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# Acute and Long Term Safety Evaluation of Grape Seed Extract and Zinc Containing Multivitamin-Mineral Nutritional Food Supplement in Rodents.

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**ABSTRACT**

To study the acute and sub-chronic toxicity associated with potential Zincovit tablets with grape seed extract among rats and mice. For acute toxicity in adult female Sprague-Dawley rats and Balb-c mice, the Acute Toxicity Class method (OECD 423 guideline) was employed. Animals were observed individually after dosing daily for a total of 14 days. Sub chronic toxicity was investigated in normal control (2% gum acacia, 1 ml/kg) and Zincovit tablets with grape seed extract at 50, 500 and 1250 mg/kg/day individually for 3 months in adult Sprague-Dawley female rats (4 groups, n= 6). Clinical signs, hematological and biochemical parameters were assessed. During the acute toxicity study, there were no behavioral changes or mortality observed even at 2000 mg/kg of Zincovit tablet with grape seed extract among rats and mice. There was no significant change in their body weight. LD50 cut-off for mice and rat was found to be above 5000 mg/kg and the drug came under the category 5 according to Globally Harmonized Classification System (OECD 423 guidelines). During the 90 days of sub-chronic toxicity study, treatment with combined formulation of grape seed extract and Zincovit tablets among Sprague-Dawley rats, the lowest-observed-adverse-effect levelsand no-observed-adverse-effect levels was observed as 500 mg/kg and 50 mg/kg respectively. The present study revealed that the single combined formulation of grape seed extract and Zincovit tablet is the potential functional nutritional food supplements that could offer a novel therapeutic opportunity against oxidative stress associated disorders in Sprague-Dawley rats.

**Keywords:** Grape seed extract, Zincovit tablets, Antioxidants, Safety

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## INTRODUCTION

Now a day, the huge concernis raised for the possibility that major diseases that affect humans worldwide may be preventable by the simple expedient of improving the dietary intake of antioxidant nutrients. Zincovit tablet is an advanced combined formulation of vitamins, minerals and grape seed extract. Zincovit releases a stream of anti-oxidant benefits. One of the studies suggests that long-term daily administration of grape seed extract offers enhanced antioxidant potential and protection against tissue lipid peroxidation and protein oxidation [1]. The biologically active constituents of grape seed extracts are proanthocyanidins, which represent a variety of polymers of flavan-3-ol, such as catechin and epicatechin and have a strong antioxidative effect in aqueous systems [2]. Multivitamin and mineral supplements are the most commonly used dietary supplements in the United States. According to the National Health and Nutrition Examination Survey 1999–2000, 35% of adults reported recent use of multivitamin supplements. Most persons use multivitamin and mineral supplements to ensure adequate intake and to prevent or mitigate diseases. Hence a study was planned to evaluate the acute and long term safety and to determine the clinical significance of Zincovit tablets with grape seed extract administered orally in rodents.

## MATERIALS AND METHODS

### Drugs and Reagents

Single combined formulation of grape seed extract and Zincovit tablet was obtained as kind gift from Apex Laboratories Private Ltd., Chennai (India). The diagnostic kits for alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TB) and direct bilirubin (DB), Lactate dehydrogenase (LDH) and creatinine were obtained from Aspen Laboratories, New Delhi (India). Sodium chloride and all other chemicals were obtained from Merck Chemicals, Mumbai (India). The reagents were equilibrated at room temperature for 30 minutes before use, either at the start of analysis or when reagent containers were refilled.

### Preparation of aqueous solution of Zincovit tablets for oral administration

Zincovit tablet is a single combined formulation of vitamins, minerals and grape seed extract. Each tablet of Zincovit weighs 850 mg. Ten tablets of Zincovit were crushed and fine powder form was dissolved in 100 ml of distilled water containing 2 g gum acacia (2% gum acacia). The aqueous solution of Zincovit tablets was stored in an amber colored bottle at 4$^{\circ}$C in refrigerator.

### Animals

Adult female Sprague-Dawley rats (6 to 8-weeks-old) and Balb-c mice (6 to 8-weeks-old), nulliparous and non-pregnant were selected for the study, which were bred locally in the central animal house of Manipal University, Manipal. They were housed in separate polypropylene cages (3 animals in each cage), maintained under standard conditions with temperature (22–24$^{0}$C), 12-h light/12-h dark cycle and relative air humidity 40–60%. The animals were acclimatized to the laboratory conditions for one week before the start of the experiment. The animals were provided with a normal pellet diet (Amrit Feeds Ltd., Pune, India) and water ad libitum. Animals described as fasted were deprived of food for 16-h but had allowed free access to water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC/KMC/35/2012) and experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines.

### Experimental design

### Acute toxicity study

For acute toxicity study, both rats and mice were divided individually into four groups (n=3).

Group IA: 300 mg/kg of Zincovit tablet with grape seed extract
Group IB: 300 mg/kg of Zincovit tablet with grape seed extract

Group IIA: 2000 mg/kg of Zincovit tablet with grape seed extract
Group IIB: 2000 mg/kg of Zincovit tablet with grape seed extract

The acute toxicity class method (OECD 423) was employed for the acute toxicity study of combined formulation of grape seed extract and Zincovit tablets[3]. In this method, Zincovit tablet with grape seed extract was administered through oral route at a single dose level to a group of experimental animals and a sequential testing procedure with three animals per group was used. Animals were fasted prior to dosing (e.g. with the rat and mice food was withheld over-night and for 3-4 hours respectively but water ad libitum). Following the period of fasting, the animals were weighed and the drug was administered. After the administration of Zincovit tablet with grape seed extract in rats or 1-2 hours in mice. The time interval between treatment groups was determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose was delayed until one was confident of survival of the previously dosed animals. Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. All observations were systematically recorded with individual records being maintained for each animal. Observations included were changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activities and behavior patterns. Attention was directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. At the end of the test surviving animals will weighed and humanely killed.

**Sub chronic toxicity study**

For sub chronic toxicity study, rats were divided into 4 groups (n=6). The corresponding doses of drugs were administered orally till 90 days as follow-

Group I: Normal control (2% gum acacia, 1 ml/kg))
Group II: 50mg/kg of Zincovit tablet with grape seed extract
Group III: 500mg/kg of Zincovit tablet with grape seed extract
Group IV: 1250mg/kg of Zincovit tablet with grape seed extract

**Collection of blood samples**

At the end of the experimental period, the animals were anesthetized with ketamine(80 mg/kg; *i.p.*) following a 12 h fast. Blood was collected from retro-orbital plexus through capillary tube. Serum was obtained by centrifugation of blood at 3,000 rpm for 20 min at 4ºC using a refrigerated centrifuge (MIKRO 22R, Andreas Hettich GmbH & Co. KG, Germany). The resulting supernatant (serum) was stored at-20˚C.

***Hematological parameters***

0.5 ml of blood from each animal was collected in EDTA containing vacutainer. Further RBC, WBC, differential leukocytes, platelet count and amount of hemoglobin was measured with the help of veterinary automatic blood cell counter.

***Biochemical parameters***

Blood glucose level was estimated in the fasting blood samples by glucose oxidase-peroxidase reactive strips (Accu-chek, Roche Diagnostics, USA).Serum was analyzed further for assay of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TB), direct bilirubin (DB), creatinine, lactate dehydrogenase (LDH), triglyceride (TG), total cholesterol (T-CHO) and high-density lipoprotein cholesterol (HDL-C) according to the standard protocols given along with respective kits (Aspen Laboratories, New Delhi, India). Low-density lipoprotein cholesterol (LDL-C) and Very low-density lipoprotein cholesterol(VLDL-C) was calculated by using Friedewald's equation:

$$VLDL\text{-}C = Triglycerides\ (TG)/5 \quad LDL\text{-}C = Total\ cholesterol - (HDL\text{-}C + VLDL\text{-}C)$$

**Statistical analysis**

Using Statistical Package for the Social Sciences (SPSS version 20.0;SPSS Inc., Chicago, USA), normally distributed data were expressedas mean ± standard deviation and analyzed by one way analysis of variance (ANOVA) followed by post hoc Tukey test. Data with non-uniform distribution were expressed as median, Quartile (Q1, Q3) and analyzed by non-parametric K Independent samples test followed by Kruskal-Wallis H test. A level for $p \leq 0{\cdot}05$ was considered to be statistically significant.

## RESULTS

**Acute toxicity study**

There were no behavioral changes or mortality observed even at 2000 mg/kg of Zincovit tablet with grape seed extract among rats and mice. There was no significant change in their body weight. LD50 cut-off for mice and rat was found to be above 5000 mg/kg and the drug came under the category 5 according to Globally Harmonized Classification System (OECD 423 guidelines).

**Sub-chronic toxicity study**

During the 90 days of treatment with combined formulation of grape seed extract and Zincovit tablets among Sprague-Dawley rats, the lowest-observed-adverse-effect levels (LOAELs) and no-observed-adverse-effect levels (NOAELs) was observed as 500 mg/kg and 50 mg/kg respectively.

**Effect on hematological parameters**

There were no significant changes in RBC, Platelets, WBC, Differential leukocyte count and amount of hemoglobin in the test drug treatment groups in comparison with the normal control (untreated) group.

**Effect on biochemical parameters**

**Table 1: Effect of combined formulation of grape seed extract and Zincovit tablets on fasting blood glucose (mg/dl) and ALP (U/L)**

| Groups (n=6) | Glucose | ALP |
|---|---|---|
| I- Normal control (2% gum acacia) | 108.50±11.37 | 37.09 (16.67, 52.31) |
| II- ZVT (50 mg/kg/day) | 87.50±7.47*** | 619.00 (483.82, 736.02)** |
| III- ZVT (500 mg/kg/day) | 93.80±5.76* | 430.90 (359.35, 461.90)** |
| IV- ZVT (1250mg/kg/day) | 100.67 (74.39, 116.28)** | 430.00 (394.95, 448.77)** |

n, number of rats in each group;ZVT, zincovit tablets with grape seed extract; ALP, alkaline phosphatase. Values are mentioned as mean± standard deviation for glucose and median (Quartile 1, Quartile3) for ALP.***indicates statistically significant difference compared with normal control (p< 0.001), **indicates statistically significant difference compared with normal control (p< 0.01), *indicates statistically significant difference compared with normal control (p< 0.05).

**Table 2: Effect of combined formulation of grape seed extract and Zincovit tablets on HDL-cholesterol (mg/dl) and lactate dehydrogenase (IU)**

| Groups (n=6) | HDL-C | LDH |
|---|---|---|
| I- Normal control (2% gum acacia) | 58.44 (55.71, 71.36) | 150.40 (120.22, 177.47) |
| II- ZVT (50 mg/kg/day) | 50.13(47.98, 51.28) | 164.50 (131.33, 181.05) |
| III- ZVT (500 mg/kg/day) | 69.98 (53.47, 78.99)** | 120.50(110.30, 156.95)* |
| IV- ZVT (1250mg/kg/day) | 100.67 (74.39, 116.28)** | 107.65(101.10,107.65)* |

n, number of rats in each group; ZVT, zincovit tablets with grape seed extract; HDL-C, high-density lipoprotein cholesterol; LDH, lactate dehydrogenase. Values are mentioned as median (Quartile1, Quartile3). **indicates statistically significant difference compared with normal control (p< 0.01), *indicates statistically significant difference compared with normal control (p< 0.05).

There were no significant changes in ALT, AST, Triglycerides, Total cholesterol, VLDL-cholesterol, LDL-cholesterol and Creatinine ($p > 0.05$) among the combined formulation of grape seed extract and Zincovit treated groups when compared with the normal control (untreated) group. Whereas the significant increase in ALP ($p = 0.002$), HDL-cholesterol ($p = 0.002$) and decrease in Lactate dehydrogenase ($p = 0.050$), fasting blood glucose($p = 0.001$) were observed especially in 50 and 500 mg/kg treatment group when compared with the normal control group (Table 1 and 2).

## DISCUSSION

In the present study, there were no significant changes observed in the hematological parameters between the normal control and the Zincovit treated groups which suggests that the Zincovit tablet with grape seed extract may not be toxic as they did not affect either the circulating red cells, nor the hematopoiesis that could otherwise have caused a megaloblastic anemia, nor changes differential leukocyte count, platelets count and hemoglobin.

The liver and heart release ALT and AST and an elevation in their plasma concentrations are indicators of liver and heart damage [4, 5]. However, ALT is more specific to the liver and is thus a better parameter for detecting liver injury [6]. The non-significant changes in ALT and AST reveal no hepatotoxicity with the combined formulation of grape seed extract and Zincovit tablets treatment. The significant increase in the level of alkaline phosphatase (ALP) may be as a result of increased osteoblastic activity due to presence of vitamin D as one of the constituent of Zincovit tablet or congestion/obstruction of biliary tract, which may occur within the liver. The ALP activity on the other hand is related to the functioning of hepatocytes and an increase in its activity may be due to its increased synthesis in the presence of increased pressure [7]. The hypoglycemic effect of Zincovit tablet with grape seed extract may be attributed to enhanced antioxidant potential and protection against tissue lipid per oxidation and protein oxidation due to long-term daily administration of grape seed extract and could therefore be associated with reduced glucose levels in diabetic rats [1].

The increase in HDL-C observed in groups treated with Zincovit tablet with grape seed extract may be attributed to release of a stream of anti-oxidant benefits including proanthocyanidins from the drug. might be attributed to the synergistic interplay of constituents of Zincovit tablets, such as-grape seed extractproanthocyanidins which comprise only procyanidins [subunits constituted of (+) catechin (C) and (−)-epicatechin (EC)], Vitamins A, B, C, D, E, folic acid, biotin and minerals like zinc, copper, selenium, magnesium, manganese, chromium and molybdenum mainly, which are promoters of antioxidant activity and act against oxidative stress.

The drug had no adverse effect on the serum concentration of creatinine. This is suggestive of no kidney damage specifically by renal filtration mechanism or probably indicates that Zincovit tablet with grape seed extract did not interfere with the renal capacity to excrete its metabolites.

The significant decrease in lactate dehydrogenase (LDH) with higher dose treatment group observed in the present investigation apparently indicated the protective membrane stabilizing effect of Zincovit tablet with grape seed extract.

The hind limb weakness among few animals was seen in higher dose (above 500 mg/kg) treatment group lead to immobility, starvation and finally their death. This may be attributed to excess of pyridoxine (Vitamin $B_6$) and zinc present in Zincovit tablets with grape seed extract. One of the studies suggests that high and intermediate doses of pyridoxine resulted in sensory ataxia, diminished distal limb position and vibration sense with injury progressing to necrosis of the affected ganglionic neurons and associated sensory myelinated axonopathy and fiber degeneration [8]. Zinc pyrithione given orally has been observed to produce hind limb weakness [9].

However, all the surviving animals were healthy as shown by the normal appearance of respiratory pattern, color of body surfaces, frequency and nature of movement, marked involuntary contraction or seizures of contraction of voluntary muscle, and loss of reflex.

Thus, the present study demonstrates that the single combined formulation of grape seed extract and Zincovit tablet is the potential functional nutritional food supplements that could offer a novel therapeutic

opportunity against oxidative stress associated disorders in Sprague-Dawley rats. The therapeutic effect seen in animal studies cannot always be entirely extrapolated to humans. Hence, clinical evaluation should be performed to precisely define the safety of Zincovit tablets with grape seed extract in humans.

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