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Phytochemistry, Biological Activity and Toxicity of Botanical Dietary Supplement: KL21

Çinel Kösal Karayıldırım[1], Adem Güner[2], Gürkan Yiğittürk[3], Altuğ Yavaşoğlu[4], N. Ülkü Karabay Yavaşoğlu[1]*

[1]Ege University, Science Faculty, Biology Department, İzmir, Turkey
[2]Giresun University, Science and Literature Faculty, Biology Department, Giresun, Turkey
[3]Muğla Sıtkı Koçman University, Medicine Faculty, Histology and Embryology Department, Muğla, Turkey
[4]Ege University, Medicine Faculty, Histology and Embryology Department, Izmir, Turkey
* ulku.karabay@ege.edu.tr
* Orcid: 0000-0002-7483-0184

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Abstract

The use of nutritional supplements has grown conspicuously over the last decades. The present study aimed to design, develop, and optimize the toxicological and biological procedures to perform the quantitative determination of botanical dietary supplement KL21 which is a novel product containing extract form 21 plant species in different amounts. The concentrations of phenolics in KL21 were 11.90 and 258.58 mg for ethanol extract, 3.58 and 86.42 mg for methanol extract, respectively. Carvacrol is a major component of the extracts according to GC-MS analysis. DPPH activity of methanol extract was higher than that of the ethanol extract. Both extracts showed similar relative antioxidant activity according to ABTS+ assay. Only methanol extract exhibited antimicrobial activity. KL21 ethanol extract exhibited inhibitory activity with 50% growth inhibitory concentration of 92.09 µg/mL, 95.47 µg/mL and 44 µg/mL on MCF-7, HeLa and HEK-293 cells respectively. KL21 demonstrated no mutagenic activity with the Salmonella strains. The in vivo toxicity test results indicated that KL21 had no significant adverse effects. Dose-depended KL21 treatment seemed nontoxic due to biochemical, hematological values. No histological damage in the liver was observed in the tissues. These results suggested that KL21 polyherbal formulation is a naturel source of antioxidant with antimicrobial activities and has therapeutic potential in a safe range.

Keywords: Antioxidant activity, Biochemical and hematological analyses, Dietary supplement, Phenolic and flavonoid contents, Toxicity

1. Introduction

Herbal drugs have been used for thousands of years in the worldwide especially they were extensively gained attention on last decades [1, 2]. Numerous herbal ingredients are now used to treat various diseases or health problems [3]. Additionally, there are many polyherbal formulations which can widely have used such as dietary supplements and treatment agents. Moreover, these formulations are source of vitamins, minerals and antioxidant compounds [4]. Because of herbs are source of polyphenolic compounds these formulations are widely used for anti-carcinogenic, anti-viral, anti-inflammatory effects. Also, the quality of polyherbal formulations depends on the composition and concentration of relevant natural compounds. For these reasons, the quality control studies and toxicological investigations are most important parameters to determine the safety use of polyherbal formulations [5]. Nowadays, many studies that focused on adverse effects of herbal drug combinations results regarding the risk of herbal drug combinations induced adverse effects such as liver injury because of these herbs were contaminated by heavy metals, microbial toxins, pesticides, polycyclic aromatic hydrocarbons and fumigants. These factors can accumulate during the production and manufacturing of herbs and may have adverse effects on consumer health. Recently, the practice on herbal medicine is increasingly used in various diseases with the expectation of reducing drug toxicity, alleviating adverse effects and minimizing recruitment periods. Considering this situation,

reliability and efficiency of these herbal formulations are one of the important health problems for last decade. It is important to find new combinations and also to determine their toxicological level to improve the outcome for patients.

In this study, KL21 was used as a prototype which consisting of *Achillea millefolium* L. (Asteraceae), *Equisetum arvense* L. (Equisetaceae), *Urtica dioica* L. (Urticaceae), *Thymus vulgaris* L. (Lamiaceae), *Viscum album* L. (Viscaceae), *Acorus calamus* L. (Acoraceae), *Rosmarinus officinalis* L. (Lamiaceae), *Solidago vigaurea* L. (Asteraceae), *Silybum marianum* L. (Astreraceae), *Curcuma longa* V. (Zingiberaceae), *Lavandula stoechas* L. (Laminaceae), *Fumaría officinalis* L. (Papaveraceae). *Taraxacum officinale* L. (Asteraceae), *Cichorium intybus* L. (Asteraceae), *Zingiber officinale* R. (Zingiberaceae), *Peganum harmala* L. (Nitrariaceae), *Juniperus communis* L. (Cupressaceae), *Nigella sativa* L. (Ranunculaceae), *Hypericum perforatum* L. (Hypericaceae), *Valeriana officinalis* L. (Valerianaceae), *Melissa officinalis* L. (Lamiaceae). Standardized KL21 product is approved as a botanical dietary supplement by The Republic of Turkey, Ministry of Agriculture and Forestry. However, there is no information about chemical composition profile and biological activities of KL21. The aim of this study was to assess the total phenolic and flavonoid content, antioxidant capacity and antimicrobial efficacy of KL21 polyherbal formulation. Additionally, it was aimed to analyze the toxicological profile and to investigate histological changes in liver. It allowed acquiring data about this product's therapeutic potential in a safe range.

2. Materials and Methods

2.1. Plant material

KL21 is a novel product containing extract form 21 plant species in different amounts. It is a mixture of *A.millefolium* L. (63 mg), *U.dioica* L. (63 mg), *E.arvense* L. (63 mg), *T.vulgaris* L. (99 mg), *V.album* L. (13 mg), *A.calamus* L. (13 mg), *R.officinalis* L. (27), *S.vigaurea* L. (13 mg), *S.marianum* L. (50 mg), *C.longa* V. (27 mg), *L.stoechas* L. (27 mg), *F.officinalis* L. (27 mg). *T.officinale* L. (27 mg), *C.intybus* L. (27 mg), *Z.officinale* R. (27 mg), *P.harmala* L. (13 mg), *J.communis* L. (27 mg), *N.sativa* L. (13 mg), *H.perforatum* L. (4 mg), *V.officinalis* L. (4 mg), *M.officinalis* L. (13 mg) in a hydroxypropylmethyl cellulose capsule (amount of 1 capsule). Merely, all quality control studies of KL21 have been done by Naturin Nutraceuticals Products Company, Izmir, Turkey. Ethanol and methanol extracts of KL21 were separately prepared.

2.2. Determination of total phenolic and flavonoid contents

The total phenolic compound was determined spectrophotometrically utilizing Folin–Ciocalteu reagent [6]. The total flavonoid compound content was measured as quercetin equivalence [7]. Volatile constituents were determined by Shimadzu QP 2010 plus Gas chromatography Mass spectrometry equipped with Rtx-CL capillary standard non-polar column compose of 100% dimethyl polysiloxane. Approximately 1 g of KL21 extracts were taken into a screw cap vial and 10 mL of acetone was added then 1 µL ethanolic and methanolic solutions were analyzed by GC-MS. The identification of the extracts was performed by Wiley 7 Library data provided by the software of the GC-MS.

2.3. Total antioxidant capacity

The antioxidant capacity of KL21 extracts were determined according to DPPH radical-scavenging activity and $ABTS^+$ (Sigma-Aldrich Co., USA) radicalcation decolorisation assays. DPPH assay was performed with 1000 µL of the extracts and 4 mL methanol solution of DPPH. After 30 minutes of incubation at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of a free radical by DPPH in percent was calculated. According to decolorisation assays, ABTS with potassium persulfate generates blue/green $ABTS^+$. The radical formed shows a maximum absorbance at 734 nm. The total antioxidant activity percentage (TAA%) was calculated [8].

2.4. Minimum inhibitory concentration test

The bactericidal activity test was evaluated using the following four-gram negative test organisms which are *Escherichia coli* ATCC 11230, *Klebsiella pneumonie* ATCC 13883, *Salmonella typhimurium* CCM 583, *Pseudomonas aeruginosa* ATCC 27853 and four-gram positive test organisms which are *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228**,** *Bacillus cereus* ATCC 7064, *Enterococcus faecalis* ATCC 29212. Moreover, the fungicidal activity test was performed using the vegetative cells of *Candida albicans* NCPF 3179 and the spores of *Aspergillus brasiliensis* NCPF 2275. Strains were obtained from Biomerieux® (France) and Microbiologics® (USA). Gentamycin (CAS: 1405-41-0, Sigma Aldrich Co., USA) and Nystatin (CAS: 1400-61-9, Sigma Aldrich, Co., USA) were used as a positive control (NCCLS, 2015).

2.5. Cytotoxicity test

According to cell viability, modified MTT test was performed to determine the cytotoxic effects of KL21 extracts [9]. Human breast adenocarcinoma, Human lung adenocarcinoma, Human colon adenocarcinoma, Human cervix adenocarcinoma and Human embryonic kidney cell lines were maintained in flasks at 37 °C in incubator. The broth was treated with different dilutions (0.1 mg/mL, 1 mg/mL and 10 mg/mL) of KL21 and incubated for 72 hours. Inhibition of cell growth was calculated as IC_{50} (50 % effective concentration). The results were measured at 570 nm with an UV spectrophotometer. GraphPad Prism (San Diego, USA) was used for the calculation of KL21 causing a 50 % inhibition in comparison to untreated controls.

2.6. The Ames Salmonella/microsome mutation test

Ames MPF^{tm} mutagenicity assay (Xenometrix Inc. Switzerland) was conducted on 4 strains of *Salmonella typhimur*ium, tester strains TA98, TA100, TA 1535 and TA 1537 according to the OECD Guideline 471 [10]. TA98 and TA 1537 strains are used for the detection of frameshift mutations and TA100 and TA1535 for base pair substitutions. Genotypes of the strains were checked by Xenometrix Inc. Study design was performed w/wo S9, strain-specific positive control chemicals and bacteria are exposed to 6 concentrations of ethanol and methanol extracts of KL21. Bacterial growth which is mutagenicity is measured colorimetrically by a color change (pH drop) from purple to yellow.

2.7. Animals and experimental design

The study was approved by the Ege University, Local Ethical Committee of Animal Experiment (24.02.2012, 2012-032). Ethical guidelines for investigation of experimental pain unconscious animals were considered all *in vivo* experiments [11]. Mice purchased from the KOBAY Laboratory (Izmir, Turkey). During the study, all animals were weighed daily.

2.7.1. Single dose oral toxicity test

Up-and-Down-Procedure (UDP) was conducted on 5 animals according to OECD Guidelines No: 425 [12]. Doses of KL21 which were applied 175, 500 and 2000 mg/kg. During the lethality period, body weight data and toxicity signs were observed individually. The total observations period was 14 days according to method.

2.7.2. Subacute toxicity test

Repeated dose 28-day oral toxicity study was conducted on male (n=20) and female (n=20) Swiss albino mice according to OECD Guidelines No: 407 [13].

After acclimation period healthy male and female mice were assigned randomly to groups (300, 600 and 900 mg/kg KL21 and control). All mice were sacrificed end of the study and liver weights were recorded after; liver/ body weight ratios were calculated. Blood samples were collected before and end of the study for hematological and biochemical analyses. Routine protocols were performed for evaluation of histological analyses.

2.8. Statistics

All experimental results were the means of experiments performed in triplicate and the data in the tables and figures represent the mean values ±standard deviations (n=3). $p<0.05$ was considered to be statistically significant.

3. Results

3.1. Total phenolic and flavonoid compounds analysis

Total phenolic and flavonoid compound contents of KL21 extracts were demonstrated. The highest contents of these compounds were seen in KL21 ethanol extract (Table 1). GC-MS analysis of the KL21 extracts led to identification and quantification of compounds. Nine compounds were identified constituting 99.9% of the total extracts (Table 2; Fig 1 and 2). Carvacrol has been reported a common major component (45.091% for ethanol extract; 42.414% for methanol extracts).

Table 1. Content of flavonoid and total phenolic compounds of KL21 extracts

Extracts	Phenolic Content (GAE mg /g)	Flavonoid Content (QE mg /g)
Ethanol	258.58 ± 0.02	11.09 ± 0.02
Methanol	86.42 ± 0.03	3.58 ± 0.02

* GAE: Gallic acid equivalent; QE: Quercetin equivalent

Table 2. Chemical composition of the KL21 extracts

Retention Time (min.)	Compounds	Composition of KL21 Extracts (%)	
		Ethanol	Methanol
7.32	Carvacrol	45.091	42.414
7.98	Eugenol	0.954	0.974
8.57	α-Curcumine	1.495	1.479
8.89	β-Sesquiphellandrene	0.615	0.508
10.94	α-Asarone	8.665	6.230
11.36	β-Tumerone	17.721	11.780
11.75	α-Tumerone	9.143	6.398
15.33	Hexadecanoic acid	6.056	12.409
18.30	Linolenic acid	10.259	17.807

The other most plentiful compounds were identified as β-Tumerone (17.721%), Linolenic acid (10.259%), α-Tumerone (9.143%), α-Asarone (8.665%) and Hexadecanoic acid (6.056%) for ethanol extract. Similarly, Linolenic acid (17.807%), Hexadecanoic acid (12.409%), β-Tumerone (11.780%), α-Tumerone (6.398%) and α-Asarone (6.230%) were identified for most abundant components of methanol extract.

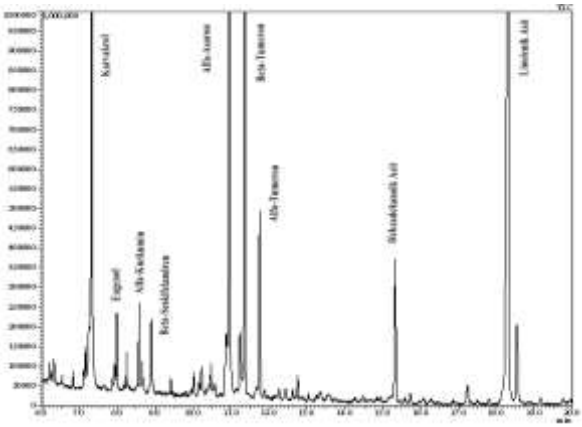

Figure 1. GC-MS chromatogram of KL21 ethanol extract

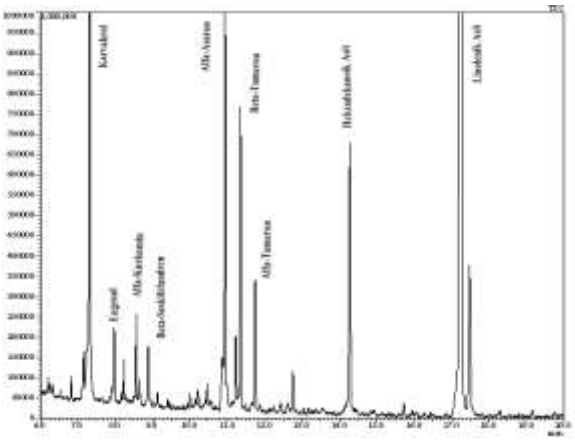

Figure 2. GC-MS chromatogram of KL21 methanol extract

3.2. Antioxidant capacity test

The antioxidant activities of KL21 extracts are demonstrated in Tables 3 and 4. According to DPPH method, methanol extract exhibited higher antioxidant activity than ethanol extract. The extract (0.25 mg/mL) displayed 40.82 % inhibition in the test. Additionally, it has exhibited similar antioxidant effect of α-tocopherol (36.89 mg/mL). Besides the IC_{50} value of methanol extract of KL21 was calculated as 0.294 mg/mL. However, IC_{50} value of ethanol extract was determined as 14.173 mg/mL. These data were demonstrated that lower IC_{50} value indicates higher antioxidant activity (Table 3). Also, both extracts showed similar relative antioxidant activity with antioxidant capacity according to $ABTS^{+}$ assay (Table 4).

Table 3. DPPH radical scavenging activity of KL21 extracts

Extract (mg/mL)		Inhibition (%)	a-Tocopherol equivalent antioxidant activity values (µg/mL)	IC_{50} (mg/mL)
Methanol	0.25	40.82 ± 0.04	36.89 ± 0.01	0.294
	0.50	74.86 ± 0.03	67.66 ± 0.01	
	1	90.66 ± 0.04	81.94 ± 0.03	
Ethanol	0.25	0.41 ± 0.05	22.31 ± 0.04	14.173
	0.50	2.92 ± 0.01	32.22 ± 0.02	
	1	4.67 ± 0.02	42.16 ± 0.03	

* Data expressed as mean ± SD, IC50: concentrations of extracts for 50% inhibition of DPPH free radicals

Table 4. Total antioxidant activity of KL21 extracts by ABTS assay

Extract (0.125 mg/mL)	ABTS Inhibiton (%)	RRA
Ethanol	72.80 ± 0.02	0.53 ±0.02
Methanol	71.70 ± 0.02	0.52 ± 0.02

* Data expressed as mean ± SD, RAA, relative antioxidant activity (TAA% extract/TAA % standard antioxidant compound)

3.3. Antimicrobial activity

In general, there was major differences activity between methanol and ethanol extracts. Methanol extract was active against the tested microorganism with MIC values ranging from 32 to 64 µg/mL. However, ethanol extract had no activity at the highest concentration (256 µg/mL) (Table 5).

Table 5. Minimum inhibitor concentration values of KL21 extracts

Microorganism	MIC (mg/mL)			
	Extract		Gentamicin	Nystatin
	Methanol	Ethanol		
S. aureus	32	128	1	-
S. epidermidis	32	128	1	-
S. typhimurium	32	128	1	-
E. coli	32	256	1	-
B. cereus	64	256	4	-
K. pneumonie	64	256	4	-
E. faecalis	32	256	16	-
P. aeroginosa	32	128	2	-
C. albicans	64	256	-	4
A. brasiliensis	64	256	-	8

3.4. Cytotoxicity test

The MTT results indicated that only ethanol extract inhibits cancer cell proliferation in a dose-dependent manner. The ethanol extract's IC_{50} values were found to be 92.09 µg/mL and 95.47 µg/mL for 48 h treatment on MCF-7 and HeLa cell lines respectively. 44 µg/mL of ethanol extract and 49 µg/mL dose of methanol extract showed marked cytotoxicity on HEK-293 cell line.

3.5. The *Salmonella*/microsome mutagenicity assay

The mean number of positive yellow wells per 6 doses was calculated from the triplicates and the fold increases above the baseline were determined for each dose of KL21 polyherbal formulation extracts. According to results no mutagenic evidence was determined for maximum dose 5000 µg/mL of KL21 ethanol and methanol extracts (Fig 3 and 4).

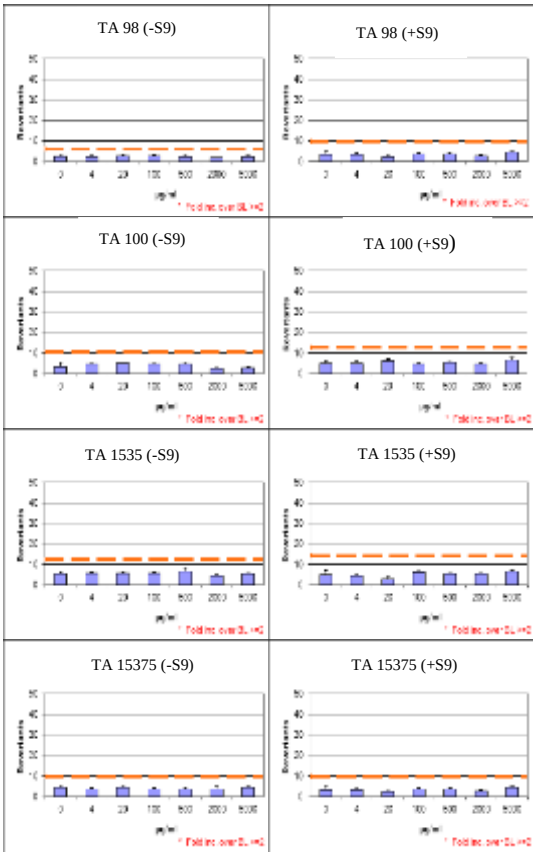

Figure 3. *S. typhimurium* mutagenicity test results of KL21 ethanol extract

3.6. *In vivo* toxicity test results

Each animal was observed daily throughout the entire 14-day single-oral-dose toxicity test period. There is no abnormal clinical signs or lethality, as well as the observation day and time were recorded (Table 6).

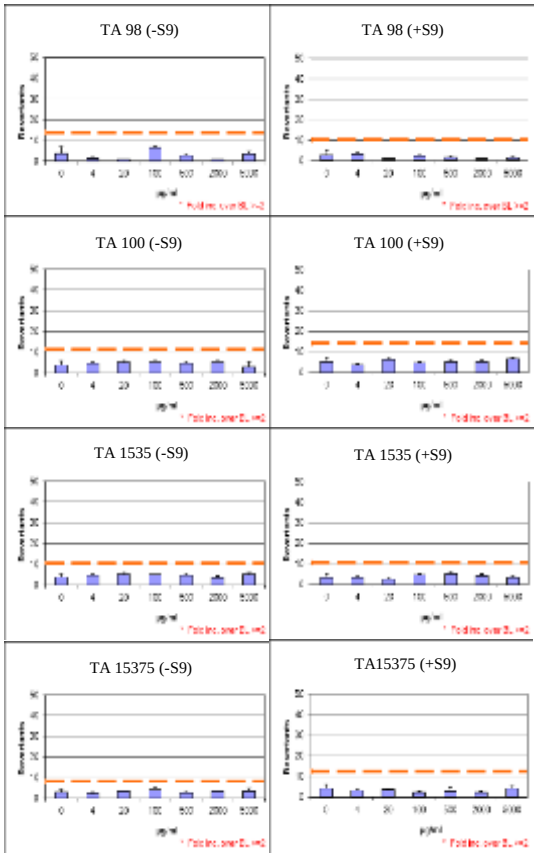

Figure 4. *S. typhimurium* mutagenicity test results of KL21 methanol extract

Table 6. Dose-dependent single dose oral toxicity test result

Step	Include (I) Exclude (E)	Dose (mg/kg)	Response (X) Non-response (O)	Log_{10} Dose
1	I	175	O	2.2430
2	I	550	O	2.7404
3	I	2000	O	3.3010
4	I	2000	O	3.3010
5	I	2000	O	3.3010
6	E			
7	E			
8	E			
Calculated maximum LD_{50} value ≥ 2000 mg/kg				

The LD_{50} is greater than 2000 mg/kg because more than three animal survive.

According to subacute toxicity test results, there was no significant change in body weight gains of the treatment groups in comparison with control groups (Table 7). There were also no important differences in absolute and relative weights of organs of KL21 treated mice when compared with control ($p<0.05$) (Table 8). There were no significant differences in both biochemical and hematological parameters compared to KL21 treated and control groups in male and female mice ($p<0.05$). According to comparison between the pre-treatment (day 0), post-treatment (day 28) and control values of female and male mice, it was demonstrated that there were no toxicological significant differences ($p<0.05$) (Table 9-12).

Hematoxylin-eosin-stained sections of liver tissue were examined for routine histological parameters. According to results histological appearance of liver tissue was evaluated normal in all groups. Regular appearance of vena centralis, vena interlobular, hepatic artery, bile ducts, sinusoids, eosinophilic cytoplasm, hepatocytes formations and nucleus alterations were also observed in the liver of all treated mice. Additionally, there were no numeric increases of Kupffer cells which were observed (Fig 5 and 6).

Table 7. Body weights of male and female mice of control and KL21 treated groups

Groups	Sex	Body Weight (g) (mean ± SD)				
		Day 0	Day 7	Day 14	Day 21	Day 28
Control	M	24.2 ± 1.16	24.6 ± 1.53	25.1 ± 1.28	28.8 ± 2.61	29.1 ± 2.56
	F	24.1 ± 1.02	25.7 ± 0.74	25.8 ± 0.70	28.5 ± 1.18	28.6 ± 1.15
300 mg/kg	M	24.3 ± 0.99	24.8 ± 1.44	25.0 ± 1.46	28.5 ± 1.69	28.7 ± 1.71
	F	24.4 ± 0.84	25.7 ± 0.57	25.7 ± 0.48	26.9 ± 0.82	27.2 ± 0.85
600 mg/kg	M	24.0 ± 1.22	25.9 ± 0.89	26.4 ± 0.43	27.4 ± 2.70	27.8 ± 2.29
	F	23.4 ± 0.54	24.4 ± 1.33	24.8 ± 1.14	26.5 ± 1.99	27.1 ± 1.59
900 mg/kg	M	24.6± 1.51	25.3 ± 0.88	25.8 ± 0.48	27.7 ± 1.09	27.8 ± 0.61
	F	24.4 ± 0.59	25.7 ± 0.91	26.1 ± 0.50	26.1 ± 1.78	26.5 ± 1.56

* Data expressed as mean ± SD, Statistically significant from the control ($p<0.05$)

Table 8. Organs weights and relative organs weights of male and female mice of control and KL21 treated groups

Groups	Sex	Liver Weight (g)	Relative Liver Weight (g)	Kidney Weight (g)	Relative Kidney Weight (g)	Testicle (g)	Relative Testicle Weight (g)
Control	M	1.54± 0.18	0.0528 ± 0.0026	0.38 ± 0.05	0.0131 ± 0.0012	0.16 ± 0.04	0.0055 ± 0.0011
	F	1.47 ± 0.22	0.0517 ± 0.0087	0.32 ± 0.02	0.0109 ± 0.0006	-	-
300 mg/kg	M	1.55 ± 0.15	0.0543 ± 0.0083	0.41 ± 0.01	0.0145 ± 0.0011	0.16 ± 0.01	0.0056 ± 0.0004
	F	1.45 ± 0.11	0.0535 ± 0.0038	0.32 ± 0.06	0.0117 ± 0.0011	-	-
600 mg/kg	M	1.57 ± 0.05	0.0564 ± 0.0028	0.40 ± 0.05	0.0142 ± 0.0015	0.14 ± 0.02	0.0051 ± 0.0006
	F	1.43 ± 0.07	0.0529 ± 0.0056	0.30 ± 0.06	0.0108 ± 0.0016	-	-
900 mg/kg	M	1.56 ± 0.10	0.0561 ± 0.0083	0.39 ± 0.02	0.0139 ± 0.0011	0.15 ± 0.02	0.0053 ± 0.0004
	F	1.46 ± 0.12	0.0553 ± 0.0070	0.39 ± 0.03	0.0112 ± 0.0016	-	-

* Data expressed as mean ± SD, Statistically significant from the control ($p<0.05$)

Table 9. Biochemical profile of female mice

Parameters	Pre-treatment period (Day 0)				Post-treatment period (Day 28)			
	Control	300 mg/kg	600 mg/kg	900 mg/kg	Control	300 mg/kg	600 mg/kg	900 mg/kg
ALB (g/dL)	3.9±0.6	3.7±0.4	4.1±0.1	4.1±0.2	3.3±0.1	3.0±0.1	3.0±0.4	3.4±0.1
ALP (U/L)	81.2±4.7	82.2±5.2	81.6±9.1	80.6±4.1	87.2±2.7	86.4±6.6	84.8±5.7	86.0±2.4
ALT (U/L)	61.0±7.5	57.0±12.9	58.4±16.1	60.6±14.6	57.4±2.0	56.6±2.5	55.6±4.3	55.8±3.4
AMY (U/L)	841±204	833±190	800±198	802±167	918±67	916±76	906±97	909±78
TBIL (mg/dL)	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.2±0.1	0.2±0.05	0.2±0.1
BUN (mg/dL)	12.0±1.8	12.2±1.6	12.6±3.2	12.8±2.7	10.2±1.6	10.8±1.3	10.6±0.8	10.8±1.6
Ca (mg/dL)	10.3±0.8	10.4±0.6	10.0±0.8	9.9±0.4	10.6±0.3	10.2±0.5	9.8±0.4	9.7±0.1
Phos (mg/dL)	8.3±1.4	8.1±1.6	8.2±1.3	8.3±1.4	8.1±0.7	8.2±1.6	8.1±0.8	8.8±0.5
Cre (mg/dL)	0.2±0.1	0.2±0.1	0.3±0.0	0.2±0.0	0.1±0.1	0.2±0.1	0.2±0.1	0.2±0.1
GLU (mg/dL)	214±53	216±19	214±16	215±54	223±8	227±17	226±9	222±12
Na (mmol/L)	136.4±3.7	135.2±1.7	135.8±1.9	135.0±3.2	142.0±1.5	142.2±5.2	142.4±4.6	142.0±5.0
K (mmol/L)	6.1±1.6	6.1±1.8	6.0±1.4	6.0±2.0	5.2±0.1	5.2±0.1	5.2±0.3	5.1±0.4
TP (g/dL)	5.4±0.4	5.3±0.5	5.7±0.4	5.5±0.5	6.2±0.2	6.2±0.4	6.3±0.2	6.2±0.3
GLOB (g/dL)	2.2±0.7	2.3±0.6	2.4±0.5	2.8±0.4	2.8±0.5	2.8±0.3	2.8±0.4	2.7±0.1
BA (umol/L)	3.8±2.1	4.0±1.5	3.4±1.8	3.6±2.4	3.2±0.3	3.8±0.3	2.4±0.8	2.0±0.7
Chol (mg/dL)	90.2±9.5	92.8±4.5	96.4±9.4	89.4±16.5	94.8±6.4	95.0±9.1	130.0±11.9	125.8±3.4

* Data expressed as mean ± SD, Statistically significant from the control ($p<0.05$)

Table 10. Biochemical profile of male mice

Parameters	Pre-treatment period (Day 0)				Post-treatment period (Day 28)			
	Control	300 mg/kg	600 mg/kg	900 mg/kg	Control	300 mg/kg	600 mg/kg	900 mg/kg
ALB (g/dL)	3.4±0.6	3.7±0.4	3.6±0.4	3.5±0.5	3.1±0.1	2.8±0.2	2.8±0.1	3.0±0.2
ALP (U/L)	88.8±7.9	85.2±4.9	89.6±12.2	86.6±7.4	87.6±14.3	89.4±7.8	87.6±3.3	88.4±2.7
ALT (U/L)	64.0±8.2	63.2±6.9	61.8±10.1	58.6±8.9	63.8±3.4	61.2±3.7	62.2±1.7	62.0±2.5
AMY (U/L)	826±78	821±57	818±11	827±30	919±75	917±81	911±90	914±92
TBIL (mg/dL)	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
BUN (mg/dL)	11.6±3.1	11.0±2.5	11.4±2.6	11.8±4.0	11.4±1.5	11.0±1.0	11.0±1.8	10.6±1.6
Ca (mg/dL)	10.4±0.6	10.4±0.4	10.2±0.4	10.1±0.3	10.4±0.4	10.1±0.3	10.1±0.4	9.9±0.3
Phos (mg/dL)	8.3±1.2	8.2±1.5	8.5±1.3	8.1±1.6	8.1±0.6	8.20±1.07	8.7±0.7	7.9±1.2
Cre (mg/dL)	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.20±0.07	0.2±0.0	0.1±0.1
GLU (mg/dL)	213±9	212±8	213±9	211±13	227±5	226±11	227±11	288±8
Na (mmol/L)	137.8±1.1	136.0±1.4	135.6±2.1	136.0±2.5	141.4±2.3	136.0±1.4	142.4±2.3	144.0±23.3
K (mmol/L)	5.3±0.8	5.2±0.4	5.2±0.4	5.2±0.3	5.3±0.4	5.2±0.4	5.3±0.3	5.3±0.2
TP (g/dL)	5.7±0.3	5.5±0.4	5.7±0.5	5.6±0.6	6.3±0.1*	5.5±0.4	6.3±0.1	6.3±0.1
GLOB (g/dL)	2.2±0.6	2.2±0.5	2.3±0.5	2.3±0.5	2.9±0.2	2.2±0.5	2.9±0.1	2.8±0.3
BA (umol/L)	4.0±1.5	3.8±1.3	4.2±1.4	4.0±2.0	4.1±0.1	3.8±0.2	4.0±0.7	3.3±0.1
Chol (mg/dL)	89.8±5.4	89.0±2.5	86.0±6.5	85.0±11.6	89.4±5.7	88.2±1.9	132.0±6.7	138.0±16.1

* Data expressed as mean ± SD

Statistically significant from the control ($p<0.05$)

Table 11. Hematological profile of female mice

Parameters	Pre-treatment period (Day 0)				Post-treatment period (Day 28)			
	Control	300 mg/kg	600 mg/kg	900 mg/kg	Control	300 mg/kg	600 mg/kg	900 mg/kg
WBC (10^9 cell/L)	8.7±2.3	8.7±2.1	8.5±1.81	8.4±1.5	8.6±1.1	8.2±1.1	8.3±1.1	8.5±1.3
LYM (10^9 cell/L)	5.4±1.3	5.5±1.2	5.3±1.32	5.1±1.4	5.9±0.8	5.8±0.9	5.9±1.4	5.7±1.11
MON (10^9 cell/L)	0.2±0.1	0.3±0.1	0.2±0.2	0.2±0.1	0.3±0.1	0.2±0.1	0.2±0.2	0.2±0.1
NEU (10^9 cell/L)	1.3±0.8	1.4±0.8	1.7±1.0	1.4±1.0	1.7±1.02	1.7±0.9	1.7±1.0	1.7±1.0
RBC(10^{12} cell/L)	8.1±0.9	8.1±1.1	8.1±1.1	8.2±0.9	8.5±1.3	8.6±1.2	8.3±1.3	8.5±1.0
HGB (g/L)	14.5±0.5	14.5±1.1	14.1±1.2	14.2±1.1	13.9±1.3	13.9±1.1	13.6±1.3	13.8±1.1
HCT (%)	38.6±3.6	40.1±2.6	39.5±3.6	37.9±3.9	39.4±3.9	39.9±4.1	38.1±2.8	38.9±3.6
MCV (fl)	48.0±3.3	47.4±2.7	47.8±1.9	46.8±2.1	48.6±2.9	48.4±2.8	48.8±3.3	48.2±2.6
MCH (pg)	12.2±0.7	12.1±0.8	11.8±0.7	11.9±0.8	11.9±0.7	11.8±0.7	11.9±0.2	11.7±0.6
MCHC (g/dL)	28.1±2.1	28.1±2.3	27.5±2.2	28.0±2.4	28.4±1.1	28.8±0.8	28.2±2.5	28.0±1.5
RDWc (%)	18.5±0.8	18.3±0.4	18.5±0.5	18.2±0.7	18.9±0.4	18.7±0.4	18.7±0.6	18.9±0.9
PLT (109 cell/L)	294.6±25.1	292.0±33.4	282.4±85.4	293.2±69.1	343.8±45.1	311.8±35.8	358.6±42.0	381.8±21.8
PCT (%)	0.1±0.1	0.1±0.1	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.0
MPV (fl)	7.4±0.7	7.5±0.9	7.3±0.7	7.2±1.1	7.4±0.5	7.3±1.1	7.3±0.7	7.2±1.1
PDWc (%)	36.6±2.7	34.2±2.3	34.3±2.1	34.1±1.5	34.5±0.6	34.9±1.1	34.9±1.0	34.2±1.6

* Data expressed as mean ± SD

Statistically significant from the control ($p<0.05$)

Table 12. Hematological profile of male mice

Parameters	Pre-treatment period (Day 0)				Post-treatment period (Day 28)			
	Control	300 mg/kg	600 mg/kg	900 mg/kg	Control	300 mg/kg	600 mg/kg	900 mg/kg
WBC (10^9 cell/L)	8.8±1.5	8.9±0.2	8.6±0.4	8.6±0.5	9.8±1.1	9.9±0.3	9.6±0.3	9.9±0.3
LYM (10^9 cell/L)	5.4±0.9	5.5±0.6	5.4±0.6	5.9±0.6	6.3±0.8	6.4±0.8	6.5±0.9	6.3±0.92
MON (10^9 cell/L)	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1
NEU (10^9 cell/L)	1.8±0.6	1.8±0.1	1.8±0.2	1.8±0.5	1.5±0.5	1.5±0.4	1.5±0.3	1.5±0.3
RBC (10^{12} cell/L)	8.9±0.4	8.8±0.1	8.8±0.1	8.7±0.3	9.4±1.1	9.5±0.9	9.4±0.8	9.4±0.5
HGB (g/L)	13.8±0.2	13.7±0.2	13.7±0.3	13.9±0.4	13.6±1.1	13.4±0.8	13.3±1.1	13.4±0.8
HCT (%)	39.7±2.3	38.9±1.9	39.3±1.4	39.1±3.2	40.8±1.5	40.4±1.6	40.8±1.9	40.5±1.5
MCV (fl)	79.2±1.4	49.0±2.5	49.2±5.4	49.0±1.5	48.0±1.3	48.0±2.1	48.0±2.2	48.2±1.9
MCH (pg)	11.9±0.4	11.6±0.4	11.6±0.4	11.6±0.4	11.7±0.3	11.8±0.4	11.8±0.3	11.7±0.4
MCHC (g/dL)	29.9±1.5	29.6±1.2	29.6±1.0	28.7±2.1	30.5±1.1	30.6±0.8	30.4±1.2	30.7±1.6
RDWc (%)	18.9±1.6	18.5±0.9	18.5±0.9	18.5±0.9	20.6±1.9	20.1±1.5	20.3±1.5	20.1±1.0
PLT (109 cell/L)	287.2±7.3	287.2±6.4	288.2±8.7	288.8±8.5	286.8±45.8	289.0±7.0	287.0±9.2	287.8±8.2
PCT (%)	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
MPV (fl)	7.1±0.5	7.2±0.3	7.1±0.3	7.24±0.5	7.3±1.7	7.8±2.1	7.9±1.8	7.7±1.6
PDWc (%)	35.4±1.4	35.8±2.5	35.3±2.9	35.6±2.2	34.9±2.3	35.3±1.8	35.7±2.5	35.4±1.7

* Data expressed as mean ± SD

Statistically significant from the control ($p<0.05$)

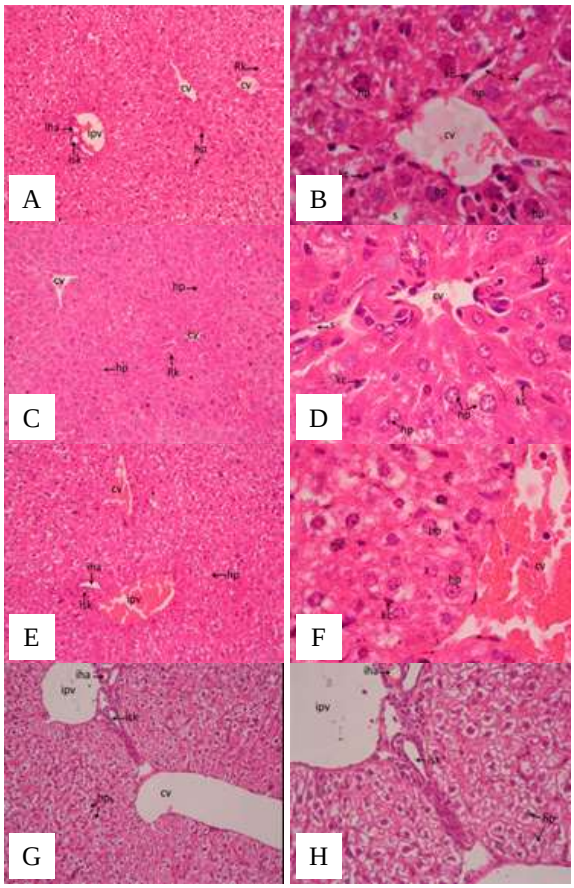

Figure 5. Histologic effects of Polyherbal formulation KL21 on liver of female mice. The liver sections of mice were stained with haematoxylin and eosin (H & E). Liver section of normal control group **(Ax20, Bx100)** and dose groups which were 300 mg/kg **(Cx20, Dx100)**, 600 mg/kg **(Ex20, Fx100**) and 900 mg/kg **(Gx20, Hx100)** showing normal visible central veins (cv), sinusoids (sn), hepatocytes (hp), remark cords (Rk), interlobular portal vein (ipv), interlobular hepatic artery (iha), interlobular bile ducts (isk) and sinusoids (s). kc: Kupffer cells.

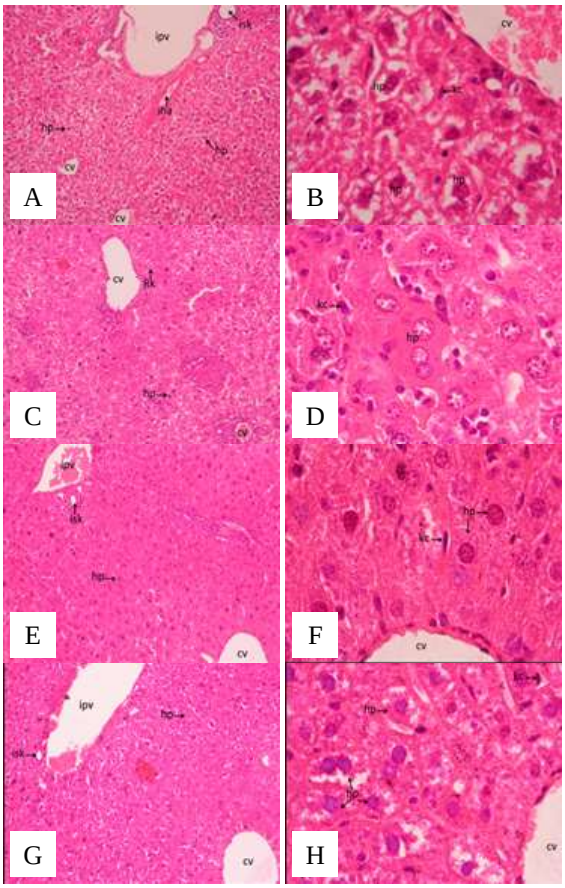

Figure 6. Histologic effects of Polyherbal formulation KL21 on liver of male mice. The liver sections of mice were stained with haematoxylin and eosin (H & E). Liver section of normal control group **(Ax20, Bx100)** and dose groups which were 300 mg/kg **(Cx20, Dx100)**, 600 mg/kg **(Ex20, Fx100**) and 900 mg/kg **(Gx20, Hx100)** showing normal visible central veins (vc), sinusoids (sn), hepatocytes (hp), remark cords (Rk), interlobular portal vein (ipv), interlobular hepatic artery (iha), interlobular bile ducts (isk). kc: Kupffer cells.

4. Discussion

New trends in drug discovery emphasize on natural sources with biological effective properties. Many people prefer to use them because they are safe which are derived from phenolic and flavonoid compounds [14]. Advantages of herbal drugs are decreased adverse effects, short time efficacy, induced immune system and positive economic outcomes on health expenditure [15]. Therefore, they are associated with conventional drugs or utilized significantly instead of them. For all these reasons it is necessary to determine their toxicological profile and verification for common implementation of safety data before marketing. The first step of this study was determined the phenolic and flavonoid contents, biological properties such as antioxidant and antimicrobial activities of KL21 extracts

Quercetin equivalent flavonoid content and gallic acid equivalent phenolic content of the ethanol extract was calculated as 11.09 and 258.58 mg/g respectively, while 86.42 mg/g and 3.58 mg/g were calculated in methanol extract. The results clearly demonstrated that ethanol extract had a high flavonoid and phenolic compound in comparison with methanol extract. It is already known that phenolic and flavonoid compounds are the major plant component with a high antioxidant capacity [8]. Therefore, considering the important role of polyphenolic and flavonoid compounds in the prevention and therapeutically of diseases, it is important to determine herbal formulations ingredients before activity studies. Bioactive ingredients are very important because of efficacy and safety of herbal formulations depends on phytochemical composition [16]. According to GC-MS analysis in our study, the major seconder compound in both extracts was

carvacrol. These results were in agreement with those reported in previous published papers that phenolic compounds. Phenolic compounds especially carvacrol demonstrate various pharmacological activities such as anti-inflammatory, anti-angiogenic and anti-cancer activities [17]. The most commonly used antioxidant methods are $ABTS^{+}$ and DPPH conducted on both extracts. The results indicated that both extracts exhibited higher antioxidant capacity according to two analyzed methods. The phenolic components show multi-different biological activities [18]. According to MIC values of antimicrobial activity test, only methanol extract exhibited antimicrobial efficacy. Conversely, ethanol extract did not display antimicrobial activity against all bacteria included fungi which are tested microorganisms. This data suggested that the solvent type used in the extraction had a great impact on MIC values. There are experiments which emphasized it is important to consider that both polar and nonpolar compounds may find in extracts; thus, the microbial efficacy may be due to presence of components with variable polarities [19]. In the other hand, higher amount of linolenic acid in methanol extract was detected in comparison with ethanol extract [20].

Cytotoxicity of the KL21 extracts was tested on MCF-7, A549, CaCo-2, HEK-293 and HeLa cells. The tested extracts had no cytotoxic effects on A549 and Caco-2 cell lines. Our data showed that the 48 h IC_{50} of ethanol and methanol extracts were 44 and 49 µg/mL on HEK-293 human kidney cells respectively. Additionally, it was demonstrated that ethanol extract was significantly toxic for cancer cell lines which were MCF-7 (92.09 µg/mL) and Caco-2 (95.47 µg/mL). Cytotoxic effects on cancer cell lines were affected by different solvent types of the polyherbal formulation including polar and non-polar constituents. This allows also the different phytochemical profile and biological activities due to extraction solvent [21]. Previously there were suggested that many herbal formulations exhibited cytotoxicity against various cancer cell lines [22]. Genetic toxicity testing has moved towards the earlier stages of drug discovery in order to identify genotoxic liabilities of new compounds in the pipeline.

Ames test is designed to detect genetic damage such as gene mutations which may reflect pharmaceuticals, including herbal preparations. According to test, both extracts of KL21 had no genotoxic potential in four *Salmonella* strains. This data exhibited specific approach to perform reduced risk assessment of KL21. However, Gumiganghwal-tang (GGT) is an herbal prescription made from nine different herbs and its extract acted as a genotoxic material [23]. Similarly, Pyungwi-san (PWS) is a mixture of six herbs and PWS extract exhibits genotoxicity. As a result of this it is important to determine herbal medicinal products (HMPs) genotoxicological profile and this may be a pre-condition for registration or marketing authorization. The single dose oral toxicity up and down procedure permits estimation of an LD_{50} with a confidence interval and the results allow a substance to be ranked and classified according to the Globally Harmonised System for the classification of chemicals which cause acute toxicity. The concept of the up-and-down animal testing approach was first step to determination of toxicity of the test material. According to the oral acute toxicity test, mice administered KL21 doses up to 2000 mg/kg did not exhibit mortality in 24 h. This result exerted the orally administered KL21 could be considered practically non-toxic. Likewise, Ojeok-san (OJS) is a widely used herbal formula in traditional Korean Japanese herbal medicine. The lethal dose of OJS with a 50 % mortality rate was over 2000 mg/kg [24]. Kai-Xin-San (KXS) is traditional Chinese medicine (TCM) formula and LD_{50} of KXS was over 32.59 g/kg for mice which is relatively safe for oral medication [25]. In the assessment and evaluation of the toxic characteristics of the herbal formulation, the determination of oral toxicity using repeated doses carried out after initial information on toxicity has been obtained by single dose oral toxicity test. Herbal formulations may cause various side effects due to their complex chemical compounds. Because of this reason it is important to determine their toxicological background. The subacute toxicity study allowed identifying KL21 chemical compounds with toxic potential such as organ toxicity, which may warrant further in-depth investigation. Therefore, this study provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time. However, after 28 days' observation period, it was identified that KL21 has no adverse effects in the highest volume used. There is not any sign of observable toxicity was detected during the experimental period. During study, there was no any significant change in body and absolute/relative weights of organs compared to the normal groups. This result is important evidence to evaluate the toxicity of KL21 because of body weight changes such as decreases or increases are associated with toxic effects of chemicals, synthetic or herbal drugs. Moreover, hematological and biochemical analysis of metabolites was not significantly altered between control and treatment groups. Previously it has been reported to there are many acute, subacute and chronic toxicity studies to exerted safety evaluation of herbal formulations [26, 27]. The effects of KL21 were evaluated by histopathological examination of hepatic tissue sections using H&E staining. The highest dose of KL21 (900 mg/kg) did not causes any hepatic damage results in fibrotic changes in the hepatic tissues.

5. Conclusion

In conclusion, the results provide important information regarding the biological activity of KL21, thus, this data offer information critical for herbal drug development and emphasize to importance of determining the quality

criteria of natural product formation stage after manufacturer process. These findings indicated that KL21 due to its antioxidant capacities may be useful in the obviation of aging-related and various inflammatory diseases for human welfare. It was also undertaken to evaluate *in vitro* and *in vivo* toxicological profile of KL21 polyherbal formulation as a botanical dietary supplement. It is important to provide scientific evidence showing that the formulation is safe and efficacious in human's welfare.

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Author's Contributions

Çinel Köksal Karayıldırım: Performed the project, research design all experiments and wrote the manuscript.
N. Ülkü Karabay Yavaşoğlu: Helped to data interpretation and statistical analysis and wrote the manuscript.
Adem Güner: Supported the antioxidant test
Gürkan Yiğittürk and Altuğ Yavaşoğlu: Supported the histological tests

Ethics
There are no ethical issues after the publication of this manuscript.

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