.

TJPS-92678
Safety Assessment of Vanillic Acid: Subacute Oral Toxicity Studies in Wistar Rats
Vanilin Asitinin Güvenlik Değerlendirmesi: Wistar Sıçanlarında Subakut Oral Toksisite Çalışmaları

Anwarbaig Chandbaig Mirza1, Shital Sharad Panchal2
1Anjuman-i- Islams's Kalsekar Technical Campus, School Of Pharmacy, Department Of Pharmacology, Navi Mumbai, Maharashtra, India
2Institute Of Pharmacy, Department Of Pharmcology, Nirma University, Ahmedabad, Gujarat, India

Abstract:
Objective: Vanillic acid is a flavoring agent, phenolic acid and an intermediate product in the process of production of vanillin from Ferulic acid. It has been investigated for diverse pharmacological actions and used in Chinese medicine since decades but the safety and/or mechanism of toxicity with long term use is lacking in the present literature. The subacute toxicity study will add value to its pharmacological profile and support its exploration as a future medicine. Therefore, it was decided to conduct a subacute toxicity study.
Material and methods: According to OECD TG407 (OECD, 2008), rats were divided into 3 groups of 12 animals (6 male and 6 female). The dose of vanillic acid for the subacute toxicity study was decided after conducting limit test. The vanillic acid (1000 mg/kg/day, p.o for 14 days) was administered to the first group of rats, whereas an equal volume of vehicle was given to the control group. In order to access reversibility, satellite groups were given vanillic acid (1000 mg/kg/day, p.o for 14 days) and were kept for another 14 days post-treatment. The toxic signs, mortality and the body weight changes were recorded. On 15th and 29th day, the rats were anesthetized to collect blood for estimation of hematological and biochemical parameters than after sacrificed to collect internal body organs for weighing, gross necropsy and histopathological studies.
Results: The hematological parameters of satellite groups were found to be increased; the serum sodium level decreased in treatment and satellite groups with no other major change in biochemical parameters as compared to the control groups. Relative organ weight, gross necropsy and histopathological structure of internal body organs were found with no major alteration.
Conclusion: Vanillic acid has no adverse effect on the process of erythropoiesis, leucopoiesis and on internal body organs which was confirmed by evaluating various hematological, biochemical parameters, gross necropsy and histopathological studies. Decrease in serum sodium level was observed which may not be considered as severe or major toxic effect.
Key words: subacute, toxicity, vanillic acid, polyphenol, safety.
Öz
Amaç: Vanillik asit, bir lezzetlendirici madde, fenolik asit ve ferulik asitten vanilin üretimi sürecinde bir ara üründür. Çeşitli farmakolojik etkiler araştırılmış ve Çin tıbbında on yıllardan beri kullanılan ancak uzun süreli kullanımda toksisite mekanizması mevcut literatürde bulunmadığından, akut toksisite çalışması yapılması kararlaştırılmıştır.

Gereç ve Yöntemler: OECD TG407'ye göre (OECD, 2008), sıçanlar 12 hayvandan oluşan 3 gruba (6 erkek ve 6 dişi) ayrıldı. Subakut toksisite çalışması için vanillik asit dozuna limit testi yapıldıktan sonra karar verildi. Birinci sıçan grubuna vanilya asidi (1000 mg / kg / gün, p.o 14 gün boyunca) uygulanmış, kontrol grubuna eşit miktarda taşıt verilmiştir. Tersinirliğe erişebilmek için, uydu gruplarına vanilinik asit (1000 mg / kg / gün, p.o 14 gün boyunca) verildi ve işlemden sonraki 14 gün boyunca tutuldu. Toksik belirtiler, mortalite ve vücut ağırlığı değişiklikleri kaydedildi. 15. ve 29. günlerde, hematolojik ve biyokimyasal parametrelerin tahmininde kan toplamak için kan ve anestezi, tartım ve histopatolojik çalışmalar için iç vücut organlarını toplamak için kurban edildikten sonra.

Bulgular: Uydu grubunun hematolojik parametreleri artmış bulundu; Tedavi grubu ve uydu grubunda serum sodyum düzeyi kontrol grubuna göre diğer biyokimyasal parametrelerde önemli bir değişiklik olmadan azaldı. Göreceli organ ağırlığı ve iç vücut organlarının histopatolojik yapısı büyük bir değişiklik olmadan bulundu.

.

Sonuç: Vanilin asidinin eritropoezi, lökopezi süreci ve çeşitli biyokimyasal parametreler ve histopatolojik çalışmalar değerlendirilerek doğrulanan iç vücut organları üzerinde olumsuz etkisi yoktur. Ciddi veya majör toksik etki olarak kabul edilemeyecek serum sodyum seviyesindeki düşüş gözlendi.

Anahtar kelimeler: subakut, toksisite, vanillik asit, polifenol, güvenlik.

1. Introduction:

Vanillic acid (VA) [4-hydroxy-3-methoxybenzoic acid] has been a frequently used flavoring agent. It is an oxidized form of vanillin and an intermediate in the production of vanillin from ferulicacid.[1,2] Majorly found in the roots of Angelica Sinensis,[3] fruits of Euterpeoleracea,[4] wine, vineg[5] and used in Chinese medicine since decades[6]. It was investigated for diverse pharmacological actions in experimental animals and proved to have antidiabetic activity,[7,8] anti-inflammatory effect,[9,10] strong antioxidant effect,[11,12]cardioprotectivity,[13,14,15] thermal tolerance,[16] and inhibitory effects on Neuro-2Acells,[17]. In addition, ameliorates glomerulonephritis,[18] had the cognitive effect in diabetic mice[19] and its presence was reported in cerebospinalfluid[20]. It was reported to inhibit carbonic anhydrase isozyme III[21] and snake venom 5'-nucleotidas[22]. It controls transgene expression in mammalian cells and mice[23]. It showed a protective effect on ulcerative colitis,[24] liver toxicity[25] and has proved analeptic effect[26]. Further, it acts as an anti-filarial agent[27] and respiratory stimulant[28].

In mid of 20th century, clinical studies were conducted on excretion of VA in urine. In one of the study, VA was found to be a metabolic product of 4-hydroxy-3-methoxyphenylglycol and 4-hydroxy-3-methoxymandelicacid[29]. It was reported as a metabolite of photochetchuic acid which is stable at alkaline pH of urine[30]. In addition, excretory studies were reported on healthy volunteers, patients with circulatory and liver disorder, smokers, non smokers and during stress.[31- 33]

VA is supposed to administer for long durations to treat chronic disorders, therefore assessment of safety or risk of occurrence of toxic effects becomes essential. In addition, current scientific reports showed toxic effects of phytochemicals or plant extracts in experimental animals at high doses such as carcinogenic (capsaicin, chili powder, safrole), neurotoxic (Papaver somniferum, erytroxylum sp. and cannabis sativa), genotoxic (thymol and carvacro), teratogenic (pyrrolizidine alkaloid monster, heliotrine), cytotoxic (withianiasomniferum, safrole-2', 3'-oxide, etc.), nephrotoxic (aristolochic acid, turmeric etc.) hepatotoxic (Cimicifugaracemosa etc.), and gastrointestinal effects (red pepper, fennel etc.)[34]. Brown, (2017) documented case studies of twenty one herbs and twelve dietary supplements which posed a possible risk for liver injuries in certain individuals. These reports are alarming and compelling researchers to explore the safety assessment of phytochemicals on long term use. Research in the toxicological domain (such as acute, subacute and chronic studies) not only add value to the ethannopharmacology of phytochemicals but also ensures the safe use to avoid the occurrence of any untoward effects which were considered a major obstacle to the long term use of synthetic drugs.[36] As per the OECD TG 407(2008)[37], subacute toxicity studies are usually carried out after availability of initial information on acute toxicity studies. It provides information on possible health hazards likely to arise from repeated exposure of drugs/chemical over a limited period of time and it is considered as a preliminary to a long-term study[38]. In addition, subacute toxicology studies in rodents are considered to be an obligatory step to support the progression to clinical trials and the eventual marketing of drug molecules[39].

Acute toxicity studies on VA are reported in literature and LD50 value was reported as 5020 mg/kg, i.p in rats and 2691 mg/kg, i.p in mice.[40-42] However, no reports of subacute toxicity of VA are available in the literature. Hence, it was decided to conduct a subacute toxicity study to find out safety aspect of VA on vital internal organs such as liver, brain, heart, kidney and sciatic nerve, etc., in wistar rats.

2. Material and Methods:

2.1: Chemicals:

Vanillic acid (HSN-29189900) was purchased from Sigma (St. Louis, MO, USA). All required biochemical kits are of analytical grade and purchased from the Lab-Care Diagnostics Pvt Ltd and Transasia Bio-Medicals Ltd, India.

2.2: Experimental Animals:

Healthy young adult male and female wistar albino rats were obtained from National Institute of Bioscience, Pune. They were housed under standard environmental conditions of temperature at 22±1 ^{0}C under a12 h-light: 12 h-dark cycle and allowed free access to drinking water and standard pelleted diet. The Institutional Animal

.

Ethics Committee of Kalsekar Technical Campus, School of Pharmacy, Navi Mumbai, India has approved all experimental protocols.

2.3: Limit Test:

As per the OECD TG 407 (2008)[37], limit test was conducted to decide the dose of subacute toxicity study by the administration of a single oral dose of VA (2000 mg/kg) to wistar rats. Animals were observed critically for any morbidity and mortality.

2.4. Subacute Toxicity:

According to OECD TG407 (2008)[37], rats were divided into 3 groups of 12 animals (6 male and 6 female). The VA (1000 mg/kg/day, p.o for 14 days) was administered to the first group of rats, whereas an equal volume of vehicle was given to the control group. In order to access reversibility, satellite groups were given VA (1000 mg/kg/day, p.o for 14 days) and kept for another 14 days post-treatment. The toxic signs, mortality, food intake, water consumption and the body weight changes were recorded. On day 15 the rats were anesthetized by isoflurane. The blood samples were collected in tubes coated with ethylenediaminetetraacetic acid (EDTA) to determine the complete blood count, which includes red blood cell count, platelet count and red cell indices. The non-heparinized blood samples were collected for biochemical estimations. After collection of blood samples, all rats were sacrificed and internal vital body organs were dissected for gross necropsy and to determine relative organ weight. All tissues were stored in 10% buffered formaldehyde solution for histological studies.

2.5: Statistical Analysis:

Data analysis was carried using Graph Pad Instat 3.0 version (GraphPad, San Diego, CA). Data were expressed as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). Dunnett's multiple range test was applied for post hoc analysis. A value of $P < 0.05$ was considered to be statistically significant.

3: Results:

3.1: Limit Test:

A single dose of 2000 mg/kg/p.o of VA did not show any mortality and sign of toxic effects; hence as per the guideline of OECD-407 (2008), 1000 mg/kg dose was selected for subacute toxicity study.

3.2: Clinical Observations, Body Weight, Food and Water Consumption:

No signs of toxicity and mortality were reported during and after the study period in treatment and satellite groups. Body weights, food intake and water consumption were recorded on a weekly basis and it was found that there was a normal increase in body weight, food and water intake of treatment and satellite group animals as compared to normal control animals.

3.3: Hematological Examination:

The results of hematological examination are presented in Table 2 & 3. The treatment group retained normal increase in hemoglobin (HGB), red blood cell count (RBC), packed cell volume (PCV), mean corpuscular cell volume (MCV), platelet count (PLT), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) in male and female wistar rats but significant increase in all hematological parameters were observed in rats of satellite group. In addition, white blood cells (WBC) count of rats in treatment and satellite groups were observed to be in normal range and had no significant difference as compared to normal control groups although the neutrophil count was slightly increased in male rats of treated group which was reversed after 14 days in satellite group (Table 3). The eosinophil, monocyte and lymphocyte count in treated and satellite groups showed no significant change as compared to normal animals.

3.4. Blood Biochemistry:

The findings of clinical biochemistry analysis are shown in Table 4. It involves estimation of blood glucose (BG), blood urea nitrogen (BUN),creatinine (CR), total protein(TP), albumin (ALB), total bilirubin (TB),direct bilirubin (DB), serum glutamic-oxaloacetic transaminase (SGOT), serumglutamine-pyruvic transaminase (SGPT), alkaline phosphatase (ALP), electrolytes [calcium (Ca), potassium (K), chloride (Cl) and phosphate (P)], lactate dehydrogenase (LDH), lipid profile [high density lipoprotein (HDL), total cholesterol (TC) and triglycerides (TG)]. Treatment of VA in male and female animals showed no change in all biochemical parameters as compared to normal control animals except statistically significant decreased sodium levels in male treatment group which did not recover to normal levels in satellite group.

.

3.5: Gross Necropsy and Histopathological Findings:
Macroscopic findings of vital organs and/or systems of treatment and satellite groups did not show any irregularities in gross anatomical features as compared to control group animals.

Microscopically, sciatic nerve tissue sections showed normal histomorphological features of nerve tissue with an intact length of nerve fibers and normal cellular detail. Brain section revealed normal neuronal histomorphological features with intact supporting matrix. Section of heart tissues showed normal features of cardiac muscle fibers, cardiac fibers were arranged in dense and compact fashion with intact length and normal cell striation and nuclei. Examination of kidney tissue sections showed normal glomeruli and renal tubules in the cortex and medulla. Liver tissue showed intact hepatic parenchyma comprised of hepatocytes, portal triad and central vein. Hepatocytes were arranged in cords with intact nucleus and cellular borders of hepatocytes. No inflammatory or metabolic changes were observed in all histological sections of vital body organs. Representative sections of histological studies are shown in Figure 1.

4. Discussion:
VA is a flavoring agent and investigated for various pharmacological effects.[7-28] Safety of VA on long term use is a major concern specifically to decide the optimum dose and to avoid untoward effects, but in the current available literature no such information is available. Subactue toxicity study is preliminary to chronic study.[37,43] In addition, the results of the current study will add value to pharmacological profile and support its exploration as future medicine. In the light of these facts, we have conducted subacute toxicity study. A limit test was performed using a single dose (2000 mg/kg p.o) to decide the dose of subacute toxicity study. Rats neither showed any sign of toxicity nor mortality. Hence, It can be concluded that 1000 mg/kg dose of VA may be safe to conduct a subacute toxicity study.
The study was designed by referring OECD TG 407 (2008)[37]. All essential and crucial examinations have been included in the study such as daily observation for toxic signs, mortality, measurement of body weight, food consumption, water intake, all hematological evaluations, biochemical estimations in the blood (BG, BUN, CR, TP, ALB, TB, DB, SGOT, SGPT, ALP, CA, K, Cl, P, LDH, HDL, CHL and TG), gross necropsy and histopathological studies of vital internal organs. Though, we would like to declare that not all examination mentioned in the OECD TG 407 have been included the study (such as urine analysis, estimation of T3, T4, TSH hormone and cholinesterase etc)".

Toxic signs, food and water intake, if taken together may indicate malaise or covert toxicity long before overt signs; if the animal does not feel well it will not eat sufficient food and/or drink the usual amount of water. Hence, body weight, food and water intake are the indicator of general health and relative organ weight indicates target organ toxicity which can be revealed by hypertrophy and/or hyperplasia. Therefore, these parameters are crucial to assess the safety of the drug during subacute toxicity study[43-45]. Rats of treatment and satellite group received 1000 mg/kg/day p.o of VA for 14 days and they were observed daily during the dosing period for any mortality and signs of toxicity such as changes in skin, fur, eyes, mucous membranes, occurrence of secretions, excretions and autonomic activities (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behaviour was also monitored.[37] No signs of toxicity and mortality were observed in treated and in satellite groups as compared to normal control groups. Results showed a normal increase in body weight, food consumption and water intake. Table 1 showed the effect of VA on relative internal body organ weight. A significant increase in liver weight was observed in male treated group as compared to a normal control group but it was reversed in satellite group. Increase in liver weight was considered as an adaptive and non-adverse reaction[46]. Other internal body organ in treated group did not show any significant effect on internal body organ weight.

Biochemical estimations in the blood and hematological evaluations are important indicators in finding out the mechanism of toxic effect[47-49] and the parameters have been selected not only to detect target organ toxicity but also to detect pretoxic changes that might predict impending toxicity.[38,43] The results are shown in Table 2, 3 & 4. The treatment group showed no significant increase in the HGB, RBC, PCV, MCV, PLT, MCH, MCHC in

.

male and female wistar rats. Significant increases in all hematological parameters were observed in rats of satellite group which were within the normal range and cannot be considered as a toxic effect of VA.[50] This can be justified by considering contrast situation of decrease in the HGB, RBC count and other related parameters which showed anemic effect and adverse effect of VA on erythropoiesis. In addition, WBC count of rats in treated and satellite groups was observed to be in normal range and had no significant difference as compared to normal control groups although the neutrophil count was slightly increased in male and female rats of satellite groups. This, however, cannot be considered as consequences of infection and inflammation. The eosinophil, monocyte and lymphocyte count in treated and satellite groups showed no significant changes as compared to the normal groups. Therefore, it might be conclude that the VA is nontoxic and did not alter functioning and efficacy of red bone marrow.

Effect of VA on biochemical parameters were presented in Table 4, plasma level of BG, BUN, CR, TP, ALB, TB, ALP, Ca, K, P, Cl and lipid profile was found unaltered. But few of the biochemical parameters showed non-significant alteration in male and female rats, which includes DB, SGOT, SGPT and Na levels. DB, SGOT, SGPT and ALB levels are important indicators of liver function[51]. In male wistar rats, the DB was found to be non-significantly increased in both treatment and satellite group as compared to the control group, but no major alteration was reported in other liver function test (SGOT & SGPT). In female rats, DB level was reported to be non-significantly increased in the treatment group and reversed in the satellite group with slight increased in SGPT and SGOT level in treatment and satellite group. All the alterations are statistically non-significant and not in coordination with the macroscopic and histopathological findings on liver (Fig 1), conducted to correlate the biochemical findings of liver[39,43]. Further albumin levels were not significantly altered in male and female groups. Based on these findings, it can be concluded that VA does not have any subacute toxic effect on the liver.

Effect of VA on renal function was assessed by estimation of BUN, CR, TP and LDH levels.[52,53] No statistically significant difference was observed in the all renal functional parameters of male and female treatment and satellite group. The above evaluations showed that oral administration of VA may not alter the renal function which is in concurrence with normal renal macroscopic and histopathological findings (Fig 1) of male and female treatment and satellite groups.

Serum electrolytes (Na, K, Ca and P) were evaluated during subacute toxicity study. [54,55] In our study K, Ca and P levels were found to be in the normal range of treatment and satellite groups as compared to normal control male and female rats. In male treatment and satellite group, Na level was found to be significantly decreased while in the female rats, change is non-significantly decreased as compared to a normal control group. Based on the available knowledge of electrolyte balance, we can propose the probable mechanism behind reduced serum Na level. Firstly, it could be increased urinary loss and high cellular uptake [56]. Secondly, the process may be mediated by antidiuretic hormone and atrial natriuretic peptide [56]. But this could not be considered as a severe toxic effect because the Na level below 20 mEq/L can only indicate Na leakage from damaged renal tubules or hypovolemia[57] or this effect can be recognized as a side effect as in the case of all classes of antidepressant drugs.[58] Repeated administration of VA does not affect the lipid profile of both male and female rats. Therefore, it is apparent that the oral administration of VA does not affect the electrolyte balance and the process of lipid metabolism.

Gross necropsy and histopathological studies represents cornstone in the process of safety assessment before they can be tried in human patients, essential to find out any relationship and relevance of treatment related finding.[39,43] In our study, toxic effect on internal vital organs (i.e.liver, brain, heart, kidney and sciatic nerve) was evaluated with the help of gross necropsy and histopathological studies (Fig 1) which showed no abnormality in any vital major body organs. Therefore, the results suggest that the VA is fairly nontoxic at selected subacute dose.

4. Conclusion:

.

VA is a polyphenol with diverse pharmacological actions; but no information on subacute toxicity studies is available in current literatures. To enrich its pharmacological profile and encourage its evidence based pharmacotherapeutic use, a subacute toxicity study was conducted using experimental rats. To summarize, no mortality and clinical signs of toxicity were observed during study consisting of normal increase in body weight and internal body organ weight. The processes of erythropoiesis, leukopoiesis and physiology of internal body organs were not altered and no structural abnormalities were observed in gross necropsy and histopathological studies. The serum Na level was found to be decreased in male wistar rats with no major changes in levels of the other electrolytes, so this cannot be categorized as a major toxic effect. Hence, it can be concluded that the VA is safe in experimental rats during subacute toxicity studies. Further, chronic toxicity studies are required to explore the details of safety.

Acknowledgement: Authors would like to acknowledge the support provided by AI's Kalsekar Technical Campus to complete the study.
Funding: Author would like to acknowledge the financial assistance provided by Mumbai University to complete the research work.
Conflict(s) of interest: None

Legends of figures and tables:
Figure 1: Effect of VA on histomorphology of vital body organs of rat.
Representative histological observations of hematoxylin and eosing stained sections.
VA^t is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
VA^s is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed
Table 1: Effect of VA on relative body organ weights of wistar rats.
Values are expressed as mean ± SD. * significantly different from control, $p<0.05$
VA^t is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
$VA^s$is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed

Table 2: Effect of VA on hematological parameters at the termination of study.
Values are expressed as mean ± SD. * significantly different from control, $p<0.05$
VA^t is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
$VA^s$is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed
Table 3: Effect of VA on differential leucocyte count at the termination of study.
Values are expressed as mean ± SD. * significantly different from control, $p<0.05$
VA^t is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
$VA^s$is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed.
Table 4: Effect of VA on biochemical estimations in wistar rats.
Values are expressed as mean ± SD. * significantly different from control, $p<0.05$
VA^t is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
$VA^s$is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed.

References:
1. Lesage ML, Delattre M, Haon M, et al. A two-step bioconversion process for vanillin production from ferulic acid combining Aspergillusniger and Pycnoporuscinnabarinus. JBiotechnol. 1996; 50 (2–3): 107-13.

2. Civolani C, Barghini P, Roncetti AR, Ruzzi M, SchiesserA.Bioconversion of ferulic acid into vanillic acid by means of a vanillate-negative mutant of Pseudomonas fluorescens strain BF13.Appl EnvironMicrobiol.2000;66 (6): 2311–17.

.

3. Duke JA. Database of Phytochemical Constituents of GRAS Herbs and Other Economic Plants. Boca Raton, FL: CRC Press, 1992.

4. Pacheco LA, MertensTS, Talcott ST. Chemical composition, antioxidant properties, and thermal stability of a phytochemical enriched oil from Acai (Euterpeoleracea Mart.). J Agric Food Chem. 2008; 56 (12): 4631–36.

5. Galvez MC, BarrosoCG., Carmelo G, Pérez B, Juan A. Analysis of polyphenolic compounds of different vinegar samples. J.A.ZLebensmUntersForch.1994;199: 29-31.

6. Pranav KP, Mukesh D. Effect of natural products on commercialoralantidiabetic drugs in enhancing 2-deoxyglucose uptake by 3T3-L1adipocytes.TherAdvEndocrinolMetab.2011; 2(3):103-14.

7. Chang WC, Wu JS, Chen CW. Protective Effect of Vanillic Acid against Hyperinsulinemia, Hyperglycemia and Hyperlipidemia via Alleviating Hepatic Insulin Resistance and Inflammation in High-Fat Diet (HFD)-Fed Rats. Nutrients. 2015; 7(12):9946-59.

8. Klinger W, Kersten L. The effect of vanillic acid diethylamide on the blood sugar in rabbits and ascorbic acid in the liver and adrenal glands in rats. ActaBiol Med Ger. 1963.10:689-90.

9. Calixto CC, Carvalho TT, Hohmann, MS.Vanillic Acid Inhibits Inflammatory Pain by Inhibiting Neutrophil Recruitment, Oxidative Stress, Cytokine Production, and NFκB Activation in Mice. J Nat Prod.1963.28;78(8):1799-808.

10. Kim MC, Kim SJ, Kim DS.Vanillic acid inhibits inflammatory mediators by suppressing NF-κB in lipopolysaccharide-stimulated mouse peritoneal macrophages. ImmunopharmacolImmunotoxicol. 2011.;33 (3):525-32.

11. Tai A, Sawano T, Ito H. Antioxidative properties of vanillic acid esters in multiple antioxidant assays. BiosciBiotechnolBiochem.2012; 76(2):314-8.

12. Chou TH, Ding HY, Hung WJ, Liang CH.Antioxidative characteristics and inhibition of alpha-melanocyte-stimulating hormone-stimulated melanogenesis of vanillin and vanillic acid from Origanumvulgare. ExpDermatol. 2010; 19(8):742-50.

13. Dianat M, Radmanesh E. Badavi M, Goudarzi G, Mard SA. The effects of PM(10) on electrocardiogram parameters, blood pressure and oxidative stress in healthy rats: the protective effects of vanillic acid. Environ. Sci. Pollut. Res. Int. 2016; 23 (19),19551-19560.

14. Dianat M, Hamzavi GR, Badavi M, Samarbaf-zadeh A. Effect of vanillic acid on ischemia-reperfusion of isolated rat heart: Hemodynamic parameters and infarct size assays. Indian J Exp Biol. 2016;53(10):641-6.

15. Stanely PP, Dhanasekar K, RajakumarS.Vanillic acid prevents altered ion pumps, ions, inhibits Fas-receptor and caspase mediated apoptosis-signaling pathway and cardiomyocyte death in myocardial infarcted rats.ChemBiol Interact. 2015. 5;232:68-76.

16. Yemiş GP, Pagotto F, Bach S, Delaquis P. Thermal tolerance and survival of Cronobactersakazakii in powdered infant formula supplemented with vanillin, ethyl vanillin, and vanillic acid. J. Food Sci.2012; 77 (9):M523-7.

17. Huang SM, Hsu CL, Chuang HC. Inhibitory effect of vanillic acid on methylglyoxal-mediated glycation in apoptotic Neuro-2A cells. Neurotoxicology. 2008; 29(6):1016-22.

.

18. Motiram KR, Tejaswini G, Patil MA, Dinesh KB, Diwan PV. Vanillic Acid Ameliorates Cationic Bovine Serum Albumin Induced Immune Complex Glomerulonephritis in BALB/c Mice. Drug Dev Res. 2016; 77(4):171-9.

19. Singh JC, Kakalij RM, Kshirsagar RP. Cognitive effects of vanillic acid against streptozotocin-induced neurodegeneration in mice. Pharm Biol. 2015; 53(5):630-6.

20. Ebinger G, Verheyden R. On the occurence of vanillic acid in human brain andcerebrospinal fluid. J Neurol.1976;212(2):133-8.

21. Alzweiri M, Al-Hiari Y. Evaluation of vanillic acid as inhibitor of carbonic anhydrase isozyme III by using a modified Hummel-Dreyer method: approach for drug discovery. Biomed. Chromatogr. 2013; 27(9):1157-61.

22. Dhananjaya BL, Nataraju A, Raghavendra CD, Sharath BK, D'Souza CJ. Vanillic acid as a novel specific inhibitor of snake venom 5'-nucleotidase: a pharmacological tool in evaluating the role of the enzyme in snake envenomation. Biochemistry (Mosc).2009; 74(12):1315-9.

23. Gitzinger M, Kemmer C, Fluri DA. The food additive vanillic acid controls transgene expression in mammalian cells and mice. Nucleic Acids Res. 2012; 40(5):e37.

24. Kim SJ, Kim MC, Um JY, Hong SH. The beneficial effect of vanillic acid on ulcerative colitis. Molecules. 2010. 19;15(10):7208-17.

25. Itoh A, Isoda K, KondohM.Hepatoprotective effect of syringic acid and vanillic acid on CCl4-induced liver injury. Biol Pharm Bull. 2010; 33(6):983-7.

26. Klinger W, Kersten L. The analeptic effect of vanillic acid diethylamide.ActaBiol Med Ger. 1962; 9:67-78.

27. Varma RS, Shukla A, Chatterjee RK. Evaluation of vanillic acid analogues as a new class of antifilarial agents. Indian J Exp Biol. 1993; 31(10):819-21.

28. Fruhmann G, Specht H. Respiratory stimulation with vanillic acid diethylamide in chronic bronchitis and respiratory insufficiency. Med Klin. 1970. 4;65(36):1570-6.

29. .Mårdh G. Vanillicacid, a metabolite of 4-hydroxy-3-methoxyphenylglycol and 4-hydroxy-3-methoxymandelic acid in man. J Neurochem. 1984; 43(2):522-5.

30. Price J. The dependence of vanillic acid excretion on urinary pH.ClinChimActa. 1969; 26(3):413-8.

31. Schmid E, Tautz NA, Bauersachs E, Krautheim J. The excretion of vanilmandelic acid, vanillic acid, homovanillic acid and 5-hydroxyindoleacetic acid by urine in smokers and non-smokers. Arzneimittelforschung. 1968 ;18(7):819-21.

32. Brisse B, Bender F. Excretion of vanillin mandelic acid and vanillic acid in healthy subjects and patients with circulatory and liver diseases. Verh. Dtsch.Ges. Inn. Med.1967;73:1114-6.

33. Smith P, Bennett AM.Vanillic Acid Excretion During Stress. Nature. 1967;181(4610):709.

34. Guldiken, B., Ozkan, G., Catalkaya, G., Ceylan, F.D., Yalcinkaya, I.E. and Capanoglu, E., 2018. Phytochemicals of herbs and spices: Health versus toxicological effects. Food ChemToxicol. 119, 37-49.

.

35. Brown, A.C., 2017. Liver toxicity related to herbs and dietary supplements: Online table of case reports. Part 2 of 5 series. Food ChemToxicol, 107,472-501.

36. Tabasum, S., Khare, S. and Jain, K., 2019. Subchronic Toxicity Assessment of Orally Administered Methanol (70%) Seed Extract of Abrusprecatorius L. in Wistar Albino Rats. Turk J Pharm Sci. 16(1), 88-95.

37. The Organisation of Economic Co-operation and Development (OECD-2008),Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris,pp 1-13.

38. Hayes,A.W. Principles and methods of toxicology(5th ed). Boca Raton, FL; 2007 : 1223-1245.

39. Greaves,P. Histopathology of preclinical toxicity stdies: intrpetation and relevance in drug safety evaluation (4th ed). USA; 2012: 1-2.

40.Comptes Rendus Hebdomadaires des Seances, Academie des Sciences. Vol. 243, Pg. 609, 1956.
41.Yakugaku Zasshi. Journal of Pharmacy. Vol. 104, Pg. 793, 1984.
42. ChemID*plus* chemical Data Bank [Internet]. Bethesda (MD): National Library of Medicine (US); 2019[updated 2019 Apr 4; cited 2019 Apr 13]. Vanillic acid; UNII Number: GM8Q3JM2Y8; [about 3 p.]; Available from: https://chem.nlm.nih.gov/chemidplus/sid/0000121346.

43. Ecobichon, D.J.The Basis of Toxicity Testing (2nd ed). Boca Raton, FL; 1997: 94-100.

44.Wang J, Sun F, Tang S.Safety assessment of vitacoxib: acute and 90-day sub-chronic oral toxicity studies. Regul. Toxicol. Pharmacol.2017; 86: 49-58.

45.Thanabhorn S, Jaijoy K, Thamaree S, Ingkaninan, K, Panthong A. Acute and subacute toxicity study of the ethanol extract from *Lonicera japonica* Thunb.,J.Ethnopharmacol. 2006;107: 370–73.

46.Hall AP, Elcombe CR, Foster JR. Liver Hypertrophy: A Review of Adaptive (Adverse and Non-adverse) Changes–Conclusions from the 3rd International ESTP Expert Workshop. Toxicol. Pathol. 2012;40(7):971-94.

47.Wang J, Sun F, Tang S, Zhang S, Cao X. Acute, mutagenicity, teratogenicity and subchronic oral toxicity studies of diaveridine in rodents. Environ ToxicolPharmacol.2015; 40 (2):660–70.

48.Nyarkoa AK, OkinebLKN, WedzibRK, Addoa PA, Ofosuhenea M. Subchronic toxicity studies of the antidiabetic herbal preparation ADD-199 in the rat: absence of organ toxicity and modulation of cytochrome P450. J. Ethnopharmaco. 2005;97: 319–25.

49.Chung TH, MyoungJK, Seol HM. Acute and 28-Day Subacute Toxicity Studies of Hexane Extracts of the Roots of Lithospermumerythrorhizonin Sprague-Dawley Rats. Toxicol Res. 2015;31 (4): 403-14.

50.Kingsley CK, Solomon NI, Odudu A. Hematological ,biochemical and antioxidant changes in wistar rats exposed to dichlorovos based insecticide formulation

.

Organ weight (g% body weight)			
Treatment group			
Organs	**Control**	**VA^{t}**	**VA^{s}**
Male			

used in southeast

Nigeria.Toxics. 2016; 4(4):28.

51.Mathew S, Dushyant S. Interpretation of abnormal liver function tests. Hosp. Med.Clin. 2014; 3 (1):e139-48.

52.William MK. Renal function test as indicator of kidney injury in subacute toxicity studies. ToxicolApplPharmacol. 1981; 57 (3):414-24.

53.Bhargava AS, Khater AR, Gunzel P. The correlation between lactate dehyrogenase activity in urine and serum and experimental renal damage in the rat. ToxicolLett. 1978; 1(5-6): 319-32.

54.Unuofin JO, Otunola GA, Afolayan AJ. Evaluation of acute and subacute toxicity of whole-plant aqueous extract of VernoniamespilifoliaLess. inWistar rats. J Integr Med.2018;16(5):335-41.

55.Amresh G, Singh PN, Rao CV. Toxicological screening of traditional medicine Laghupatha (Cissampelospareira) in experimental animals. J.Ethanopharmcol. 2008 28;116(3):454-60

56.Mark G, Denise C. The electrylytes in hyponatremia. Am J Kidney Dis.1991; 18(5): 527-45.

57.Agostino P, Paolo DC. Simon E. Neonatal physiology and metabolic considerations. In: Pediatric Surgery. 7th ed. 2012 89-107.

58.Satyakam M, Mnas RS. Neel madhav R. Amitryptaline induced hyponatremia. Delhi Psychiatry Journal. 2014; 17 (1): 209-10.

Table 1: Effect of VA on relative body organ weights of wistar rats.

.

Lung	0.47+0.01	0.46+0.01	0.48+0.01
Heart	0.37+0.01	0.39+0.05	0.38+0.01
Liver	3.71+0.04	3.76+0.05*	3.69+0.01
Spleen	0.37+0.01	0.37+0.01	0.38+0.01
Kidney	0.47+0.001	0.45+0.02	0.48+0.001
Testis	0.63+0.02	0.61+0.03	0.60+0.03
Adrenal	0.017+0.001	0.016+0.001	0.016+0.001
Female			
Lung	0.49+0.001	0.48+0.004	0.49+0.01
Heart	0.31+0.02	0.26+0.05	0.28+0.01
Liver	2.92+0.11	2.78+0.23	2.82+0.17
Spleen	0.25+0.01	0.23+0.02	0.22+0.02
Kidney	0.46+0.07	0.39+0.03	0.39+0.06
Ovary	0.11+0.01	0.09+0.005	0.091+0.01
Adrenal	0.016+0.001	0.014+0.001	0.016+0.001

Values are expressed as mean ± SD. * significantly different from control, $p<0.05$
VA^t is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
VA^s is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed

Table 2: Effect of VA on hematological parameters at the termination of study.

Gender & Parameters	**Groups**		
Male	**Control**	**VA^t**	**VA^s**
Hb (gm%)	13.00 ± 0.44	12.42 ± 0.63	14.53 ± 0.58*
RBC (x 10^6/cmm)	7.34 ± 0.23	6.82 ± 0.12	8.62 ± 0.43*
WBC (x 10^3/cmm)	11.88 ± 3.74	11.30 ± 1.61	11.30 ± 3.89
PLT(x 10^5/cmm)	9.48 ± 1.20	7.37 ± 1.15	9.50 ± 0.96
PCV (%)	33.86 ± 1.29	29.40 ± 5.06	46.71 ± 2.31*
MCV(fl)	46.06 ± 0.56	50.50 ± 1.17*	54.20 ± 0.91*
MCH (pg)	17.61 ± 0.23	18.93± 0.79	16.80 ± 0.38*
MCHC (gm/dl)	38.33 ± 0.42	37.93 ± 1.97	31.08 ± 0.65*
Female			
Hb (gm %)	12.35 ± 0.80	12.46 ± 0.58	14.25 ± 0.52*

.

RBC (x10^6/cmm)	6.89 ± 0.40	6.79 ± 0.27	7.90 ± 0.38*
WBC (x10^3/cmm)	10.13 ± 2.91	10.45 ± 2.77	11.78 ± 3.60
PLT (x10^5/cmm)	8.87± 1.79	7.94 ± 0.59	9.38 ± 1.81
PCV (%)	32.73 ± 1.90	33.61 ± 1.61	45.31± 2.03*
MCV(fl)	47.58 ± 0.75	49.58± 1.23*	57.38 ± 1.17*
MCH (pg)	17.88 ± 0.33	18.30 ± 0.37	18.01 ± 0.41
MCHC (gm/dl)	37.66 ± 0.75	37.05± 0.95	31.4 ± 0.42*

Values are expressed as mean ± SD. * significantly different from control, p<0.05
VA^t is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
VA^s is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed

Table 3: Effect of VA on differential leucocyte count at the termination of study.

Gender & Parameters	**Group**		
	Control	**VA^t**	**VA^s**
Male			
WBC (x 10^3/cmm)	11.88 ± 3.74	11.30 ± 1.61	11.30 ± 1.61
Neutorophil %	44.66 ± 8.80	45.83 ±10.34	59.12 ± 7.88*
Eosinophil %	2.83 ± 2.85	6.00 ± 7.45	1.00 ± 0.89
Lymphocyte %	51.00 ± 9.71	47.50 ± 15.78	39.00 ± 6.95
Monocyte %	1.50 ± 1.76	0.66 ± 0.51	0.83 ± 0.75
Female			
WBC (x10^3 /cmm)	10.13 ± 2.91	10.45 ± 2.77	11.78 ± 1.47
Neutorophil %	44.00 ± 17.29	47.33 ± 11.72	60.00 ± 5.93
Eosinophil %	6.00 ± 4.60	4.00 ± 4.33	0.66 ± 0.81
Lymphocyte %	49.33 ± 21.22	48.00 ± 14.79	38.66 ± 5.35
Monocyte %	0.66 ± 0.51	0.66 ± 0.51	0.66 ± 0.51

Values are expressed as mean ± SD. * significantly different from control, p<0.05

.

VA^{t} is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
VA^{s} is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed

Table 4: Effect of VA on biochemical estimations in wistar rats.

Gender & Parameters	Group		
	Control	**VA^{t}**	**VA^{s}**
Male			
BG (mg/dl)	89.26 ± 10.42	97.22 ±9.83	91.58 ±7.18
BUN (mg/dl)	20.24 ± 1.94	21.44 + 2.12	20.82 ± 1.72
CR (mg/dl)	0.51 ± 0.04	0.52 ±0.02	0.50 ±0.09
TP (g/dl)	6.48 ± 0.76	7.21 ±0.42	7.18 ±0.41
ALB (g/dl)	4.97 ± 0.23	5.17±0.22	5.02 ±0.21
TB (mg/dl)	0.21 ± 0.03	0.23 ±0.03	0.22 ±0.04
DB.(mg/dl)	0.062 ± 0.01	0.093 ±0.07	0.086 ±0.04
SGOT (U/L)	84.32 ± 7.32	83.62 ±3.63	81.17 ±7.23
SGPT (U/L)	32.81±3.73	34.33±3.05	32.58±2.11
ALP (U/L)	262.48 ± 6.69	256.58 ±31.28	250.24 ±33.79
Ca (mg/dl)	11.21 ± 0.78	11.19 ±0.63	10.74 ±1.09
K (mEq/L)	7.54 ± 1.89	7.41 ±0.46	8.08 ±1.18
Cl (mEq/L)	103.76 ± 5.69	110.53 ±14.61	106.79 ±7.19
Na (mEq/L)	86.03 + 4.90	63.66+7.46*	58.33±10.24*
P (mg/dl)	5.93 + 1.87	5.38+ 0.69	5.19±0.62
LDH (U/L)	361.44+82.50	408.53+78.92	384.54±34.89
HDL (mg/dl)	20+3.53	20.41+3.68	21.25±4.67
CHL (mg/dl)	81.44+7.92	79.23+6.26	82.27±7.61
TG (mg/dl)	110.66+6.22	113.39+7.29	112.88±5.45

.

Gender & Parameters	Group		
	Control	**VA^{t}**	**VA^{s}**
Female			
BG (mg/dl)	98.23 ± 13.75	96.35 ± 8.04	97.52±11.86
BUN (mg/dl)	22.07 ±11.67	20.16 ±4.61	21.96 ±2.28
CR (mg/dl)	0.49 ±0.10	0.50 ±0.07	0.52 ±0.05
TP (g/dl)	7.37 ±0.48	7.24 ±0.19	7.19 ±0.35
ALB (g/dl)	4.99 ±0.39	5.05 ±0.36	5.12 ±0.26
TB (mg/dl)	0.24 ±0.04	0.22 ±0.04	0.25 ±0.04
DB.(mg/dl)	0.081 ±0.05	0.11±0.05	0.09 ±0.04
SGOT (U/L)	73.08 ±9.29	77.67 ± 4.96	80.121 ±7.20
SGPT (U/L)	28.80 ±3.94	30.26 ±3.77	32.58 ±3.05
ALP (U/L)	152.77±12.80	156.4±10.98	150.50 ±18.81
Ca (mg/dl)	11.38 ±1.02	11.15 ±1.06	10.99 ±1.01
K (mEq/L)	8.75± 0.85	7.95±0.78	7.62 ± 1.64
Cl (mEq/L)	106.77 ±7.27	107.5 ±6.62	110.60 ±9.49
Na (mEq/L)	82.33 ± 15.46	71.79±9.86	70.17±5.85
P (mg/dl)	6.11±0.73	6.28±0.95	6.27±1.19
LDH (U/L)	473.90±117.43	424.96±11.68	397.71±29.38
HDL (mg/dl)	21.66±4.37	20±3.53	21.25±4.67
CHL (mg/dl)	87.96±4.14	85.83±6.58	86.73±5.66
TG (mg/dl)	108.37±8.08	106.12+5.54	105.27+4.53

Values are expressed as mean ± SD. * significantly different from control, $p<0.05$
VA^{t} is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and then sacrificed.
VA^{s} is a group of animals treated with vanillic acid (1000mg/kg) for 14 days and no treatment for another 14 days and then sacrificed

.

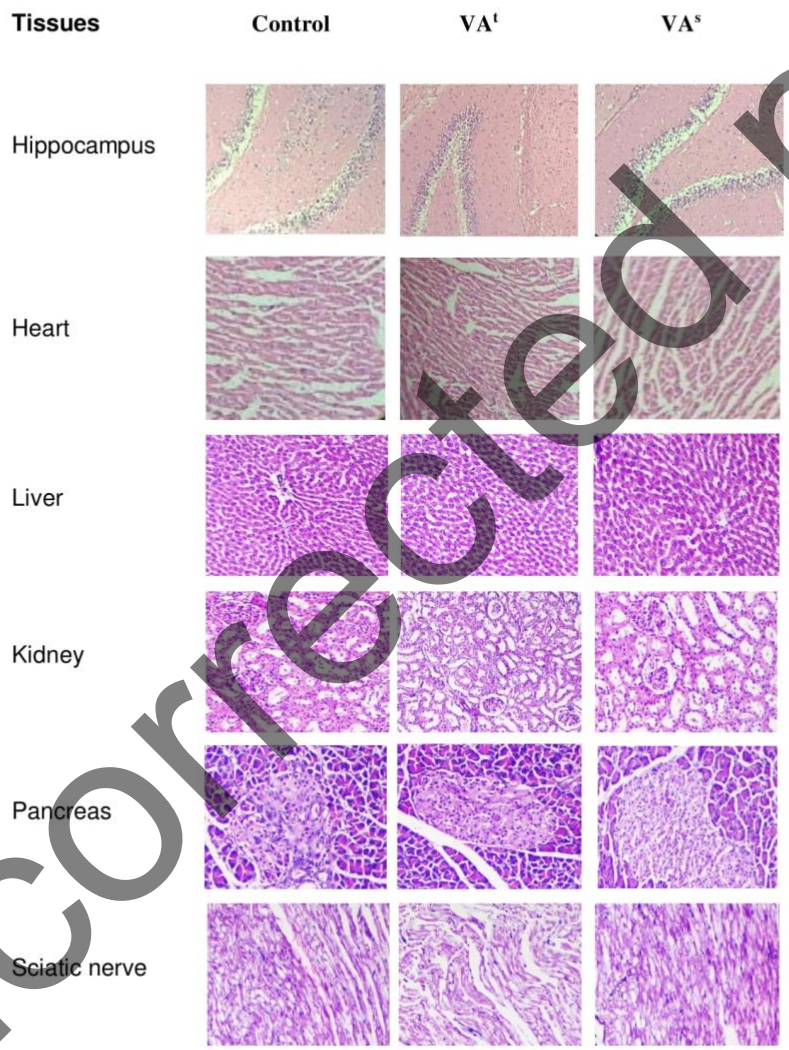

Figure 1: Effect of vanillic acid on histomorphology of vital body organs of rat.

.