Subacute Oral Toxicity Assessment of a Herbomineral Formulation with Shilajit, Swarna Bhasma and other Ingredients

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ABSTRACT

Background: An Ayurvedic herbomineral formulation containing Shilajit, Swarna bhasma, and other ingredients intended to be used as a rejuvenator for increasing strength stamina and endurance was investigated for its subacute oral toxicity in Wistar rats.

Materials and methods: The study was conducted as per the Organization for Economic Cooperation and Development (OECD) guidelines. Test item (TI) was administered to three groups at three dose levels (250, 500, and 1000 mg/kg body weight BW) for 28 consecutive days. Animals in one group were taken as high-dose satellite reversal along with one control group and one satellite control group that received Milli-Q water (10 mL/kg BW). Any clinical sign of toxicity/mortality was observed. Signs of delayed onset of toxicity was observed in satellite groups for the next 14 days.

Results: No mortality or clinical sign of toxicity was observed. All animals from the treated and satellite groups showed similar weight gain and feeding as of control group during dosing and recovery periods. Hematological and biochemical parameters were within the range of normalcy in treated and control groups. Necropsy examination did not reveal any significant gross pathology changes. Organ weights in treated group were comparable to control group. Histopathology of high-dose-treated animals and control animals was also comparable.

Conclusion: Tested formulation containing Shilajit, Swarna bhasma, and other ingredients was found to be nontoxic at tested doses in both the sexes of Wistar rats on subacute administration. Results of the current study show that TI has no observed adverse effect level (NOAEL) of 1000 mg/kg BW on subacute oral exposure to Wistar rats for 28 days.

Keywords: Ayurveda, Herbometric, Herbomineral, Organization for Economic Cooperation and Development guidelines, Repeated dose 28-day oral toxicity, Shilajit, Subacute oral toxicity, Swarna bhasma.

Introduction

Shilajit is a blackish brown herbomineral exudation made from humus and degradation of organic matter that gets deposited naturally over rocks in the high-altitude mountains, from the Himalayas to the Andes. The treasure trove of Ayurveda describes Shilajit as a rasayana or a rejuvenator. Many Ayurvedic formulations combine health benefits of Shilajit with ingredients such as kewanch, gokshur, ashwagandha, safed musli, and calcined gold and silver (bhasma), etc., that are being used since ages as rejuvenators, restoratives, aphrodisiacs, to remedy conditions such as general weakness and fatigue, and to increase strength and stamina. The present study investigated the safety profile of one such herbomineral formulation following a 28-day repeated dose oral administration.

Materials and Methods

Study Product

The herbomineral formulation containing Shilajit, Swarna Bhasma, and other ingredients coded as DRDC/AY/8055 and marketed as Shilajit Gold (Mfd. Dabur India Limited, Ghaziabad, India) with Batch No. 0037, manufactured 03/10, and expiry 5 years from manufacturing were used in this study. The composition details and therapeutic properties of its ingredients are given in Table 1.

Experimental Animals and Housing Conditions

Animals of both sexes aged 5–9 weeks weighing 120.2–173.1 g (male) and 115–169.2 g (female) obtained from the animal facility.

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Conflict of interest: Arun Gupta, Satyendra Kumar, Pankaj Gupta are employed with Dabur India Ltd. that manufactures and markets Shilajit Gold of Dabur Research Foundation, Ghaziabad, Uttar Pradesh and Mahaveera Enterprises, Hyderabad, Telangana, India, were used in this study. Polycarbonated cages were used for housing animals. Five animals were housed in each cage and acclimatized (5 days for male, 6 days for female, temperature 19.0–23.5°C, relative humidity: 55–68%, 12 hours L/day cycle). Conventional pelleted feed (Golden
Feeds, New Delhi, India) and filtered pure drinking water were provided ad libitum to animals.

Standard temperature and humidity maintained for animal housing at 21.25 ± 2.25°C and 61.5 ± 6.5% respectively, with 12 hours standard light and dark cycles were maintained. Logs were maintained to record temperature and humidity. Clean water was supplied unlimited and conventional pellet diet was used for feed

### Ethical Approvals
The facility and study20,21 were approved by the Institutional Animal Ethics Committee of Dabur Research Foundation vide (IAEC/DO/74, Dated: April 20, 2010).

### Chemicals/Reagents
Diethyl ether (Sisco Research Laboratories), EDTA (Sigma), formaldehyde (MERCK), potassium phosphate (Dibasic) (Qualigens), and potassium phosphate (Monobasic) (MERCK).

### Selection of Doses
Three doses selected for the study were 250, 500 and 1000 mg/kg BW. Low dose was (250 mg/kg) selected based on human therapeutic dose converted allometrically by applying the standard conversion factor22 to rat dose, considering average human BW (60 kg). Low dose was one fourth of the highest dose. Subsequently mid (500 mg/kg) and high dose (1000 mg/kg) were selected.

### Preparation of TI
The TI was prepared by suspending the test formulation in Milli-Q water to obtain final concentrations of 100, 50, and 25 mg/mL. Fresh TI was prepared daily prior to dosing.

### Experimental Design
The subacute toxicity study was carried out according to the OECD Guidelines No. 407 for the “Repeated Dose 28-Day Oral Toxicity Study in Rodents”.21 The rats were assigned randomly to six groups and were administered TI once daily by gavage method for a period of 28 days (Table 2). Control animals were administered vehicle only.

### Results

#### Table 1: Therapeutic properties of ingredients of the tested formulation

| S. no. | Ingredient | Part used | Botanical name | Therapeutic uses |
|--------|------------|-----------|----------------|-----------------|
| 1      | Kewanch    | Seed      | Mucuna pruriens (L.) DC. | Brmgana, balakarak, vrsaya, vatanasaka⁸ |
| 2      | Shudh shilajit | Exudate  | Asphaltum | Rasayana, yogavahi, ksaya⁵ |
| 3      | Safed musli | Root      | Chlorophytum tuberosum (Roxb.) Baker | Balya, shukrameha⁶ |
| 4      | Ashwagandha | Root      | Withania somnifera (L.) Dunal | Balya, ksaya, rasayana, shukrala⁷ |
| 5      | Akarkara   | Root      | Anacyclus pyrethrum (L.) Lag. | Vatahara, pittahara, kaphahara, sukrala, vajikara, dipana, balakarka⁸ |
| 6      | Jatiphala  | Kernel/sead | Myristica fragrans Houtr. | Vajikarana, stambhana, dipana, uttejaka⁹ |
| 7      | Lavanga    | Flower buds | Syzygium aromaticum (L.) Merr. and L.M. Perry | Svasa, ksaya, agnidipaka¹⁰ |
| 8      | Gokshura   | Fruit     | Tribulus terrestris L. | Balya, pustikara, vrsya¹¹ |
| 9      | Kumkuma    | Style and stigma | Crocus sativus L. | Snigdha, balya, uttejaka¹² |
| 10     | Tvak/dalchini | Bark    | Cinnamomum zeylanicum Blume | Balya, shukrala, dipana, stambhana¹³ |
| 11     | Varahi     | Root tuber | Dioscorea bulbifera L. | Shukra-ayu-svara-agnihala vivardhani, rasayanị¹⁴ |
| 12     | Vidari     | Root tuber | Pueraria tuberosa (Willd.) DC. | Brmgana, rasayana shukradha, jivaniya, bala-varna-da¹⁵ |
| 13     | Rajata bhasma | –        | Calcined silver | Snigdha, rucivardhaka, vrsya, rasayana, balya, ksayahara¹⁶ |
| 14     | Swarna bhasma | –        | Calcined gold | Balavardhaka, poshaka, rasayana, kantivardhaka¹⁷ |
| 15     | Yasada bhasma | –        | Calcined zinc | Bala-virya vardhaka, kshma-avsada shamanam¹⁸ |
| 16     | Kapoor     | Sublimed extract | Cinnamomum camphora (L.) J. Presl | Vrsya, uttejaka, deepana¹⁹ |

All animals were observed daily for signs of any ill health, treatment response, or mortality. Skin, fur, eyes, and mucous membranes and behavior pattern were observed for any changes. Cage-side observations were made to detect mortality and general activity of the animals.

### Body Weight
It was recorded at study initiation, randomization, weekly intervals, and at the end of the study. Percentage changes were calculated using the formula:

\[
\text{% Change in animal body weight} = \frac{\text{Final body weight (g)} - \text{Initial body weight (g)}}{\text{Initial body weight (g)}} \times 100
\]

### Feed Consumption
The quantity of feed consumed by each dose group was recorded on a weekly basis throughout the study period.

### Laboratory Investigations
Blood samples were collected from retro-orbital sinus of overnight fasted and anesthetized rats for hematological and biochemical examinations prior to sacrifice. Hematology samples were collected into the tubes containing anticoagulant (EDTA), while the blood without anticoagulant was collected for clinical biochemistry study.

### Hematological Investigations
Hematological parameters analyzed were hemoglobin (Hb) levels, erythrocyte count (red blood cell RBC), total leukocyte count (TLC), differential leukocyte count, packed cell volume (PCV/hematocrit), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and platelets.

### Blood Biochemistry
Blood without anticoagulant were allowed to stand for 30 minutes for clotting and centrifuged at 4,000 rpm at 4°C for 10 minutes to
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Obtain serum. Serum was stored at −20°C until analyzed for the following biochemical parameters glucose, alanine aminotransferase (ALT), aspartate aminotransferase, total cholesterol, triglycerides, serum creatinine, alkaline phosphatase (ALP), blood urea nitrogen (BUN), total bilirubin, total proteins, albumin, globulin, sodium, potassium, chloride, calcium, and phosphorus (measured by EM 200 from Transasia).

Necropsy

After the withdrawal of blood on day 28, the main group animals were administered ether in overdose and sacrificed, except for the satellite group animals, which were sacrificed on completion of day 14 post dosing period. Necropsy included the gross examination of the external surfaces of body; all orifices; the cranial, thoracic, and abdominal cavities and their contents; and the organs and tissues (viz., brain, adrenal, heart, kidneys, liver, lungs, spleen, spinal cord, trachea, thyroid, thymus, stomach, duodenum, jejunum, colon, thigh muscle, lymph node, urinary bladder, testes/ovaries, epididymis/uterus, prostate, and seminal vesicles).

Absolute weights of adrenal, brain, heart, testes/ovaries, epididymis/uterus, kidneys, liver, thymus, lungs, and spleen were recorded while conducting necropsy and the relative organ weights

Table 2: Treatment schedule in groups

| Group | Test item          | Dose mg/kg | No. of animals/group |
|-------|--------------------|------------|----------------------|
| GI    | Control            | Milli-Q water | 10       | Male | Female |
| GII   | Low dose           | TI         | 250       | 5    | 5      |
| GIII  | Mid dose           | TI         | 500       | 5    | 5      |
| GIV   | High dose          | TI         | 1,000     | 5    | 5      |
| GIS   | Satellite reversal | TI         | 1,000     | 3    | 3      |
| GIS   | Satellite control  | Milli-Q water | 10 mL/kg | 3    | 3      |

Figs 1A and B: Effect of subacute administration of test item for 28 days on feed consumption of Rats. (A) Female; (B) Male. Data are expressed as mean ± standard deviation. *Significant value (p < 0.05)

Figs 2A and B: Effect of subacute administration of test item for 28 days on body weight Rats. (A) Female; (B) Male. Data are expressed as mean ± standard deviation
Table 3A: Hematological findings from male and female study group, the control and reversal groups in rats treated with tested formulation measured during the 28 days subacute toxicity study (measured by KX 21 Sysmex, Japan)

| Parameter | Male Control | Male Low (250 mg/kg) | Male Mid (500 mg/kg) | Male High (1000 mg/kg) | Female Control | Female Low (250 mg/kg) | Female Mid (500 mg/kg) | Female High (1000 mg/kg) |
|-----------|--------------|----------------------|----------------------|------------------------|----------------|------------------------|------------------------|-------------------------|
| Hb (g/dL) | 14.7 ± 0.3   | 14.2 ± 0.3           | 14.3 ± 0.3           | 14.1 ± 0.4             | 14.4 ± 0.3   | 12.8 ± 1.3             | 14.1 ± 0.7             | 13.4 ± 0.7              |
| TLC (thousand/mm³) | 20.4 ± 2.5 | 19.0 ± 3.5           | 22.3 ± 1.1           | 21.4 ± 1.3             | 12.5 ± 2.3   | 13.1 ± 1.7             | 14.1 ± 2.4             | 18.8 ± 2.7              |
| N (%)     | 12.2 ± 0.9   | 18.4 ± 4.5           | 11.8 ± 0.6           | 24.0 ± 8.8             | 15.4 ± 1.6   | 20.6 ± 5.3             | 11.4 ± 0.8             | 10.6 ± 0.5              |
| L (%)     | 86.0 ± 1.0   | 79.2 ± 4.4           | 86.0 ± 0.3           | 74.2 ± 8.7             | 82.2 ± 1.7   | 76.2 ± 5.1             | 86.2 ± 1.0             | 88.0 ± 0.6              |
| E (%)     | 0.6 ± 0.2    | 0.8 ± 0.2            | 0.8 ± 0.2            | 0.8 ± 0.5              | 1.2 ± 0.4    | 1.8 ± 0.8              | 1.2 ± 0.5              | 0.4 ± 0.2               |
| M (%)     | 1.2 ± 0.4    | 1.6 ± 0.2            | 1.4 ± 0.4            | 1.0 ± 0.3              | 1.2 ± 0.2    | 1.4 ± 0.2              | 1.2 ± 0.2              | 1.0 ± 0.0               |
| B (%)     | 0.0 ± 0.0    | 0.0 ± 0.0            | 0.0 ± 0.0            | 0.0 ± 0.0              | 0.0 ± 0.0    | 0.0 ± 0.0              | 0.0 ± 0.0              | 0.0 ± 0.0               |
| EC (million/mm³) | 8.6 ± 0.2 | 7.8* ± 0.2           | 8.0 ± 0.3            | 8.1 ± 0.1              | 8.0 ± 0.1    | 7.6 ± 0.2              | 7.5 ± 0.4              | 7.1 ± 0.4               |
| MCV (fL)  | 54.0 ± 1.7   | 57.8 ± 1.4           | 57.4 ± 0.3           | 53.9 ± 2.2             | 55.6 ± 0.7   | 56.1 ± 0.4             | 56.2 ± 0.4             | 56.7 ± 0.6              |
| MCHC (g/dL) | 31.0 ± 0.5 | 31.7 ± 0.3           | 31.3 ± 0.3           | 28.3 ± 2.7             | 32.6 ± 0.3   | 33.0 ± 0.2             | 33.6** ± 0.3           | 33.3 ± 0.1              |
| P (×1,000/µL) | 781.8 ± 66.2 | 858.8 ± 49.7 | 892.4 ± 16.5 | 892.0 ± 84.7 | 1057.4 ± 55.9 | 1048.8 ± 72.4 | 939.6 ± 72.3 | 860.2 ± 121.9 |
| PCV (%)   | 47.4 ± 0.7   | 45.0 ± 1.1           | 45.7 ± 1.4           | 39.2 ± 6.4             | 44.2 ± 0.8   | 42.5 ± 1.1             | 41.9 ± 2.5             | 40.5 ± 2.1              |

Values are expressed as mean ± SEM; *p value is 0.025; **p value is 0.047. The result is significant at p < 0.05
### Table 3C: Hematological parameters of female Wistar rats: GIS (satellite control) and GIVS (satellite reversal) treated with tested formulation measured after completion of 14 days recovery (posttreatment period) during the study. (Measured by KX 21 Sysmex, Japan)

| Parameters | GIS (satellite control) | Animal number | GIVS satellite reversal (1,000 mg/kg) |
|------------|-------------------------|---------------|--------------------------------------|
|            | 44  | 45  | 46  | Mean | SEM | 50  | 51  | 52  | Mean | SEM |
| Hb (g/dL)  | 14.9| 14.4| 13.8| 14.5 | 0.4 | 14.7| 14.4| 14.7| 0.2  |
| TLC (thousand/mm$^3$) | 30.5| 22.0| 18.8| 23.8 | 3.5 | 10.6| 26.7| 26.4| 21.2| 5.3 |
| N (%)      | 10  | 12  | 14  | 12.0 | 1.2 | 9   | 17  | 10  | 12.0 | 2.5 |
| L (%)      | 88  | 86  | 85  | 86.3 | 0.9 | 91  | 80  | 87  | 86.0 | 3.2 |
| E (%)      | 1   | 0   | 0   | 0.3  | 0.3 | 0   | 1   | 1   | 0.7  | 0.3 |
| M (%)      | 1   | 2   | 1   | 1.3  | 0.3 | 0   | 2   | 2   | 1.3  | 0.7 |
| B (%)      | 0   | 0   | 0   | 0    | 0.0 | 0   | 0   | 0   | 0.0  | 0.0 |
| EC (million/mm$^3$) | 7.95| 8.59| 8.35| 8.3  | 0.2 | 8.64| 8.64| 8.38| 8.6  | 0.1 |
| MCV (FL)   | 55.6| 58.0| 55.3| 56.3 | 0.9 | 57.3| 56  | 55  | 56.1 | 0.7 |
| MCHC (g/dL)| 31.2| 30.7| 31.2| 31.0 | 0.2 | 29.7| 31  | 31.2| 30.6 | 0.5 |
| P ($\times 1,000$/$\mu$L) | 1,001| 808| 1,000| 936.3| 64.2| 1,017| 749| 831| 865.7| 79.3 |
| PCV (%)    | 44.2| 49.8| 46.2| 46.7 | 1.6 | 49.5| 48.4| 46.1| 48.0 | 1.0 |

Values are expressed as mean \(\pm\) SEM

Hb, hemoglobin; TLC, total leukocyte count; N, neutrophil; L, lymphocyte; E, eosinophil; M, monocyte; B, basophil; EC, erythrocyte count; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; PC, platelet count; PCV, packed cell volume; EC, erythrocyte count
### Table 4A: Biochemical findings from male and female study group, the control, and reversal groups in rats treated with tested formulation measured during the 28 days of subacute toxicity study (measured by EM 200 from Transasia)

| Parameters                     | Male Control | Low (250 mg/kg) | Mid (500 mg/kg) | High (1000 mg/kg) | Female Control | Low (250 mg/kg) | Mid (500 mg/kg) | High (1000 mg/kg) |
|--------------------------------|--------------|-----------------|-----------------|-------------------|----------------|-----------------|-----------------|-------------------|
| ALT (IU/L)                     | 68.1 ± 5.2   | 64.7 ± 4.4      | 61.7 ± 4.4      | 60.2 ± 4.1        | 34.6* ± 5.3    | 61.7 ± 6.1      | 59.0 ± 8.1      | 54.7 ± 3.8        |
| AST (IU/L)                     | 259.3 ± 17.5 | 283.9 ± 21.1    | 294.0 ± 21.1    | 259.9 ± 20.3      | 206.3 ± 52.1   | 293.3 ± 30.4    | 299.7 ± 11.4    | 295.0 ± 11.4      |
| ALP (IU/L)                     | 23.2 ± 4.7   | 20.9 ± 3.7      | 18.0 ± 3.7      | 20.1 ± 3.7        | 11.3 ± 3.7     | 11.4 ± 3.7      | 11.4 ± 3.7      | 11.3 ± 3.7        |
| Phosphorus (mg/dL)             | 6.5 ± 0.4    | 7.2 ± 0.4       | 7.0 ± 0.5       | 6.4 ± 0.3         | 6.4 ± 0.3      | 6.4 ± 0.3       | 6.4 ± 0.3       | 6.4 ± 0.3         |
| Total bilirubin (mg/dL)        | 0.1 ± 0.01   | 0.2 ± 0.02      | 0.2 ± 0.03      | 0.1 ± 0.01        | 0.1 ± 0.01     | 0.1 ± 0.01      | 0.1 ± 0.01      | 0.1 ± 0.01        |
| BUN (mg/dL)                    | 21.1 ± 2.1   | 24.6 ± 1.5      | 19.6 ± 1.5      | 21.6 ± 1.2        | 23.7 ± 2.1     | 22.3 ± 1.2      | 22.3 ± 1.2      | 22.3 ± 1.2        |
| Creatinine (mg/dL)             | 0.6 ± 0.02   | 0.6 ± 0.01      | 0.6 ± 0.03      | 0.6 ± 0.03        | 1.0 ± 0.04     | 1.0 ± 0.04      | 1.0 ± 0.04      | 1.0 ± 0.04        |
| Blood glucose (mg/dL)          | 5.3 ± 0.2    | 4.9 ± 0.2       | 5.3 ± 0.2       | 5.3 ± 0.2         | 4.7 ± 0.2      | 4.7 ± 0.2       | 4.7 ± 0.2       | 4.7 ± 0.2         |
| Triglyceride (mg/dL)           | 70.8 ± 5.7   | 89.6 ± 15.6     | 110.4 ± 20.9    | 61.8 ± 12.9       | 76.6 ± 11.1    | 97.8 ± 16.5     | 141.8 ± 68.0    | 141.8 ± 68.0      |
| TC (mg/dL)                     | 51.2 ± 3.3   | 53.0 ± 5.7      | 55.6 ± 9.4      | 43.8 ± 4.3        | 57.6 ± 5.8     | 63.8 ± 7.8      | 60.2 ± 8.1      | 71.0 ± 9.9        |
| Calcium (mg/dL)                | 90.0 ± 1.0   | 81.1* ± 0.2     | 8.5 ± 0.2       | 8.1** ± 0.2       | 8.6 ± 0.1      | 8.1 ± 0.4       | 8.4 ± 0.1       | 8.4 ± 0.1         |
| Albumin (g/dL)                 | 3.7 ± 0.1    | 3.6 ± 0.2       | 4.0 ± 0.3       | 3.4 ± 0.3         | 4.2 ± 0.1      | 3.9 ± 0.1       | 4.1 ± 0.1       | 4.1 ± 0.1         |
| Total protein (g/dL)           | 7.8 ± 0.2    | 7.7 ± 0.1       | 9.3 ± 0.1       | 7.6 ± 0.2         | 8.0 ± 0.2      | 7.3 ± 0.1       | 8.3 ± 0.2       | 8.0 ± 0.3         |
| Potassium (mEq/L)              | 5.7 ± 0.2    | 5.9 ± 0.1       | 5.6 ± 0.1       | 5.7 ± 0.1         | 6.0 ± 0.2      | 6.0 ± 0.2       | 5.9 ± 0.1       | 5.9 ± 0.1         |
| Sodium (mEq/L)                 | 150.2 ± 10.0 | 151.3 ± 11.1    | 147.5 ± 12.1    | 151.4 ± 12.1      | 154.5 ± 14.1   | 154.5 ± 14.1    | 154.5 ± 14.1    | 154.5 ± 14.1      |
| Chloride (mEq/L)               | 110.3 ± 1.0  | 103.4 ± 1.7     | 106.0 ± 1.7     | 107.9 ± 1.0       | 110.9 ± 1.3    | 110.9 ± 1.3     | 110.9 ± 1.3     | 110.9 ± 1.3       |

Values are expressed as mean ± standard deviation. ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; TC, total cholesterol; p-value < 0.05.
### Table 4B: Biochemical parameters of male Wistar rats: GIS (satellite control) and GIVS (satellite reversal) treated with tested formulation measured after completion of 14 days' recovery (posttreatment period) during the study (measured by E200, Transasia)

| Parameters           | GIS (satellite control) | Animal number | GVS satellite reversal (1000 mg/kg) | Animal number |
|----------------------|-------------------------|---------------|------------------------------------|---------------|
|                      | 41  | 42   | 43   | Mean | SEM | 47   | 48   | 49   | Mean | SEM |
| ALT (IU/L)           | 55.8| 49.7 | 65.6 | 57.0 | 4.6 | 50.5 | 71.7 | 72.5 | 64.9 | 7.2 |
| AST (IU/L)           | 244.5| 263.4 | 270.9 | 259.6 | 7.9 | 235.9 | 326.8 | 275.7 | 279.5 | 26.3 |
| ALP (U/L)            | 176 | 232  | 167  | 191.7 | 20.3 | 127  | 330  | 243  | 233.3 | 58.8 |
| Phosphorus (mg/dL)   | 7.4 | 8    | 8.47 | 8.0  | 0.3 | 6.59 | 6.93 | 7.05 | 6.9* | 0.1 |
| Total bilirubin (mg/dL) | 0.11 | 0.12 | 0.13 | 0.1  | 0.01 | 0.15 | 0.13 | 0.12 | 0.1  | 0.01 |
| BUN (mg/dL)          | 21.78 | 21.92 | 22.9 | 22.2 | 0.4 | 21.26 | 20.79 | 20.23 | 20.8** | 0.3 |
| Creatinine (mg%)     | 0.56 | 0.59 | 0.61 | 0.6  | 0.01 | 0.58 | 0.59 | 0.63 | 0.6  | 0.02 |
| Blood glucose (mg/dL)| 48.7 | 52.8 | 38.5 | 46.7 | 4.3 | 52.8 | 71.7 | 47.1 | 57.2 | 7.4 |
| Triglyceride (mg/dL) | 83  | 127  | 97   | 102.3 | 13.0 | 58   | 72   | 113  | 81.0 | 16.5 |
| TC (mg/dL)           | 44  | 58   | 63   | 55.0 | 5.7 | 49   | 55   | 52   | 52.0 | 1.7 |
| Calcium (mg/dL)      | 8.5 | 8.5  | 7.9  | 8.3  | 0.2 | 10.5 | 8.8  | 7.5  | 8.9  | 0.9 |
| Albumin (g/dL)       | 3.72 | 3.79 | 3.63 | 3.7  | 0.0 | 3.61 | 3.87 | 3.86 | 3.8  | 0.1 |
| Total protein (g/dL) | 7.04 | 7.43 | 7.89 | 7.5  | 0.2 | 6.86 | 7.8  | 6.54 | 7.1  | 0.4 |
| Globulin (g/dL)      | 3.32 | 3.64 | 4.26 | 3.7  | 0.3 | 3.25 | 3.93 | 2.68 | 3.3  | 0.4 |
| Sodium (mEq/L)       | 150.5| 165.5 | 148.7 | 150.8 | 1.3 | 148.5 | 116  | 150.3 | 130.3 | 11.1 |
| Potassium (mEq/L)    | 5.74 | 5.54 | 5.9  | 5.73 | 0.10 | 5.4  | 6.7  | 5.65 | 5.9  | 0.4 |
| Chloride (mEq/L)     | 110.5| 113.0 | 108.7 | 110.5 | 1.04 | 107.1 | 96.8 | 107.2 | 103.7 | 3.5 |

Values are expressed as mean ± SEM, ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; SGPT, serum glutamic pyruvatic transaminase; SGOT, serum glutamic oxaloacetic transaminase; TC, total cholesterol

*p value is 0.0315; **p value is 0.0355. The result is significant at p < 0.05
Table 4C: Biochemical parameters of female Wistar rats-GIS (satellite control) and GIVS (satellite reversal) treated with tested formulation measured after completion of 14 days recovery (post-treatment period) during the study (measured by E200, Transasia)

| Parameters                      | GIS (satellite control) | Animal number | GIVS satellite high (1000 mg/kg) |
|---------------------------------|-------------------------|---------------|---------------------------------|
|                                 | 44  | 45  | 46  | Mean | SEM | 50  | 51  | 52  | Mean | SEM |
| SGPT/ALT (IU/L)                 | 74  | 56.5| 65.6| 65.4| 5.1 | 61.8| 103.6| 67.2| 77.5| 13.1 |
| SGOT/AST (IU/L)                 | 263.4| 269.3| 259.6| 264.1| 2.8 | 224.1| 338.1| 340.7| 301.0| 38.4 |
| ALP (U/L)                       | 124 | 122 | 112 | 119.3| 3.7 | 135 | 157 | 135 | 142.3*| 7.3 |
| Phosphorus (mg/dL)              | 7.04| 6.24| 6.57| 6.6  | 0.2 | 7.12| 8.9  | 7.96 | 8.0  | 0.5 |
| Total bilirubin (mg/dL)         | 0.12| 0.13| 0.11| 0.1  | 0.01| 0.16| 0.21 | 0.19 | 0.2**| 0.01|
| BUN (mg/dL)                     | 25.89| 17.85| 18.97| 20.9 | 2.5 | 23.18| 19.44| 20.5 | 21.0 | 1.1 |
| Creatinine (mg%)                | 0.54| 0.59| 0.57| 0.6  | 0.01| 0.6  | 0.1  | 0.67 | 0.5  | 0.2 |
| Blood glucose (mg/dL)           | 24.2| 30.6| 30.7| 28.5 | 2.2 | 74.6 | 26.5 | 31.5 | 44.2 | 15.3 |
| Triglyceride (mg/dL)            | 80  | 74  | 75  | 76.3 | 1.9 | 80  | 126  | 89  | 98.3 | 14.1 |
| TC (mg/dL)                      | 50  | 59  | 55  | 54.7 | 2.6 | 55  | 63  | 72  | 63.3 | 4.9 |
| Calcium (mg/dL)                 | 9.3 | 9.1  | 9.3 | 9.2 | 0.1 | 8.4  | 7.8  | 8.7  | 8.3***| 0.3 |
| Albumin (g/dL)                  | 4.08| 3.95| 3.94| 4.0  | 0.05| 3.97| 4.33 | 4.06 | 4.1  | 0.1 |
| Total protein (g/dL)            | 7.52| 7.44| 7.31| 7.4  | 0.1 | 6.83| 7.79 | 8.11 | 7.6  | 0.4 |
| Globulin (g/dL)                 | 3.44| 3.49| 3.37| 3.4  | 0.03| 2.86| 3.46 | 4.05 | 3.5  | 0.3 |
| Sodium (mEq/L)                  | 149.8| 149.7| 149.1| 149.5| 0.2 | 149.2| 145.6| 149.6| 148.1| 1.3 |
| Potassium (mEq/L)               | 5.7 | 5.5  | 4.9 | 5.4  | 0.2 | 5.1  | 5.8  | 5.4  | 5.4  | 0.2 |
| Chloride (mEq/L)                | 109.5| 110 | 106.9| 108.8| 1.0 | 108.4| 104.3| 107.7| 106.8| 1.3 |

Values are expressed as mean ± SEM, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; SGPT, serum glutamate-pyruvate transaminase; SGOT, serum glutamic oxaloacetic transaminase; TC, total cholesterol

*p value is 0.0489; **p value is 0.0130, ***p value is 0.0268. The result is significant at p < 0.05
Table 5: Relative organ weight per 100 g body weight recorded at the end of the study from male and female rats treated with tested formulation measured during the 28 days of subacute toxicity study (mean ± SD)

| Groups            | Brain   | Heart   | Adrenal | Kidney  | Liver   | Thymus  | Lung    | Spleen  | Testis   | Epididymis |
|-------------------|---------|---------|---------|---------|---------|---------|---------|---------|----------|------------|
| Male rats         |         |         |         |         |         |         |         |         |          |            |
| Control           | 0.83 ± 0.070 | 0.39 ± 0.014 | 0.03 ± 0.002 | 0.86 ± 0.028 | 3.74 ± 0.153 | 0.21 ± 0.011 | 1.35 ± 0.262 | 0.46 ± 0.031 | 1.47 ± 0.092 | 0.51 ± 0.049 |
| Low dose          | 0.82 ± 0.038 | 0.39 ± 0.022 | 0.03 ± 0.005 | 0.79 ± 0.058 | 3.89 ± 0.417 | 0.20 ± 0.041 | 1.08 ± 0.246 | 0.51 ± 0.070 | 1.38 ± 0.063 | 0.45 ± 0.026 |
| Mid dose          | 0.75 ± 0.015 | 0.43 ± 0.026 | 0.02 ± 0.001 | 0.83 ± 0.053 | 3.22 ± 0.164 | 0.21 ± 0.031 | 0.84 ± 0.028 | 0.42 ± 0.022 | 1.23 ± 0.016 | 0.40 ± 0.018 |
| High dose         | 0.86 ± 0.068 | 0.38 ± 0.018 | 0.03 ± 0.005 | 0.90 ± 0.034 | 3.82 ± 0.252 | 0.22 ± 0.009 | 1.91 ± 0.701 | 0.47 ± 0.047 | 1.33 ± 0.103 | 0.47 ± 0.031 |
| Satellite control | 0.76 ± 0.045 | 0.35 ± 0.04  | 0.02 ± 0.003 | 0.66 ± 0.102 | 3.43 ± 0.142 | 0.18 ± 0.008 | 0.86 ± 0.135 | 0.30 ± 0.035 | 1.26 ± 0.031 | 0.46 ± 0.07  |
| Satellite reversal| 0.71 ± 0.007 | 0.35 ± 0.032 | 0.03 ± 0.003 | 0.76 ± 0.008 | 3.45 ± 0.387 | 0.19 ± 0.034 | 0.90 ± 0.281 | 0.36 ± 0.031 | 1.18 ± 0.038 | 0.49 ± 0.03  |
| Female rats       |         |         |         |         |         |         |         |         |          |            |
| Control           | 0.83 ± 0.029 | 0.41 ± 0.02 | 0.03 ± 0.003 | 0.67 ± 0.037 | 3.04 ± 0.111 | 0.18 ± 0.022 | 0.91 ± 0.132 | 0.32 ± 0.027 | 0.07 ± 0.004 | 0.22 ± 0.012 |
| Low               | 0.94 ± 0.029 | 0.47 ± 0.019 | 0.04 ± 0.002 | 0.76 ± 0.023 | 3.44 ± 0.101* | 0.23 ± 0.021 | 0.77 ± 0.068 | 0.30 ± 0.016 | 0.08 ± 0.008 | 0.26 ± 0.032 |
| Mid               | 0.89 ± 0.063 | 0.39 ± 0.015 | 0.04 ± 0.003 | 0.72 ± 0.023 | 3.20 ± 0.069 | 0.28 ± 0.028 | 0.75 ± 0.065 | 0.34 ± 0.020 | 0.07 ± 0.007 | 0.15 ± 0.004 |
| High              | 0.90 ± 0.041 | 0.38 ± 0.025 | 0.04 ± 0.004 | 0.74 ± 0.028 | 3.46 ± 0.142** | 0.22 ± 0.036 | 0.87 ± 0.063 | 0.36 ± 0.049 | 0.07 ± 0.006 | 0.26 ± 0.023 |
| Satellite control | 0.95 ± 0.022 | 0.40 ± 0.009 | 0.03 ± 0.003 | 0.78 ± 0.03  | 3.15 ± 0.034 | 0.15 ± 0.02  | 0.78 ± 0.044 | 0.30 ± 0.022 | 0.05 ± 0.002 | 0.22 ± 0.02  |
| Satellite reversal| 0.87 ± 0.02  | 0.39 ± 0.014 | 0.03 ± 0.003 | 0.81 ± 0.007 | 3.30 ± 0.24  | 0.20 ± 0.006 | 0.79 ± 0.02  | 0.37 ± 0.105 | 0.07 ± 0.007 | 0.24 ± 0.053 |

Control = (Milli-Q water); low = (250 mg/kg); mid-dose = (500 mg/kg); high dose = (1000 mg/kg); satellite control = (Milli-Q water); satellite reversal = (1000 mg/kg)

*p value is 0.0276; **p value is 0.046. The result is significant at p < 0.05
### Table 6: Histological findings from rats

| Groups         | Male |               |               | Female |               |               |
|----------------|------|---------------|---------------|--------|---------------|---------------|
|                | GI (control) | GIV (high dose) | GI (control) | GIV (high dose) | GI (control) | GIV (high dose) |
| Organ          |      | M1 | M4 | M5 | M32 | M34 | M35 | F06 | F07 | F08 | F36 | F37 | F39 |
| Brain          |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Adrenals       |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Heart          |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Kidney         |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Liver          |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Lungs          |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Spleen         |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Spinal cord    |      | NA  | WNL | NA  | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Trachea        |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Thymus         |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Stomach        |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Duodenum       |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Jejunum        |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Colon          |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Thigh muscle   |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Lymph node     |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Urinary bladder|      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Testis         |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Epididymis     |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Prostate       |      | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Seminal vesicles|    | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Ovaries        |      | –   | –   | –  | –   | –   | –   | –   | –   | –   | –   | –   | –   |
| Uterus         |      | –   | –   | –  | –   | –   | –   | –   | –   | –   | –   | –   | –   |

WNL, within normal limits
Subacute Oral Toxicity Assessment of a Herbomineral Formulation with *Shilajit, Swarna Bhasma*

Figs 3A to V: Histopathology of major organs. (A) Control group: brain; (B) High-dose group: brain; (C) Control group: adrenal; (D) High-dose group: adrenal; (E) Control group: heart; (F) High-dose group: heart; (G) Control group: testes; (H) High-dose group: testes; (I) Control group: epididymis; (J) High-dose group: epididymis; (K) Control group: kidney; (L) High-dose group: kidney; (M) Control group: liver; (N) High-dose group: liver; (O) Control group: lung; (P) High-dose group: lung; (Q) Control group: muscle; (R) High-dose group: muscle; (S) Control group: lymph node; (T) High-dose group: lymph node; (U) Control group: prostate; (V) High-dose group: prostate
(ROWS) were calculated using the formula below. All the collected organs were preserved in 10% buffered formalin for subsequent histopathological examination.

\[
\text{ROWS} = \left( \frac{\text{Each organ weight}}{\text{BW}} \right) \times 100
\]

Histopathological Evaluation
Fixed organ samples from representative animals were subjected for histopathology examination after paraffin embedding. Thick sections (4–6 μ) were stained using hematoxylin and eosin for microscopic evaluation (Nikon E-200 Microscope).

Statistical Analysis
Data on each parameter were summarized in tabular and suitable graphical form. All the values were expressed as mean ± standard error of the mean (SEM). All the groups were compared and tested for significance using unpaired t-test, two-way analysis of variance followed by Bonferroni posttests using Graph Pad prism software program. Level of significance was set as \( p < 0.05 \).

RESULTS AND DISCUSSION
Following oral administration of test item to male and female rats at tested dose levels for 28 days, none of the animals showed any sign or symptom of toxicity and mortality throughout the study in control and treatment groups. No signs of delayed onset of toxicity were observed in satellite groups for next 14 days.

Body Weight
Increase in body weight was comparable in all the groups, including the satellite groups. The mean percentage increase in BW in T1 groups was similar to that of the control group. However, these changes in the body weights of treated rats were not significantly different from control (Fig. 1).

Feed Consumption
The weekly feed consumption of animals was normal during the study and reversal period. No significant reduction was observed in weekly feed consumption (Fig. 2).

Hematology
The effect of subacute administration of T1 on hematological parameters is presented in Table 3. All the hematological parameters in both the sex of the treated animals were statistically nonsignificant except the significant decrease observed in erythrocyte count \( (p = 0.025) \) (GII; male animals), whereas significant increase was observed in MCHC \( (p = 0.047) \) (GII; female animals). However, these changes were not observed to be dose dependent.

Clinical Biochemistry
Calcium levels showed significant decrease in GII \( (p = 0.0097) \), GIV \( (p = 0.0124) \), and phosphorus \( (p = 0.0315) \), and BUN \( (p = 0.0355) \) in group GIVS. Female rats revealed statistically significant decrease in serum glutamic pyruvic transaminase (SGPT/ALT) in group GII \( (p = 0.0114) \) and calcium \( (p = 0.0268) \) (GIVS; female animals), whereas significant increase in ALP \( (p = 0.0489) \) and total bilirubin \( (p = 0.0130) \) in female GIVS. These changes were, however, absent in other group and were not observed to be dose dependent (Table 4).

Relative Organs Weights
Changes in ROWs in both the sexes of rats were statistically nonsignificant except significant increase observed in liver [GII \( (p = 0.0276) \) and GIV \( (p = 0.0461) \]; female animals]. Moreover, no alteration was observed in the histopathological structure (Table 5).

Gross Pathological and Histopathological Examination
Gross pathological examination was comparable between treated and control groups. Histopathological investigation of organs of animals exposed to high dose did not show treatment-related changes. No alterations in pathology examination were observed in both the sexes. Owing to the normal histology findings in representative animals, further histopathological analysis was not performed on the remaining animals of high, mid, and low, satellite control and satellite reversal dose groups (Table 6 and Fig. 3).

CONCLUSION
Subacute toxicity study on Shilajit formulation by oral administration for 28 consecutive days in Wistar rats did not show changes in BW, food intake, hematological parameters, ROWs, or histopathology of Wistar rats. Some changes were observed in the biochemical parameters; however, dose-dependent changes were not observed in organ morphology.

Based on the results of the study, tested formulation was found to be nontoxic at tested doses in both the sexes of Wistar rats when administered orally for 28 days. The NOAEL of tested formulation containing Shilajit, Swarna Bhasma, and other ingredients in male and female Wistar rats was found to be 1000 mg/kgBW, when administered orally once daily for a period of 28 consecutive days.

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हिंदी सारांश

शिलाजीत, स्वर्ण भ्रमस और अन्य घटकों से निर्मित खनिज-औषधीय योगों का उपतीक मौखिक विषाक्तता का आकलन

पृष्ठभूमि: शिलाजीत, स्वर्ण भ्रमस, और अन्य घटकों से युक्त एक आयुर्वेदिक खनिज-औषधीय जिसे शक्ति बढाने के लिए कायाकल्प के रूप में प्रयोग किया जाता है उसका विस्तार चूहों में उपतीक मौखिक विषाक्तता के लिए परीक्षण किया गया।

सामायी और पद्धति: प्रस्तुत अध्ययन आर्थिक सहयोग और विकास संगठन (ओईसीडी) के दिशा निर्देशों के अनुसार आयोजित किया गया। परीक्षण मद (टीआई) का जलीय अर्क लगातार 28 दिनों के लिए तीन खुराक स्तर (250, 500 और 1000 मिलीग्राम/किग्रा शरीर भार बीडल्ट्यू) में तीन समूहों को दिया गया था। एक समूह के जानवरों को उच्च खुराक उपयोग प्रत्यावर्तन के रूप में लिया गया, एक नियंत्रण गुप्त तथा एक उपयोग नियंत्रण समूह जिसे भिली-ब्यू वाटर (10 एमएल/किग्रा बीडल्ट्यू) प्राप्त किया गया तथा विषाक्तता मूल्य दर का नैदानिक संकेत देखा गया। अगले 14 दिनों के लिए उपयोग समूहों में विषाक्तता की शुरुआत में देशी के संकेत देखे गए।

परिणाम: विषाक्तता का कोई मृत्यु या नैदानिक संकेत नहीं देखा गया। उपचारित और उपयोग समूहों के सभी जानवरों ने खुराक और स्वास्थ्य लाभ अवधि के दौरान नियंत्रण समूह की तरह समान वजन और भोजन प्राप्त किया। हेमेटोकोजिक और जैव रासायनिक मापदंड उपचार और नियंत्रण समूहों में सामान्य स्थिति की सीमा के भीतर थी। नेक्रोप्सी परीक्षण में किसी भी महत्वपूर्ण सकल विकृति परिवर्तन को नहीं देखा गया। उपचारित समूह में अंग भार नियंत्रित समूह के तुलनात्मक था। उच्च खुराक वाले जानवरों और नियंत्रण वाले जानवरों की हिस्टोपैथोलॉजी भी तुलनात्मक थी।

निष्कर्ष: शिलाजीत, स्वर्ण भ्रमस, और अन्य घटकों से युक्त टेस्टेड फॉर्मूलेशन, उपतीक देने पर विस्तार चूहों के दोनों लिंगों में परीक्षण किए गए खुराक में अंतिक्रिया पाया गया। दृष्टिगत आयुर्वेद के परिणाम बताते हैं कि परीक्षण मद (टीआई) का 28 दिनों के लिए विस्तार चूहों पर उपतीक मौखिक संपर्क में 1000 मिलीग्राम/किग्रा बीडल्ट्यू का कोई प्रतिकूल प्रभाव स्तर (एनओएल) नहीं देखा गया है।

मुख्य शब्द: आयुर्वेद, हॉमेडिकल, खनिज-औषधीय, आर्थिक सहयोग और विकास के दिशा निर्देशों के लिए संगठन, दोहराई गई खुराक 28-दिन मौखिक विषाक्तता, शिलाजीत, उप-तीव्र मौखिक विषाक्तता, स्वर्ण भ्रमस।