney (R)	0.19±0.02	0.20±0.03	0.21±0.06	0.22±0.05
Kidney (L)	0.20±0.03	0.20±0.02	0.22±0.05	0.22±0.04
Adrenal gland (R)	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00
Adrenal gland (L)	0.01±0.01	0.01±0.00	0.02±0.03	0.01±0.00
Stomach	0.99±0.20	1.19±0.32	0.78±0.16	1.17±0.51
Stomach (empty)	0.39±0.07	0.54±0.15	0.38±0.07	2.53±2.39
Gut	3.30±2.03	3.93±0.50	3.91±0.65	2.58±2.42
Gut (empty)	2.40±1.24	2.24±0.31	2.24±0.44	2.36±0.59
Ovary (R)	0.02±0.01	0.01±0.01	0.02±0.01	0.02±0.01
Ovary (L)	0.02±0.01	0.02±0.01	0.01±0.01	0.02±0.01
Uterus	0.12±0.04	0.14±0.04	0.16±0.03	0.13±0.06

The values are mean ± SD of ten rats per group. The statistical analysis was done by ANOVA followed by Dunnett's test for all groups. * $P < 0.05$ vs. AECNL 0 mg/kg.

Body weight

The mean body weights of the male and female rats were not significantly different from that of the control group after 28-day treatment. Furthermore, no significant differences in mean body weight of female group were noted in the 90-daystudy. A significant ($P < 0.05$) reduction in mean body weight of male rats treated with

500, 1000, and 2000 mg/kg of HESCL was noted at weeks 7 and 8. At week 13 significant ($P < 0.05$) increase was recorded in the mean body weight of the male group received1000 mg/kg of HESCL compared to control group (Figure 3). The mean body weight in the other groups showed no significant difference with the control group.

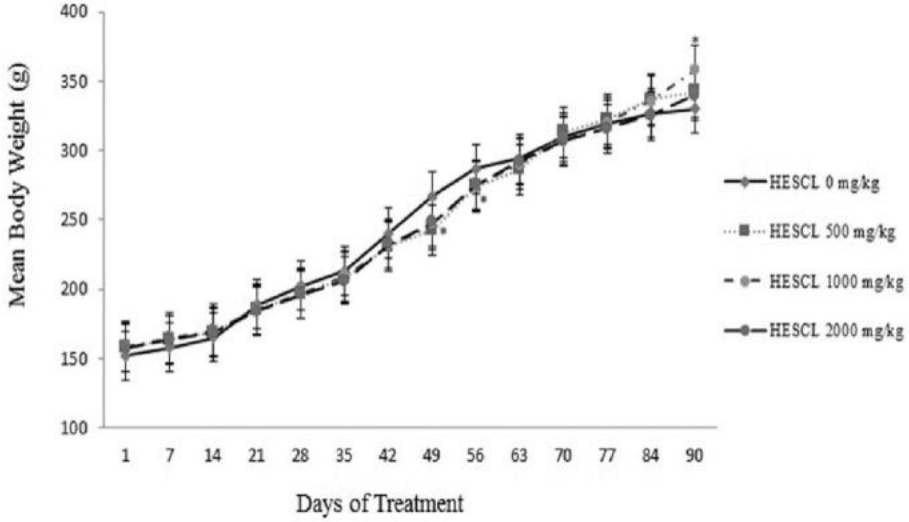

Figure 3. Mean ± SD body weight of male rats treated with 500, 1000, and 2000 mg /kg of HESCL, for 90 days. *P < 0.05 compared with the control group.

Histopathological parameters

The results of the histopathological studies illustrate the organ-related toxicological effects and provide support for the biochemical analysis results. Microscopic observation revealed no gross indications of adverse effects of the treatment on rat organs upon necropsy after 28 and 90 days of HESCL administration. (Image not presented as there were no identified treatment-related changes in the highest dose group).

Conclusion

In summary, based on the analyses the major phytochemical in HESCL is betulinic acid (60.43 mg/g.), it also contains total glycol saponins, total phenolics, total proteins, total tannins, and total flavonoids. Evaluation of mutagenicity of HESCL via Ames test provides sufficient evidence that HESCL is not genotoxic or carcinogen. Refereeing to the toxicological parameters evaluated in this article we conclude that single and repeated exposure to HESCL is particularly safe in male and female Sprague-Dawley rats. The LD_{50} is more than 5000 mg/kg and the NOEAL up to 90 days is 2000 mg/kg for HESCL. However, obtained results grant detail investigation on altered parameters.

Authors' contributions

AMSAM, ASAM, MAS and EF designed the experiments. EF, KI, and AS executed the experiment. EF design and performed the *in vitro* mutagenicity studies. ASAM, MBKA, EF, KI, and AS analyzed data and interpreted the results. EF, and ASAM, participated in drafting the manuscript. All authors have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation, also they have read and approved the final manuscript.

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Conflicts of interests

The authors declare no conflicts of interests.

References

[1]. Veiga Junior VF, Pinto AC, Maciel MAM. Medicinal plants: safe cure? Química Nova. 2005;28:519-28.

[2]. Saad B, Azaizeh H, Abu-Hijleh G, Said O. Safety of traditional Arab herbal medicine. Evid Based Complement Alternat Med. 2006;3:433-9.

[3]. Debelle FD, Vanherweghem J-L, Nortier JL. Aristolochic acid nephropathy: a worldwide problem. Kidney Int. 2008;74:158-69.

[4]. Ifeoma O, Oluwakanyinsola S. Screening of Herbal Medicines for Potential Toxicities: INTECH Open Access Publisher; 2013.

[5]. OECD. OECD Guidelines for the Testing of Chemicals. Paris: OECD Publishing; 2015.

[6]. Gad SC. Preclinical development handbook: toxicology. New York City, US: John Wiley & Sons; 2008.

[7]. Aisha A, Abu-Salah K, Darwis Y, Abdul Majid A. Screening of antiangiogenic activity of some tropical plants by rat aorta ring assay. Int J Pharmacol. 2009;5:370-6.

[8]. Aisha AF, Ismail Z, Abu-Salah KM, Siddiqui JM, Ghafar G, Majid AMSA. *Syzygium campanulatum* korth methanolic extract inhibits angiogenesis and tumor growth in nude mice. BMC Complement Altern Med. 2013;13:168-79.

[9]. Memon AH, Ismail Z, Aisha AF, Al-Suede FSR, Hamil MSR, Hashim S, et al. Isolation, Characterization, Crystal Structure Elucidation, and Anticancer Study of Dimethyl Cardamonin, Isolated from Syzygium campanulatum Korth. Evid Based Complement Alternat Med. 2014;2014:1-11.

[10]. WHO. Evaluation of certain food additives and contaminants. World Health Organ Tech Rep Ser. Geneva, Switzerland: Joint FAO WHO Expert Committee on Food Additives; 2000. p. 1.

[11]. USP USP. Method for the Microbiological Examination of Food. Washington, United State: United State Pharmacopoeia; 2009.

[12]. Farsi E, Shafaei A, Hor SY, Ahamed MB, Yam MF, Asmawi MZ, et al. Genotoxicity and acute and subchronic toxicity studies of a standardized methanolic extract of *Ficus deltoidea* leaves. Clinics (Sao Paulo). 2013;68:865-75.

[13]. Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res Genet Toxicol Environ Mutagen. 1975;31:347-63.

[14]. OECD. Test No. 423: Acute Oral toxicity - Acute Toxic Class Method. Paris: OECD Publishing; 2002.

[15]. OECD. Test No. 425: Acute Oral Toxicity: OECD Guidelines for the Testing of Chemicals. In: Chemicals OGftTo, editor. Paris: OECD Publishing; 2008.

[16]. OECD. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals. Paris: OECD Publishing; 1998.

[17]. Bernal J, Mendiola JA, Ibáñez E, Cifuentes A. Advanced analysis of nutraceuticals. Journal of Pharmaceutical and Biomedical Analysis. 2011;55:758-74.

[18]. Zhou Q, Sun S-Q, Zuo L. Study on traditional Chinese medicine 'Qing Kai Ling' injections from different manufactures by 2D IR correlation spectroscopy. Vib Spectrosc. 2004;36:207-12.

[19]. Hayes AW, Kruger CL. Hayes' principles and methods of toxicology. Boca Raton, US: CRC Press; 2014.

[20]. Hodge HC, Sterner JH. Tabulation of toxicity classes. American Industrial Hygiene Association Quarterly. 1949;10:93-6.

[21]. Evans GO. Animal Hematotoxicology: A Practical Guide for Toxicologists and Biomedical Researchers. Abingdon, US: CRC Press; 2008.

[22]. Giknis ML, Clifford CB. Clinical Laboratory Parameters for Crl CD(SD) Rats. USA: Charles River Laboratories International, Inc.; 2006.

[23]. Evans GO. Animal Clinical Chemistry: A Practical Handbook for Toxicologists and Biomedical Researchers. Second ed. Abingdon, US: CRC Press; 2009.

[24]. Hamdy RC, Lewiecki EM. Osteoporosis. Northamptonshire, US: Oxford University Press; 2013.

[25]. Naber TH, Baadenhuysen H, Jansen JB, van den Hamer CJ, van den Broek W. Serum alkaline phosphatase activity during zinc deficiency and long-term inflammatory stress. Clin Chim Acta. 1996;249:109-27.

[26]. Cho YE, Lomeda RAR, Ryu SH, Sohn HY, Shin HI, Beattie JH, et al. Zinc deficiency negatively affects alkaline phosphatase and the concentration of Ca, Mg and P in rats. Nutr Res Pract. 2007;1:113-9.

[27]. Baker HJ, Lindsey JR, Wesibroth SH. The Laboratory Rat: Biology and Diseases: Elsevier Science; 2013.

[28]. Zimmerman HJ. Hepatotoxicity: The Adverse Effects of Drugs and Other Chemicals on the Liver. Philadelphia, USA: Lippincott Williams & Wilkins; 1999.

[29]. Sharp P, Villano JS. The Laboratory Rat, Second Edition: Taylor & Francis; 2012.

[30]. Hor SY, Ahmad M, Farsi E, Yam MF, Hashim MA, Lim CP, et al. Safety assessment of methanol extract of red dragon fruit (*Hylocereus polyrhizus*): Acute and subchronic toxicity studies. Regul Toxicol Pharmacol. 2012;63:106-14.